Photodissociation at 193 nm of some singly protonated peptides and proteins with \( m/z \) 2000–9000 using a tandem time-of-flight mass spectrometer equipped with a second source for delayed extraction/post-acceleration of product ions

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A tandem time-of-flight mass spectrometer was built for photodissociation (PD) of singly protonated peptides and small proteins generated by matrix-assisted laser desorption/ionization. PD was performed in a second source after deceleration of precursor ions. The delayed extraction/post-acceleration scheme was used for the product ions. For the PD at 193 nm of small singly protonated peptides, the present instrument showed much better sensitivity and resolution for product ions than the previous one (Moon JH, Yoon SH, Kim MS, Bull. Korean Chem. Soc. 2005; 26: 763) even though the overall spectral patterns obtained with the two instruments were similar. The present instrument was inferior in precursor ion selection and background noise level. PD was achieved for precursor ions as large as the singly protonated ubiquitin (\( m/z \) 8560.63), indicating that the photoexcitation is capable of supplying a sufficient amount of internal energy to dissociate large singly protonated proteins. As the precursor ion \( m/z \) increased, however, product ion signals deteriorated rather rapidly. As in the PD of small peptide ions with \( m/z \) around 1000, the types of the product ions generated from singly protonated peptides with \( m/z \) in the range 2000–4000 were mostly determined by the positions of arginine residues. Namely, \( \alpha_n \) and \( \delta_n \) ions dominated when an arginine residue(s) was near the N-terminus while \( \upsilon_n \), \( \omega_n \), \( \chi_n \) and \( \eta_n \) dominated when the same residue(s) was near the C-terminus. In addition, \( \delta_n \), \( \upsilon_n \) and \( \omega_n \) ions were generated according to the correlation rules previously observed in the collisionally activated dissociation. Isoleucine and leucine isomers could be easily distinguished based on the \( \omega_n \) and \( \delta_n \) ions. Copyright © 2007 John Wiley & Sons, Ltd.

With the establishment of various techniques to generate gas-phase ions from condensed-phase samples, tandem mass spectrometry\(^1\) (MS/MS) is widely used to obtain detailed structural information on large biological molecules. Most of the ion activation techniques used for this purpose are those developed previously to induce dissociation of relatively small molecular ions\(^2\)–\(^8\). An example is collisionally activated dissociation (CAD),\(^2\)\(^,\)\(^3\) which is probably the most popular among these techniques. One of the difficulties in CAD, which is common to many other ion activation techniques, is that the amount of energy transferred in each activation event cannot be determined and controlled precisely. The failure to supply a large amount of internal energy is another difficulty. The latter difficulty is especially critical to the study of large biological molecules because the theory of mass spectra predicts that the energy needed for dissociation of a precursor ion increases almost in proportion to its molecular mass.\(^9\) A large amount of internal energy may be supplied via multiple activation events, such as multiple collisional activation. However, such schemes can cause various difficulties especially for precursor ions under the beam condition.

Photodissociation\(^10\)\(^,\)\(^11\) (PD) has been widely used to study the structure and dissociation dynamics of small molecular ions. The fact that the amount of energy supplied and the excited state accessed can be controlled easily is its main advantage. Its additional advantages in MS/MS of large biological molecules are the possibility to supply a large amount of internal energy via multiphoton absorption, when needed, and the fact that the beam condition is not disturbed by photoabsorption. Under the multiphoton absorption condition, a precise control of the energy supplied would
not be possible. Even in such a case, a chromophore responsible for photoexcitation can be selected. In spite of the above potential merits, PD has not been widely used for MS/MS of biological molecules, probably because the quality of the photofragment spectra, or PD spectra, was not as good as that of the CAD spectra recorded with commercial instruments. Recent reports of high-quality PD spectra recorded with improved instruments are expected to change the situation.

This laboratory has been building photodissociation tandem time-of-flight (PD-TOF/TOF) mass spectrometers and evaluating their performance in the UV PD of singly protonated peptides generated by matrix-assisted laser desorption/ionization (MALDI). In the initial instrument, the usual delayed extraction was used to time-focus a precursor ion beam at an intermediate focal point where the PD laser was irradiated. By synchronizing the PD laser pulse with a precursor ion beam pulse, monoisotopomeric selection of the precursor ion was achieved. A reflectron with a linear-plus-quadratic potential inside was used to analyze PD product ions. Good resolution for the product ions could be achieved without switching the reflectron voltage, which is needed when the usual linear potential reflectron is used. Recently, a deflection system was installed between the ion source and the intermediate time focus to reduce the interference from the PD of the post-source decay (PSD) products. However, the instrument was not adequate both in resolution and in sensitivity to handle singly protonated peptides with m/z larger than 2000.

To study the PD efficiency for large peptide ions, a new PD-TOF/TOF mass spectrometer was constructed. The design goal was to improve the resolution and sensitivity for the product ions. A design with a second source for delayed extraction/post-acceleration of product ions was adopted. The monoisotopomeric selection capability for precursor ions was sacrificed. In this paper, details of the instrument, its performance in the PD of singly protonated peptides and proteins with m/z in the range 2000–9000 and general characteristics of the PD spectra will be presented.

**EXPERIMENTAL**

**Instrument and operation**

Figure 1 shows a schematic of the PD-TOF/TOF mass spectrometer equipped with a second source for post-acceleration of PD product ions. It consists of a MALDI source with delayed extraction, a flight tube to time-separate the ions generated by MALDI, a second source for PD, and a second stage mass spectrometer equipped with a reflectron. Two dual multichannel plate (MCP) detectors (APTOF 40, Burle, Lancaster, PA, USA) are installed, one at the end of reflectron to detect ions in the linear mode of operation and the other in the middle of the instrument to detect reflected ions.

The first ion source consists of a sample plate (S), an intermediate electrode (E1), a ground electrode (G1), and an einzel lens. The distances between S and E1 and between E1 and G1 are 5 and 16 mm, respectively. Even though the ion source can tolerate 30 kV of acceleration potential, 17.8 kV is normally used as DC potential (V) in the MS mode. In addition, a 1.8 kV pulse is applied (∆V) to S for delayed extraction using a high-voltage pulser (PVX-4140, Directed Energy, Fort Collins, CO, USA) and an AC-coupling circuitry. In the MS/MS mode, 12 kV DC and 1.3 kV AC potentials are used. The first time focal positions in the MS and MS/MS modes are at 644 and 382 mm from G1 as calculated by SIMION. In our previous PD-TOF/TOF instrument, an ion gate was installed immediately in front of the first time focal point where a PD laser was irradiated. In the present instrument, an ion gate is located 39 mm ahead of the first time focal point to make room for an ion deceleration system. Accordingly, its mass resolving power is not good. A nitrogen laser (VSL-337ND-S, Laser Science, Franklin, MA, USA) is used for MALDI.

The second source consists of a grid/electrode assembly for deceleration, a PD cell, a delayed extraction/post-acceleration system and an einzel lens. Ion deceleration...
Two equipotential grids E2 and E3 separated by 31 mm form a grid system inside the reflectron. The first grid E1 is located at 364 mm, while the second grid E2 is at 70 mm from the PD cell. The distance between the two grids is 117.5 mm, and the diameter of each grid is 100 mm. The reflectron is 242.5 mm long, and a grid is located at 117.5 mm from the PD cell. Grounded apertures (20 mm) are installed between the two grid systems to eliminate chemical noises as much as possible. Potentials of 17.7 kV through delayed extraction/post-acceleration may occur at any position between the exit electrode of the reflectron and eventually to the final detector, only the product ions generated in field-free regions can form peaks in this spectrum. In contrast, ions generated inside field regions may occur at any position between the exit electrode of the reflectron and eventually to the final detector, only the product ions generated in field-free regions can form peaks in this spectrum. For example, the instrument reported by Morgan and Russell25 the inside and outside kinetic energies are 7 and 15 keV, respectively.

A dual-stage reflectron27 is used in the second stage mass spectrometer. It consists of 24 parallel ring electrodes (i.d. = 70 mm, o.d. = 100 mm, thickness = 5 mm). Its total length is 242.5 mm. A grid is located at 117.5 mm from the entrance. The axis of the reflectron is 20 mm off from the ion-optical axis of the first stage. A deflector is used to guide the ion beam into the reflectron and eventually to the final detector. The entrance of the reflectron is located at 775 (d1) and 699 (d2) mm from the first time focus and the final detector, respectively. Grounded apertures (20 mm × 20 mm) are installed at the entrance and exit of the reflectron to eliminate chemical noises as much as possible. Potentials of 23.5 and 19.2 kV are applied to the final electrode of the reflectron in the MS and MS/MS modes, respectively. The penetration depth (d3) inside the reflectron is around 184 mm. There is another way to record a tandem mass spectrum with this instrument, namely the linear MS/MS mode. In this mode, the reflectron voltage is turned off and ions are detected with the MCP detector located after the reflectron end.

Timings for electrode pulsing and laser firing are controlled by a personal computer through two delay generators (DG353, SRS, Sunnyvale, CA, USA). MCP output is digitized by an A/D card (DC-252, Acqiris Inc., Geneva, Switzerland) and transferred to the same computer. The method used to treat the data was reported previously.26

RESULTS AND DISCUSSION

Figure 2 shows the [M+H]+ (monoisotopic m/z of 5730.61) region of the MALDI spectrum of bovine insulin recorded in the MS mode. The resolving power (m/Δm) determined using the full width at half maximum was 12000. The resolving power determined from the same ions in the MS/MS mode was worse, around 7000.

The PSD spectrum of the protonated RLLAPITAY (m/z 1017.61) is shown in Fig. 3(a). Curve smoothing was not done in all the spectra shown in this paper. Even though the metastable ion decomposition (MID)30 of the precursor ion may occur at any position between the exit electrode of the first ion source and the final detector, only the product ions generated in field-free regions can form peaks in this spectrum. In contrast, ions generated inside field regions such as inside the deceleration region and inside the reflectron form broad background, or chemical noise. The

The method used to treat the data was reported previously.26

Samples

The peptide RLLAPITAY was purchased from Peptron (Daejeon, Korea). The peptide and protein samples, ACTH fragment 18-39, melittin, β-casein, bovine insulin and ubiquitin, were purchased from Sigma (St. Louis, MO, USA). The matrices, α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA), and CNBr were purchased from Sigma also. Some peptide samples were prepared via CNBr digestion of β-casein as follows: 1 mg of β-casein and 2 mg of CNBr were dissolved in 0.1 mL of 80% trifluoroacetic acid (TFA). The mixture was kept in the dark for 20 h at room temperature, diluted with 0.5 mL of deionized water and lyophilized. The digestion products were separated using a high-performance liquid chromatography (HPLC) system (model 1100, Agilent Technologies, CA, USA) and collected. Matrix solution was prepared daily using acetonitrile and 0.1% TFA, which was mixed with the peptide solution. The final peptide concentration prepared for the PD experiment was around 10 pmol/μL; 1 μL of the solution was loaded on the sample plate.

Figure 2. The molecular ion region of the MALDI spectrum of insulin recorded in the MS mode. SA was used as the matrix in MALDI.
The most important field-free region in the present instrument is the one between the electrodes E₂ and E₄. Accordingly, the mass scale in the spectrum was established for the product ions generated in this region. The product ions generated in this region appeared with an excellent resolution such as b₄, y₅ and b₈H₂O ions at m/z 454.31, 564.30 and 854.55, respectively, as intended in the instrument design. However, the intensities of these PSD peaks were weak because the length of this field-free region was relatively short compared to the overall dimension of the instrument.

The PSD product ions formed between the second source exit and the reflectron entrance can appear also in the spectrum. As is well known, the resolution for such product ions decreases rapidly as the m/z decreases. Hence, only those product ions with m/z close to that of the precursor ion can form peaks with reduced resolution in a spectrum recorded without switching the reflectron voltage.

According to our calculations with the present instrumental configuration and experimental condition, the PSD product ions with m/z very close to that of the precursor ion, such as [M+H–NH₃]⁺ in the present case, generated in this region appear at nearly the same time-of-flight as the same ions generated inside the cell. As the product ion m/z gets lower, the PSD peaks generated in the two different field-free regions begin to separate, the inside-cell product appearing as a sharp peak while the post-cell product appears as a broad band. An example is the band contour of b₈H₂O at m/z 854.55 in which a high mass band accompanies a sharp isotopic cluster. As the product ion m/z decreases further, the post-cell component broadens more and becomes difficult to recognize. There is another field-free region in the instrument, namely between the first source exit (G₁) and the deceleration region entrance (G₂). Since the potential applied to the deceleration system was 12.5 kV while the maximum
The kinetic energy of the direct ions were 13.3 keV in the MS/MS mode, the PSD product ions generated in this region with m/z less than 94% of the precursor ion m/z could not pass the deceleration system. Further discrimination against such ions was made at the time of the delayed extraction in the second source. Namely, these ions formed peaks only when they were present between E2 and E4 at the time of the second source pulsing. Such ions could be easily identified by changing the second source tuning and by changing PD laser irradiation time. It is obvious that the PSD product ions generated outside the cell contribute to the background level. However, it seems that this alone cannot explain the strong background level appearing over the entire mass range. Some ions and neutrals generated via unwanted scattering and sputtering processes may also be responsible for the chemical noise. Several very broad features appeared in the PSD spectra obtained at the early stage of the instrument development. The intensities of these features and the overall background level were significantly suppressed when apertures were installed at the reflectron entrance and exit. Overall, the present instrument is not adequate for the acquisition of PSD spectra with high sensitivity.

Figure 3(b) shows the MS/MS spectrum recorded simultaneously with the PSD spectrum in Fig. 3(a), but with the 193 nm PD laser pulse (around 1.2 mJ per pulse was used throughout the experiment) synchronized with the precursor ion pulse. The intensities of all the isotopomers of the precursor ion were reduced to 50% of those measured in the absence of the PD laser. Isotopomeric discrimination was not observed because the resolution of the ion gate was poor, the ion beam was not well time-focused at the PD laser irradiation point, and the PD laser itself was not spatially focused. In contrast, the product ion peaks were well resolved, as demonstrated in the inset, much better than in the spectra obtained with the previous instrument\(^{17,18}\) that did not utilize the delayed extraction/post-acceleration scheme. Also, a factor of 10 difference in the y-magnification in the product ion region of the PSD and PD spectra in Fig. 3 is to be noted. Namely, the PD spectrum is superior to the PSD spectrum in sensitivity. The intensities of strong peaks in the PD spectrum are a few % of the original precursor ion intensity measured in the absence of the PD laser. In terms of the relative intensities of PD product ions, this is the most sensitive PD spectrum of a protonated peptide ever recorded with a TOF/TOF instrument. The relative intensities of strong PD product ions detected with the present instrument are higher than those with the previous instrument\(^{20}\) by a factor of 5–10. There are two obvious reasons for the improvement in the apparent sensitivity achieved with the present instrument. First is the improvement in the time resolution for the product ions as mentioned above. Second is the enhanced quantum efficiency for ion detection. Both of these effects, arising from the post-acceleration, are thought to enhance low mass ion signals more than high mass ion signals. The fact that the distance between the PD laser irradiation point and the reflectron exit is longer in the present instrument may be another reason for the apparent enhancement of the product ion yield. Due to the metastable ion decomposition occurring in this region, the intensity of a precursor ion measured by the final detector may be less than that at the laser irradiation position, more so in the present instrument than in the previous one. With photoabsorption, some of the precursor ions which would have been lost from the PSD spectrum via the above process now generate product ion signals, contributing to the apparent enhancement of the PD yield. However, background noise level is higher in the PD spectrum recorded with the present instrument than that with the previous one. The higher background level is not simply due to the use of PD, because the background level in the PSD spectrum recorded simultaneously with the PD spectrum is comparable. In addition, the monoisotopomeric selection is not possible with the present instrument unlike with the previous one. Even though the presence of isotopic clusters may help in identifying fragment ions, it is more or less a nuisance in MS/MS of high mass ions. Various difficulties may be encountered when one attempts monoisotopomeric selection for high mass precursor ions such as m/z 5000. Compared with the 193 nm PD spectrum\(^{20}\) of the same precursor ion recorded with the previous instrument, the present spectrum shows higher intensities in the low mass region for the reasons described already. However, the product ions appearing in the two spectra are quite similar. \(a^+_n (n = 1–8), a^+_n-NH_3 (n = 1 and 2), c^+_n\) and \(d^+_n (n = 2, 3, 6a, 6b and 7)\) were the major product ions in the previous spectrum. The situation is similar in the present spectrum even though other ions can be identified also, most notably \(b^+_n (n = 1–4)\) and \(b^+_n-NH_3 (n = 1–4)\).

The 193 nm PD spectrum of the singly protonated ACTH fragment 18-39 ([RPVKVYPNGADESAEAPPLEF+H]+, m/z 2465.20) is shown in Fig. 4. The degree of photodepletion was 55% at 1.2 mJ per pulse of 193 nm laser. Here again, the product ion intensities were strong in the low m/z region and decreased as the m/z increased. It was found by Reilly and coworkers\(^{25}\) that singly protonated peptides with an arginine residue at the N-terminus generated contiguous series of \(a^+_n\) ions and some \(d^+_n\) ions by PD at 157 nm while those with the arginine residue at the C-terminus generated contiguous series of \(x^+_n\) ions and some \(v^+_n\) and \(w^+_n\) ions. Our own PD studies at 193 nm showed the same trend. In agreement with this correlation, complete \(a^+_n (n = 1–21)\) ion series appeared in the spectrum even though \(a^+_4–a^+_21\) were very weak. In addition, \(d^+_n\) ions appeared for \(n = 3–5, 8, 11–13, 16, 20\) and 21. \(d^+_3\) and \(d^+_4\) must have been generated by cleavages at the valine sites, \(d^+_11, d^+_13, d^+_16\) and \(d^+_21\) at the glutamic acid site, and \(d^+_20\) at the leucine site. These are in agreement with the spectral correlation for the generation of \(d^+_n\) ions reported by Biemann and coworkers.\(^{31}\) The other two \(d^+_n\) ions were formed through cleavages at the asparagine (\(d^+_3\)) and aspartic acid (\(d^+_12\)) sites. Other product ions appearing in the spectrum were \(a^+_n-NH_3 (n = 1–6 and 14)\), \(b^+_n (n = 1–6, 8, 9, 11–13\) and 16) and \(b^+_n-NH_3 (n = 2\) and 4) almost exclusively.

Figure 5 shows the 193 nm PD spectrum (70% photodepletion) of the singly protonated melittin from honey bee venom ([GIGAVKVTTLTGPLALISWIKKRQGQH2+H]+, m/z 2845.76). In this cation, two arginine residues are present near the C-terminus. Hence, one expects to observe extensive depletion of the singly protonated melittin from honey bee venom. Due to the metastable ion decomposition occurring in this region, the intensity of a precursor ion measured by the final detector may be less than...
very weak. \( y_n \) (n = 3–16 and 22–24), \( v_n \) (n = 7–10, 16, 17, 19, 22 and 25) and \( w_n \) (n = 3–6, 7a, 7b, 10a, 10b, 11, 13, 14, 16a, 17a, 18–22, 25a and 25b) appeared prominently. Some \( a_n, b_n, c_n, d_n \) and \( z_n \) ions appeared also. \( v_n \) appeared at the isoleucine (n = 7, 10 and 25), tryptophan (n = 8), serine (n = 9), threonine (n = 16 and 17) and valine (n = 19 and 22) sites, in agreement with the correlation rule (F, H, W, Y, I, T, S and V sites with a basic residue to the right) reported by Biemann and coworkers.\(^{31}\) \( w_n \) ions appeared more frequently than \( v_n \) ions in agreement with the less restrictive correlation rule (w-site not aromatic, a basic residue to the right) reported by the same investigators. Namely, \( w_8 \) did not appear because a tryptophan residue is present at this position. Two \( w_n \) ions, \( w_{na} \) and \( w_{nb} \) appeared at all the

Figure 4. PD spectrum of the protonated ACTH fragment 18-39 ([RPVKYPGAEDESANEAFPLEF+H]\(^+\), m/z 2465.20). Averaged over 10000 single shot spectra. CHCA was used as the matrix in MALDI.

Figure 5. PD spectrum of the singly protonated melittin from honey bee venom ([GIAGA-VLKLTTGLPALISWKIRKRQQ-NH\(_2_\)+H]\(^+\), m/z 2845.76). Averaged over 10000 single shot spectra. CHCA was used as the matrix in MALDI.
isoleucine sites (n = 7, 10 and 25) compared with one at all the leucine sites (n = 11, 14, 18 and 21). All these peaks were prominent, suggesting that the 193 nm PD is useful to distinguish isoleucine from leucine, like the high energy collisional activation technique. Also to be noted is the appearance of some $a_n$ (n = 2–5), $b_n$ (n = 2–5) and $d_n$ (n = 2b and 5) ions, which is unexpected because the arginine residues are near the C-terminus in the precursor ion. This suggests that the N-terminal side of the precursor ion has access to the proton, possibly by participating in sequestering the proton attached to one of the arginine residues. Detailed investigation will be needed to find out the causes for such violations of the phenomenological correlation rule. Finally, it is to be mentioned that the 193 nm PD spectrum of the same molecule with +4 charge was obtained by McLafferty and coworkers using a Fourier transform mass spectrometer. The major product ions in the spectrum were $c$, $z$- and $y$-types mostly, being dramatically different from the present observation. A similar pattern was observed also for ubiquitin with +5 charge. The dramatic change in the fragmentation pathways, which must have been caused by the presence of additional charges, is very interesting and needs further study to find out a proper explanation.

As other examples, the 193 nm PD spectra were recorded for two fragments from the CNBr digestion of $\beta$-casein. Figure 6 shows the PD spectrum of the protonated fragment I, ([PIQAFLLYQEPVLGYPVRGPFPIIV+H]$^+$, m/z 2663.53) recorded at 75% photodepletion. In this cation, an arginine residue is located at the n = 17 position from the C-terminus, or at the n = 8 position from the C-terminus. Accordingly, one expects to observe $a_n$ and $d_n$ ions with $n$ in the range of 17–23 and $x_n$, $v_n$ and $w_n$ with $n$ in the range of 8–23. Indeed, $a_{17}$–$a_{20}$, $a_{22}$, $d_{17}$, $d_{22}$a and $d_{23}$a appeared in the high m/z region. However, some $a_n$ (n = 2–7) and $d_n$ (n = 2a and 2b) ions appeared in the low m/z region also as in the previous case, in disagreement with the correlation. Almost complete series of the expected $x_n$ ions appeared except $x_{14}$, $v_n$, appeared at the valine (n = 9 and 13), tyrosine (n = 17), phenylalanine (n = 20) and isoleucine (n = 23) sites in agreement with the correlation reported by Biemann and coworkers. Absence of $w_{17}$ and $w_{20}$ is in agreement with the spectral correlation rule for $w_n$. However, appearance of $w_5$ violates this rule. Further investigations on a variety of protonated peptides will be needed to find an explanation for this violation. Figure 7 shows the 193 nm PD spectrum (75% photodepletion) of the protonated fragment II from $\beta$-casein ([FPQPQSVLSSQSKVLPVPQAKVPQRDh+H]$^+$, m/z 3188.75). Here h indicates the homoseryl lactone residue generated from methionine upon CNBr digestion. Many $x_n$ (n = 2–6, 8–26), $v_n$ (n = 6, 8, 13, 16, 18, 20, 22, 24 and 25) and $w_n$ (n = 4, 5, 7, 8, 10–16, 19, 22–24, 26 and 27) ions appeared in the spectrum as expected from the fact that an arginine residue is present near the C-terminus. $v_n$ appeared at the tyrosine (n = 6), valine (n = 8, 13, 16 and 24) and serine (n = 18, 20, 22 and 25) sites while $w_n$ was absent, in agreement with the correlation rule reported by Biemann and coworkers.

Since the number of chromophores in a protein, and hence the molar absorption coefficient also, increases roughly in proportion to its mass, the probability for multiphoton absorption would increase with the molecular mass. Namely, there is a possibility to supply a large amount of internal energy sufficient for dissociation of protein ions with higher mass than those investigated above via photoexcitation at a moderate laser power. To check such a possibility, the 193 nm PD spectra were recorded for the singly protonated bovine insulin (m/z 5730.61) and ubiquitin (m/z 8560.63). The PD spectra of these protein ions are shown in Figs. 8 and 9, respectively. With 1.2 mJ per pulse at 193 nm, the degree of photodepletion was around 70% for these cations. It is interesting to note that the degree of photodepletion achieved so far was rather similar, 55–75%, regardless of the precursor ion mass. From the perspective of analytical application, probably the more important is the fact that such
A high degree of photodepletion was achieved at a moderate laser energy, 1.2 mJ per pulse. The relative intensities of the strongest product ion peaks in Figs. 8 and 9 were 2–6% of the original precursor ion intensities. Compared to the PD spectra of the smaller peptide ions investigated above, the number of product ion species appearing in Figs. 8 and 9 seemed to be much less. Quite a few product ion peaks could be recognized in each spectrum. It seems that many more peaks may be found through further improvement in instrumentation such as the background suppression. Even though some product ion peaks are marked in the spectra offhandedly by referring to recent reports on CAD of insulin and ubiquitin ions, we will not attempt to identify these peaks until we obtain much better spectra through further improvement in instrumentation. At this point, we will just emphasize the fact that the PD product ions from a precursor ion as large as the singly protonated ubiquitin could be recorded with a good resolution and high apparent yield.

A general trend in the PD spectra presented so far is that the product signals get weaker compared to the background level as the precursor ion mass increases. There are a few obvious reasons for such a trend. First, it becomes more difficult to generate a large amount of precursor ions by UV-MALDI as the molecular mass increases. Second, the number of the dissociation channels also increases with the molecular mass, resulting in a weaker product ion signal for each channel. Third, collimating the ion beam tends to become more difficult at higher mass, at least with the present instrument. Namely, with the adjustment of einzel lens potentials, peak widths and heights change noticeably.

Figure 7. PD spectrum of the protonated fragment II, ([FPPQSVLSILSQKVLVPQKAVPYPQRPQDH+H]^+, m/z 3188.75), from the CNBr digestion of β-casein. Averaged over 20000 single shot spectra. CHCA was used as the matrix in MALDI.

Figure 8. PD spectrum of the singly protonated bovine insulin (m/z 5730.61). Averaged over 14000 single shot shot spectra. SA was used as the matrix in MALDI.
for low m/z ions such as the protonated RLLAPITAY while they are rather insensitive for ions with m/z larger than 3000, suggesting the possibility that the ion loss due to inadequate collimation increases with m/z. Hence, development of instrumental methods to increase the precursor ion intensity and to better guide the ion beam will be needed to obtain better PD spectra for high mass singly charged proteins. Technically speaking, one of the reasons for the difficulty in identifying product ion peaks in the PD spectra of high mass ions was the high background, or chemical noise, level in the spectra. In the PD spectra presented above, the background level tends to increase with the molecular mass. The background levels in the PSD spectra recorded simultaneously with the PD spectra are as significant and increase with the molecular mass also, suggesting that the metastable ion decomposition occurring both before and inside the reflectron is one of the major contributors to the background level. Other particles such as those due to scattering and sputtering at grids, electrodes, etc., must also contribute, as indicated in our observation that this level was reduced when apertures were installed at the entrance and the exit of the reflectron. A better scheme to further reduce this level without simultaneous reduction of the signal intensities would be needed.

CONCLUSIONS

It is well known that a peptide bond (P) absorbs efficiently at around 190 nm with molar absorptivity around 10^4 M^-1 cm^-1 due to the ΠP ← ΠP transition. That is, peptide bonds are the chromophores at 193 nm used in the present study. The number of this chromophore increases with the protein mass, and so does the molar absorptivity at this wavelength. The large magnitude of this coefficient combined with efficient internal conversion expected for large molecules suggests the possibility to supply a large amount of internal energy to proteins via repeated absorption at this wavelength. In this work, we built a tandem time-of-flight (TOF/TOF) mass spectrometer which could handle photodissociation (PD) of higher mass ions than the previous instrument and attempted to check the possibility to photodissociate large peptide and protein ions. The attempt was partially successful in the sense that the dissociation of a singly protonated protein as large as ubiquitin could be readily induced. However, the spectral quality was found to deteriorate rapidly as the protein mass increased. Hence, it is obvious that more efficient methods for ion generation, beam guidance and background suppression are needed to develop PD as a useful method for the analysis of proteins.

We recorded the PD spectra of several singly protonated peptides with m/z in the range 1000–2000 with the present instrument, even though the results are not reported here. Their quality was comparable to the spectrum shown in Fig. 3(b). It is to be mentioned that the spectral correlation in PD at vacuum ultraviolet reported by Reilly and coworkers was rather strictly followed for these relatively small peptide ions. That is, either set of product ions (an, dn) or (xn, yn, vn, wn) dominated the spectra depending on the position of an arginine residue in the peptide. As the precursor ion mass became larger, however, more exceptions to this correlation began to emerge. An example is the appearance of low mass an, bn and dn ions in the PD spectrum of the singly protonated melittin which possesses arginine residues very near the C-terminus and hence is not expected to generate such ions. This is in contradiction to the repulsive PD model suggested by Reilly and coworkers. Also, the present work has found that the correlation rules in the generation of dn, vn and wn ions established by Biemann and coworkers through high-energy CAD studies are closely followed in the PD at 193 nm also. This may be further evidence for the dissociation in the ground electronic state even though an excited state is initially accessed by absorption at 193 nm.

CONCLUSIONS

It is well known that a peptide bond (P) absorbs efficiently at around 190 nm with molar absorptivity around 10^4 M^-1 cm^-1 due to the ΠP ← ΠP transition. That is, peptide bonds are the chromophores at 193 nm used in the present study. The number of this chromophore increases with the protein mass, and so does the molar absorptivity at this wavelength. The large magnitude of this coefficient combined with efficient internal conversion expected for large molecules suggests the possibility to supply a large amount of internal energy to proteins via repeated absorption at this wavelength. In this work, we built a tandem time-of-flight (TOF/TOF) mass spectrometer which could handle photodissociation (PD) of higher mass ions than the previous instrument and attempted to check the possibility to photodissociate large peptide and protein ions. The attempt was partially successful in the sense that the dissociation of a singly protonated protein as large as ubiquitin could be readily induced. However, the spectral quality was found to deteriorate rapidly as the protein mass increased. Hence, it is obvious that more efficient methods for ion generation, beam guidance and background suppression are needed to develop PD as a useful method for the analysis of proteins.

We recorded the PD spectra of several singly protonated peptides with m/z in the range 1000–2000 with the present instrument, even though the results are not reported here. Their quality was comparable to the spectrum shown in Fig. 3(b). It is to be mentioned that the spectral correlation in PD at vacuum ultraviolet reported by Reilly and coworkers was rather strictly followed for these relatively small peptide ions. That is, either set of product ions (an, dn) or (xn, yn, vn, wn) dominated the spectra depending on the position of an arginine residue in the peptide. As the precursor ion mass became larger, however, more exceptions to this correlation began to emerge. An example is the appearance of low mass an, bn and dn ions in the PD spectrum of the singly protonated melittin which possesses arginine residues very near the C-terminus and hence is not expected to generate such ions. This is in contradiction to the repulsive PD model suggested by Reilly and coworkers. Also, the present work has found that the correlation rules in the generation of dn, vn and wn ions established by Biemann and coworkers through high-energy CAD studies are closely followed in the PD at 193 nm also. This may be further evidence for the dissociation in the ground electronic state even though an excited state is initially accessed by absorption at 193 nm.
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