Sequence- and site-specific photodissociation at 266 nm of protonated synthetic polypeptides containing a tryptophanyl residue

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Photodissociation at 266 nm of protonated synthetic polypeptides containing a tryptophanyl residue was investigated using a homebuilt tandem time-of-flight mass spectrometer equipped with a matrix-assisted laser desorption/ionization source. Efficient photodissociation of the protonated peptides was demonstrated. Most of the intense peaks in the laser-induced tandem mass spectra were sequence ions. Furthermore, sequence ions due to cleavages at all the peptide bonds were observed; this is a feature of the technique that is particularly useful for peptide sequencing. Fragmentations at both ends of the tryptophanyl residue were especially prevalent, which can be useful for location of the tryptophanyl chromophore in a peptide. Copyright © 2004 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization (MALDI) is one of the powerful techniques to generate gas-phase ions from samples in condensed phases which have revolutionized the application of mass spectrometry to biomolecules such as proteins and nucleic acids.1–3 Tandem mass spectrometry,4 which records fragment ions generated from a mass-selected precursor ion, is useful in providing further structural information on ions generated by MALDI.5,6 Using a tandem time-of-flight (TOF) mass spectrometer, the recording of post-source decay (PSD) product ions has been a popular method of tandem mass spectrometry.7,8 The internal energy that ions acquire in the MALDI process or via collisional activation in the source is thought to be responsible for PSD. To enhance the intensity and variety of fragment ion signals, collisionally activated dissociation (CAD) is often attempted5,9,10 by introducing collision gas into a cell located on the ion flight path. Introduction of collision gas into an analyzer maintained under high vacuum conditions and consequent deterioration of the ion beam quality due to the collisions usually requires elaborate instrumentation to permit acquisition of tandem mass spectra of high quality.

Tandem mass spectrometry utilizing photodissociation (PD) with ultraviolet (UV) radiation has been attempted also.11–13 Compared with CAD, PD may be considered a clean technique without difficulties related to the vacuum and thus to the beam quality. The capability to supply a large amount of energy to a precursor ion is another advantage of the UV-PD scheme. For example, absorption of one photon at 266 nm, available from commercial Nd:YAG lasers, increases the internal energy of a precursor ion by 4.66 eV. Further increases in internal energy are possible via multiphoton processes. Thus, UV-PD may be a viable alternative to CAD for tandem mass spectrometry of large molecules because the energy requirement for dissociation increases almost in proportion to the precursor ion mass14 as a consequence of the “degrees of freedom” effect.

Recently, we built a tandem TOF mass spectrometer to investigate UV-PD of biomolecular ions generated by MALDI.14 By synchronizing the PD laser pulse with an ion beam pulse, a mass peak of interest in the isotopic ion cluster of a protonated peptide could be selected and photodissociated, even to nearly complete depletion. However, a successful dissociation of a precursor ion in itself is not sufficient to render the technique a useful structural probe. It is more important that the technique generates fragment ions with structural information, such as the sequence-specific fragment ions from a protonated peptide. The latter requirement has been tested in this study using synthetic peptides containing a tryptophanyl residue.

EXPERIMENTAL

Details of the MALDI-TOF-PD-TOF instrument (Fig. 1), its operation, and the method for mass calibration can be found elsewhere.14 Only a brief account will be presented here. The instrument consists of a MALDI source with delayed extraction; the first stage TOF is a linear type, and the second stage TOF is of a reflectron design. A pulsed nitrogen laser (VSL-337ND-S; Laser Science, Franklin, MA, USA), with 300 μJ per pulse at 337.1 nm and less than 4 ns pulse width, was
used for MALDI. A DC voltage of 13 kV and an AC voltage of 1.4 kV were applied to the MALDI source. The protonated peptide and other ions in its vicinity in m/z generated by MALDI were selected by an ion gate and irradiated by a laser at the first time focus of the instrument for photodissociation (PD). The PD laser radiation used was the fourth harmonic at 266 nm of an Nd:YAG laser (Surelite III-10; Continuum, Santa Clara, CA, USA) with 5 ns pulse width. Its pulse was synchronized with the arrival at the PD laser beam (Fig. 1) of an ion of selected m/z value from the MALDI pulse, usually that of the A isotopic peak of the protonated peptide. The resolving power of the present instrument for precursor ion selection is around 2000, as estimated from the time-of-flight difference between adjacent isotopic peaks of a precursor ion and the pulse width of the photodissociation laser used.14 PD fragment ions were analyzed by the reflectron TOF stage and detected by a dual MCP detector. A digital storage oscilloscope (LT372; LeCroy, Chestnut Ridge, NY, USA) with sampling rate of 1 G samples/s and 500 MHz bandwidth was used to record the TOF spectrum. Tandem mass spectra with PD laser off and on were recorded alternatively and stored and averaged in separate channels. Typically, 20 spectra of each kind were averaged in the oscilloscope, and the results then transferred to a computer. Around 200 of these averaged spectra were collected and further averaged. To enhance the spectra by suppressing noise, a signal in each channel with intensity less than 50% of a typical single ion pulse height was reduced by factor of 3. Also, infrequent signals (appearing once in 10 spectra or less) were assigned as chemical noise and discarded.

The peptide samples with 98% purity were purchased from Peptron (Daejon, Korea), and the matrix, a-cyano-4-hydroxycinnamic acid (CHCA), from Sigma (St. Louis, MO, USA). Matrix solution was prepared daily using acetonitrile and 0.1% trifluoroacetic acid. The final peptide concentration for the PD experiments was 150 pmol μL−1 and 1 μL of the sample solution was spread and dried to give a 4 mm2 spot area.

**RESULTS AND DISCUSSION**

Among common amino acids only tryptophan, tyrosine, and phenylalanine absorb at 266 nm used for photodissociation in this work.15 Prototype peptides used in mass spectrometric studies usually include one or more of these aromatic amino acids as residues. Instead of these prototypes, we decided to use synthetic peptides containing only one chromophore, a tryptophanyl (W) residue in the present case, to avoid confusion in this preliminary investigation. Residues which are known to induce strong site-specific fragmentations such as histidine were not included for the same reason.16 Arginine (R) was attached at both ends of a peptide to facilitate observation of both the N-terminal and C-terminal fragment ions.17 The remaining residues forming the backbone were all glycine (G).

To observe the spectral change resulting from a change in the position of the tryptophanyl residue, two synthetic
peptides were studied, RGGWGGGGR and RGGG GWGGR. These will be called peptides A and B, respectively, in the rest of the paper. The protonated peptides, [M+H]⁺, have m/z of 916.45 for the monoisotopic (A) peaks.

Major sequence-specific fragment ions (sequence ions) from a protonated peptide are formed by cleavages at the peptide bonds. Even though a rigorous nomenclature for sequence ions has been proposed, its abbreviated form will be used here, as shown in Scheme 1. Amino acid residues are numbered starting from the left to differentiate a, b, and c fragment ions generated by cleavage at various peptide bonds and from the right for x, y, and z. Other sequence ions are d, v, and w for which a side-chain loss accompanies peptide bond cleavage.

The PSD spectrum (or laser-off spectrum) recorded for the protonated peptide A generated by MALDI is shown in Fig. 2(a). Since the mass resolution of the ion gate was rather poor, fragment ions generated inside the source, often called ‘prompt’ ions in the MALDI literature, with flight times close to that of [M+H]⁺, also passed the gate and resulted in non-PSD peaks such as those around m/z 885 in Fig. 2(a). Such peaks are one of the disadvantages of a PSD spectrum. Four peaks, at m/z 899.4, 898.4, 874.4, and 760.3, dominate the PSD spectrum, and can be assigned to [MH–NH₃]⁺, [MH–H₂O]⁺, [MH–CH₂N₂]⁺, and y9, respectively. Loss of CH₂N₂ presumably occurred at one of the Arg residues. The remaining low-intensity peaks are mostly due to sequence ions, namely a4, b5, b6, b7, y5, y6, y7, and y8. Other than the losses of H₂O and NH₃, the y9 peak is the most intense, suggesting that the peptide bond between G and R at the N-terminus is the most labile. Sequence ions identified from the PSD spectrum are shown as a fragmentation map in Fig. 3(a).

A tandem mass spectrum recorded with the PD laser on consists of both PD and PSD fragment ion peaks. Laser-induced changes in this spectrum can be obtained by subtracting the laser-off spectrum from the laser-on spectrum; the result will be called a PD spectrum in the following. A PD spectrum of the protonated peptide A, obtained with 4 mJ per pulse of the PD laser, is shown in Fig. 2(b). Around 45% of [M+H]⁺ was photodissociated under these conditions. Compared with the PSD spectrum, the PD spectrum displays a few striking features; monoisotopic nature of each peak, appearance of negative peaks, and rich spectral pattern. The first arises from the monoisotopic selection through synchronization of the PD laser pulse with the pulse of [M+H]⁺ consisting of the lowest mass isotopes (the A peak). Other ions passing the ion gate, which include A + 1 and higher isotopic ions of [M+H]⁺ and their PSD products, are not affected by the PD laser and hence disappear when the laser-off spectrum is subtracted from the

**Figure 2.** (a) PSD and (b) PD spectra of [M+H]⁺ ions generated by MALDI of the peptide A. The ion gate was set to transmit [M+H]⁺ with a mass selection capability of ±20 Da. The PD laser (266 nm, 4 mJ pulse energy, 5 ns pulse width, and 10 Hz) was synchronized with the lowest mass isotopic variant of [M+H]⁺. The PSD (laser-off) spectrum was subtracted from the laser-on spectrum to obtain the PD spectrum.
laser-on spectrum. An unintended benefit of this procedure is the elimination of prompt ion contaminants in the tandem mass spectrum, such as those appearing near $m/z$ 885 in the PSD spectrum (Fig. 2(a)). Most importantly, the monoisotopic nature of the precursor selection helps with recognition of different ionic species with similar masses, such as $[\text{MH–H}_2\text{O}]^+$ and $[\text{MH–NH}_3]^+$. Negative peaks arise when PSD fragments, which move with nearly the same velocity as the precursor, are strongly photodissociated. Since the same ions can also be generated by photodissociation of $[\text{M+H}]^+$, their relative intensities are often more sensitive to the laser radiation than those of other fragment ions.

Compared with the PSD spectrum, fragment ion peaks are more intense and numerous in the PD spectrum, and many of them are sequence ions. Considering prominent peaks only, $a_i (i = 3, 4, 5, 9)$, $c_3, x_9, y_i (i = 2, 3, 4, 5, 6)$, and $z_i (i = 2, 3, 4, 5, 6, 7, 9)$ could be readily assigned. Other prominent sequence ions include $a_{17}$, $a_{17}$, $b_{2}$, $b_{17}$, $b_{17}$, and $v_{7}$, which is generated via tryptophan side-chain loss from $y_7$. Other prominent ions are $b_{2}$–$\text{CH}_2\text{N}_2$ at $m/z$ 700.2 and internal fragment ions $G_3\text{W}$, $G_2\text{W}$, $G_5\text{W}$, $G_4\text{W}$, and $W$ at $m/z$ 388.2, 301.2, 266.2, 244.3, 229.4, and 159.4, respectively. More than half of the peaks with low intensities could be assigned to sequence ions. A fragmentation map is shown in Fig. 3(b), indicating the sequence ions identified from the PD spectrum.

By comparing the PD and PSD fragmentation maps, it is apparent that PD generates more fragment ions useful for sequence determination than PSD. For example, a complete set of the $y$ series is observed in the PD spectrum even though $y_9$ appears as a negative signal. Among the $b$ ions, only $b_9$ and $b_3$ are observed. Considering that the $a$ ions appear much more frequently, it seems that adding one or possibly more 266 nm photons increases the $[\text{M+H}]^+$ internal energy sufficiently that any $b$ ions generated undergo further dissociation to a ions.

Another striking feature of the PD spectrum is that the $y_6$ ion is much more abundant than any other ions in the series. Similarly, the $a_4$ ion is much more abundant than any other $a$-type ion. These are ions generated via cleavage of the peptide bond between W and the adjacent G residue on its C-terminal side. Equally prominent is the $v_7$ ion at $m/z$ 515.2 which is generated via cleavage of the peptide bond between W and the adjacent G residue on its N-terminal side accompanied by a side-chain loss. A feature common to all these fragmentations is that the peptide bonds on both sides of the tryptophanyl residue are especially labile in PD, unlike in PSD.

The PSD and PD spectra of the protonated peptide B and the fragmentation maps determined therefrom are shown in Figs. 4 and 5, respectively. General comments that can be made for these results are essentially the same as those for
Figure 4. (a) PSD and (b) PD spectra of [M+H]+ generated by MALDI of the peptide B. See the caption to Fig. 2 for details.

Figure 5. Sequence-specific dissociations observed in (a) PSD and (b) PD spectra of the peptide B. See the caption to Fig. 3 for details.
the protonated peptide A. Even though the y series observed is not complete, unlike the protonated peptide A, sequence ions are formed via cleavages of all the peptide bonds, supplying more than enough, or redundant, information for sequencing. Among the sequence ions, \(a_2\) and its \(\text{NH}_2\) loss product, at \(m/z\) 600.3 and 583.4, are especially prominent; these fragment ions arise via cleavage of the peptide bond between W and the adjacent G residue on its C-terminal side. The \(c_6\) ion appears prominently at \(m/z\) 459.2, and corresponds to fragmentation between G(6) and W(7). Even though \(a_8\) is not as abundant, the peak corresponding to \(a_8\)–17 is rather prominent. Thus, facile cleavages at both sides of the tryptophanyl residue are evident, even though in this case they are a little obscured by the presence of very intense \(b_9\)–CH\(_2\)N\(_2\) and G\(_3\)W peaks at \(m/z\) 700.3 and 415.2, respectively.

Not only the cleavages at every peptide bond but also the site-specific cleavages observed in PD can be useful structural probes for peptide sequencing. Preferential cleavages adjacent to aspartyl, glutamyl, and prolyl residues are well known in CAD of protonated peptides.\(^\text{20}\) In CAD via multiple collisions such as in an ion trap, for example, each collision is likely to transfer a small amount of energy to various parts of \([\text{M+H}]^+\), resulting in an activated cation under quasi-thermal condition. Then, preferential cleavages are those with favorable dissociation energetics for the bonds cleaved. Such an explanation is not applicable to preferential photodissociations observed in this work. For example, even though the sequence ions \(a_4\) and \(a_7\) dominate the PD spectra in Figs. 2 and 4, respectively, their counterparts are not particularly noticeable in the PSD spectra.

A simple explanation for the preferential cleavages at both ends of the tryptophanyl residue can be proposed as follows. Let us assume that the photon energy initially deposited in the tryptophanyl chromophore undergoes rapid internal conversion to vibrational energy in the same residue and then flows to other parts of \([\text{M+H}]^+\) via intramolecular vibrational redistribution. The fact that cleavages of the peptide bonds at both ends of the tryptophanyl residue are especially facile suggests that the energy flow through these bonds is not quite efficient relative to the dissociations at these bonds. In other words, the tryptophanyl residue becomes a ‘hot spot’ upon photoabsorption and fragmentations in its close vicinity, i.e., site-specific fragmentations, dominate. Energy flow to other parts of \([\text{M+H}]^+\) must occur, even though inefficiently, as manifested by appearance of other sequence ions. Their enhancement in the PD spectra compared with the PSD spectra must be simply due to the increase in the number of \([\text{M+H}]^+\) ions with high energy content.

CONCLUSIONS

Two characteristic features in the photodissociation at 266 nm of protonated synthetic peptides containing a tryptophanyl residue investigated in this work are the occurrence of sequence-specific fragmentations at all the peptide bonds and spectacular enhancement of cleavages at both ends of the tryptophanyl residue. These suggest potential utility of UV-PD as an analytical tool for peptide sequencing. Specific cleavages adjacent to aspartyl, glutamyl, and prolyl residues were observed in CAD of protonated peptides also.\(^\text{19}\) Reasons for the reaction specificity in PD and CAD seem to be different, however; the specific cleavages in CAD mentioned above seem to be favored energetically, while those in PD may indicate a non-statistical nature of the reactions. Regardless, the results may be utilized complementarily for peptide sequencing. It is worth emphasizing that PD can be implemented without disturbance of the high vacuum system and thus of the resolution and with the benefit of monoisotopic selection. In contrast, efforts are needed in CAD to overcome the difficulties related to these aspects.

We are not in a position to compare the resolution of CAD and PD tandem TOF mass spectrometry, however, because CAD is not implemented in the present instrument. We will mention only that the bandwidth of a PD fragment is only slightly broader than that of its precursor when the reflectron voltage is set to the optimum and that the mass accuracy is usually around \(\pm 0.2\) Da. From the present use of relatively large amounts of samples, one may conclude that PD tandem TOF mass spectrometry is inferior to CAD in sensitivity. However, this is mostly due to the fact that the present homemade instrument has not achieved the state-of-the-art in various aspects such as in the sample handling and data systems. In our previous report, we demonstrated that almost complete depletion of a precursor ion signal was possible by increasing the PD laser intensity; thus, PD is thought to be at least comparable to CAD in sensitivity.

Even though we demonstrated successful photodissociation of prototype peptide ions in a previous report, the work here has been confined to synthetic peptides because we wished to establish the influence of a particular chromophore, the tryptophanyl residue in this case, on the PD performance. We plan to report PD results for more realistic samples such as tryptic peptides after first assessing the chromophore effect.

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