Tandem Time-of-Flight Mass Spectrometer for Photodissociation of Biopolymer Ions Generated by Matrix-Assisted Laser Desorption Ionization (MALDI-TOF-PD-TOF) Using a Linear-Plus-Quadratic Potential Reflectron

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A tandem time-of-flight mass spectrometer for the study of photodissociation of biopolymer ions generated by matrix-assisted laser desorption ionization was designed and constructed. A reflectron with linear and quadratic (LPQ) potential components was used. Characteristics of the LPQ reflectron and its utility as the second stage analyzer of the tandem mass spectrometer were investigated. Performance of the instrument was tested by observing photodissociation of $[M + H]^+$ from angiotensin II, a prototype polypeptide. Quality of the photodissociation tandem mass spectrum was almost comparable to that of the post-source decay spectrum. Monoisotopic selection of the parent ion was possible, which was achieved through the ion beam–laser beam synchronization. General theoretical considerations needed for a successful photodissociation of large biopolymer ions are also presented. (J Am Soc Mass Spectrom 2004, 15, 1248–1259) © 2004 American Society for Mass Spectrometry

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The invention of matrix-assisted laser desorption ionization (MALDI) [1, 2] and electrospray ionization (ESI) [3] has revolutionized the application of mass spectrometry for the determination of molecular weights of biopolymers. The natural next step in this field is the tandem mass spectrometry, which detects fragmentation of a mass-selected parent ion. When the internal energy of a polyatomic ion acquired at the time of its formation is sufficient for its dissociation after exiting the source, it may dissociate unimolecularly during its flight to the detector, which is called the metastable ion decomposition (MID) [4]. A more popular way to supply additional energy is to introduce collision gas on the ion flight path such that some of parent ion translational energy is converted to its internal energy. This is called the collision-induced dissociation (CID) or collisionally activated dissociation (CAD) [2]. In the case of the tandem time-of-flight (TOF) mass spectrometry of ions generated by MALDI without the collision gas, the term post source decay (PSD) [5, 6] rather than MID has been popular because CID may also contribute to the observed fragment ion signals. When tandem mass spectra generated by PSD

are either very weak or do not contain sufficient structural information, CID [7–9] may be attempted by introducing collision gas intentionally. Excitation via multiple collisions is thought to be important in the CID tandem mass spectrometry of high mass biopolymers with many degrees of freedom. Recently, dissociation of multiply protonated molecules induced by electron capture, or electron capture dissociation (ECD) [10, 11], is attracting a lot of attention as a method to obtain site-specific information.

Photodissociation (PD) has been utilized in the field of tandem mass spectrometry mostly as a method to study the structure and dissociation dynamics of small polyatomic ions [12]. Infrared multiphoton dissociation (IRMPD) [13, 14] has been used to induce dissociation of biopolymer ions also, even though a large amount of internal energy needed for such a dissociation necessitates absorption of a large number of infrared photons. When electronic transitions of chromophores by ultraviolet radiation are utilized, the number of photons that must be absorbed can be far smaller than in IRMPD. A successful PD would have several advantages over CID. The first is that photoexcitation of an ion beam would not affect its translational energy and trajectory, which is an important advantage when preservation of a good beam quality is critical for subsequent analysis as in the tandem TOF mass spectrometry. The second advantage is that the introduction of photons to a mass spectrom-

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eter is entirely compatible with the very high vacuum requirement of the instrument unlike CID, which can make the instrumentation simpler. Thirdly, for a pulsed ion beam generated by MALDI the pulsed nature of a ultraviolet laser used for PD provides means to mass-select the parent ion of interest which has been difficult with the usual electronic ion gates. Finally, chromophore-selective excitation by a laser may provide an opportunity to obtain site-specific information on biopolymers, which is highly coveted as evident from the popularity of ECD.

The fact that large biopolymer ions can be photodissociated with pulsed ultraviolet lasers has been demonstrated by several research laboratories [15–18]. Also, there appeared some reports which presented daughter ion spectra generated by PD [19, 20]. In this paper, we present the general theoretical basis for the PD tandem mass spectrometry, details of the MALDI-TOF-PD-TOF mass spectrometer designed and built in this laboratory, and the PD results for some prototype polypeptide ions recorded with the instrument. It will be demonstrated that the quality of the PD spectra obtained is almost comparable to that of the usual PSD spectra in some aspects and is better than the latter in others such as in monoisotopic selection.

Theoretical Consideration

It is well known that mass spectra of large biopolymer ions, especially those generated by ESI, do not display rich fragment ion peaks and their tandem mass spectra are more difficult to record compared to the small molecular cases. According to the theory of mass spectra, however, it is not that they are inherently resistant to dissociation but that the internal energy needed for dissociation increases as the degree of freedom and hence the molecular mass get larger. We will make a rough estimate of the internal energy needed for dissociation of biopolymer ions to be observed on a practical time scale and relate the result to the PD laser power requirement.

Dissociation Rate Constant

Let us suppose that we are observing dissociations occurring on a 10^{-6} s time scale. Even though there has been some controversy concerning effective sampling of the complete phase space of a very large molecule [21], use of the Rice-Ramsperger-Kassel-Marcus (RRKM) [22] type formalism may be applicable to the rate calculation for such a slow reaction. Instead of the RRKM formalism, however, we will first use the simpler Kassel formula [23] for the rate constant, which is expected to provide a good qualitative expression because the internal energy involved and the number of degrees of freedom involved are very large.

$$k(E) = A[1 - (E_0/E)]^{s-1} \quad (1)$$

Here E_0 is the critical energy for dissociation, E is the

internal energy, s is the number of oscillators, and A is a proportionality constant with the magnitude comparable to a vibrational frequency. When E is much larger than E_0 and s is also very large, the following approximation is valid.

$$k(E) \sim A[1 - (E_0/E)]^s \sim Ae^{-sE_0/E} \quad (2)$$

As far as the slow dissociation of a very large molecule is concerned, the most important aspect of eq 2 is that the rate constant is scaled with s and hence is roughly scaled with the molecular weight. Namely, when A and E_0 remain constant, the internal energy resulting in the same rate constant, say 10^6 s^{-1} , is simply proportional to the parent ion mass. For example, investigation of the dissociation of a 10,000 Da parent ion requires supplying 10 times more internal energy than that of 1000 Da parent ion when the same experimental condition is used. Some rough estimation of the internal energy requirement is also useful to clarify the energetics situation involved. Let us assume that $A \sim 10^{13} \text{ s}^{-1}$ and $E_0 \sim 3 \text{ eV}$. Let us also assume that the average atomic mass in a molecule is 7 Da. Then, the internal energy required for a 1000 Da parent ion to dissociate with the rate constant 10^6 s^{-1} becomes 79 eV and that for a 10,000 Da parent ion becomes 800 eV, which are tremendous amounts of internal energy. We also carried out more reliable RRKM calculations for some model reactions and found that the energy requirements for $k = 10^6 \text{ s}^{-1}$ dissociations of 1000 and 10,000 Da parent ions are roughly 20 and 170 eV, respectively. Internal energy requirements are still tremendous. Some of these are already present in the form of the thermal energy and lower the energy requirement. For example, the average thermal energies of 1000 and 10,000 Da ions at 500 K are 2.8 and 28.6 eV, respectively.

The mass scaling of the rate constant forecasts a difficulty in preventing a cascade of consecutive dissociations occurring all the way down to very small daughter ions which are not very useful for structure determination. Namely, in the dissociation of a highly activated parent ion, the average internal energy content of a daughter ion will be proportional to its mass, which is again sufficient for its dissociation. The fact that PSD peaks are observed means either that the above statistical picture is not adequate or that they are due to daughter ions generated with the internal energy less than the average. At any event, it is to be emphasized that pumping too many photons into a parent ion might deteriorate the daughter ion signals in the high m/z range.

Probability of Photoabsorption

When a nanosecond pulsed laser is overlapped with an ion beam, saturation of a one-photon transition occurs even at rather moderate pulse energy. According to the kinetic and energetic situations described above, it is

the multiphoton absorption, rather than one-photon absorption, that is needed to dissociate a very large molecule even with an ultraviolet laser. There are various mechanisms for multiphoton absorption. Since the internal conversion (IC) is expected to occur very fast (~ 1 ps) for a very large molecule, the following sequential mechanism proposed by Freiser and Beauchamp many years ago may be the most important one [24].

Here $\dagger$ and $*$ denote electronic and vibrational excitations, respectively. Assuming that the photoabsorption cross section (σ) for a parent ion in the ground electronic state is hardly affected by its vibrational energy, the probability, q_n , for absorption of n photons can be obtained utilizing the probability theory for collisional activation reported previously [25],

$$q_n = e^{-a} a^n / n! \quad (4)$$

with

$$a = \rho \sigma l. \quad (5)$$

Here ρ is the number density of photons in the laser pulse and l is the length travelled by a photon during the laser pulse. The following alternative expression may be more useful.

$$a = \sigma N / A \quad (6)$$

Here N is the number of photons in a laser pulse and A is its spot area at its interaction with the ion beam. Average number $\bar{n}$ of photons absorbed can be readily derived using eq 4.

$$\bar{n} = \sum_{n=1}^{\infty} n q_n = a \quad (7)$$

Namely, the average number of photons absorbed is simply proportional to the laser pulse energy and is inversely proportional to the spot area. Combining this result with the previous kinetic consideration provides an important insight on PD of very large molecules; as you increase the molecular weight of the parent ions investigated, use proportionately larger laser pulse energy. Requirement of a linear rather than exponential increase of laser pulse energy suggests a possibility that even a very large ions, such as 100 kDa, can be probed by PD using commercial high power pulsed lasers. On the other hand, if the laser power is more than adequate, say by a factor of 5, one may observe low mass daughter ions mostly.

Instrumentation

Tandem TOF Mass Spectrometers: A Brief Review

A MALDI-Tandem TOF mass spectrometer consists of a MALDI source which usually utilizes the delayed extraction (DE), the first TOF to time-separate a parent ion of interest, an ion gate to select it, and the second TOF to analyze the fragment ions generated from the selected parent ion. The first TOF is usually the linear type [26] even though the use of reflectron has been reported also [19, 27]. The ion gate [28, 29] consists of electrodes to deflect away ions which are not selected. Even though substantial progress has been made in the design of the ion gate, selecting a single isotope ion is a difficult task which requires various instrumental arrangements.

The second TOF is always a reflectron type. Most popular have been the constant field types [30], either single or dual type, which will be called the linear potential, or linear, reflectron. The reflectron axis is usually tilted with respect to the incoming ion direction and a detector is located off-centered to this direction. There are two time focus positions for the parent ions in this reflectron, the first and the second, or the object and the image. The position of the ion gate corresponds to the first time focus position of the reflectron while that of the detector to the second. The second time focus positions for the parent and the daughters differ, resulting in progressive deterioration of the daughter ion resolution as its m/z gets smaller [31]. To get around this problem, daughter ion spectra are recorded at several different reflectron voltages and the results are stitched together to obtain the full spectrum [31]. An alternative is to post-accelerate the daughter ions using a pulsed voltage lifting cell and reduce the relative difference in kinetic energy between the parent and daughters [19]. Only those daughter ions generated inside the cell can be detected with a good resolution in this case while the remainder may appear as a broad background.

Time focusing can be achieved regardless of the daughter ion mass when quadratic potential is applied to the reflectron. The fact that this device, often called a parabolic reflectron, can be useful to record a PSD spectrum was noted by Cotter [32]. Both the first and second time foci of a parabolic reflectron are located at its entrance. This requires that both the ion gate and the detector must be placed as close to the reflectron entrance as possible for ideal operation, which results in various difficulties for CID and PD. Cornish and Cotter built a reflectron in which the retarding field increases with its depth, as in the parabolic reflectron. The electric field inside this curved-field reflectron (CFR) [33, 34] which would result in a good daughter ion resolution was determined through SIMION [35] calculation and realized in the actual instrument by adjusting resistances between 85 electrode pairs.

In the course of our effort to build an ideal MALDI-TOF-PD-TOF instrument, we realized that various re-

Figure 1. A diagram of a MALDI-TOF instrument equipped with DE source, reflectron, and center-holed MCP detector.

quirements could be met by applying a potential with linear and quadratic components. In fact, such a reflectron, which will be called a linear-plus-quadratic(LPQ) reflectron, was proposed by Yoshida [36] to record ordinary mass spectra generated by electron or laser ionization. Its evaluation as the second analyzer of a tandem TOF instrument has not been reported yet.

Characteristics of LPQ Reflectron

A diagram of a reflectron equipped with a center-holed MCP detector is shown in Figure 1. d_1 and d_2 are the distances of the first focus and the detector, respectively, from the reflectron entrance and d_r is the distance of the last electrode from the entrance. The entrance electrode is grounded and the potential V_r is applied on the last electrode. The potential inside the reflectron, V , has the linear and quadratic components.

$$V = c_1x + c_2x^2 \quad (8)$$

Accordingly, the voltage applied on the last electrode, V_r , can be divided into two components

$$V_r = V_1 + V_2 \quad (9)$$

with

$$V_1 = c_1d_r \quad (10)$$

and

$$V_2 = c_2d_r^2. \quad (11)$$

Following Yoshida [36], the time of flight from the first time focus point to the second for m^+ ion with the kinetic energy K becomes

$$T = (d_1 + d_2)(m/2)^{1/2}K^{-1/2} + (2m/ec_2)^{1/2}[\pi/2 - \sin^{-1}(1 + 4c_2K/ec_1^2)^{-1/2}] \quad (12)$$

Here e is the electronic charge. To find the condition for the second time focusing of m^+ , Yoshida utilized Taylor expansion. The first order time focusing condition thus obtained is not very useful, as we found through SIMION calculation, probably because the series do not

converge rapidly. An easier way is to impose the condition

$$dT/dK = 0. \quad (13)$$

which results in

$$(d_1 + d_2)/d_r = 4KV_1/(eV_1^2 + 4V_2K). \quad (14)$$

For a linear reflectron ($V_2 = 0$), $(d_1 + d_2)/d_r$ equals $4K/eV_1$ which becomes 4 when eV_1 is set at K , as is well known. For a parabolic reflectron ($V_1 = 0$), $d_1 + d_2$ becomes zero as has been mentioned earlier.

Let us now consider the time resolution for m_2^+ generated by dissociation of a parent ion m_1^+ .

$$m_1^+ \rightarrow m_2^+ + m_3 \quad (15)$$

The kinetic energy of m_2^+ , K_2 , is related to that of m_1^+ , K_1 , as follows.

$$K_2 = (m_2/m_1)K_1 \quad (16)$$

Looking at eq 14, it is obvious that an LPQ reflectron tuned for m_1^+ would not satisfy the time focusing condition for m_2^+ . For m_2^+ , one obtains

$$dT/dK_2 = -(\sqrt{2}/4)[(d_1 + d_2)/K_2^{3/2}]m_2^{1/2}[1 - \{1 + (m_3/4m_2)((d_1 + d_2)/d_r)(eV_1/K_1)\}^{-1}]. \quad (17)$$

For a parabolic reflectron ($V_1 = 0$), dT/dK_2 becomes zero, resulting in time focusing for all the fragment ions. This is not achieved for a linear reflectron unless $d_1 + d_2 \ll d_r$ which is not a desired condition as has been mentioned earlier.

Now, let us consider the kinetic aspect of detecting daughter ions. A daughter ion generated before entering the reflectron will be detected as such unless it undergoes further dissociation inside the reflectron while the one generated inside the reflectron will contribute to the chemical noise. A daughter ion formed after exiting the reflectron has the same time of flight as the parent ion and will not contribute to the daughter ion signal. Taking t_1 , t_r , and t_2 as the times spent before, inside, and after the reflectron, respectively, it is required to make t_1 as long as possible compared to $t_r + t_2$, or make d_1 as long as possible compared to $d_r + d_2$, to enhance the daughter ion intensity while suppressing the chemical background. d_2 can be set nearly to zero by installing the detector very close to the reflectron entrance. The d_1/d_r ratio can not be made very large because of the deterioration of the daughter ion resolution as can be seen from eq 17. One may judge from eq 17 that the daughter ion resolution would be improved by keeping the linear component small. Then, the situation becomes comparable to the parabolic case and $(d_1 + d_2)/d_r$ decreases. Namely, a good signal-to-noise (S/N) ratio and a good resolution for daughter ion

Figure 2. A diagram of the MALDI-TOF-PD-TOF instrument built in this work. See text for details.

signals cannot be achieved simultaneously. Either a compromise must be made or one must make the second analyzer very long. Such a compromise is not needed for PSD because the dissociation occurring before the ion gate (the first focus) can contribute to the daughter ion signals also.

Finally, it is to be mentioned that we also investigated the situation when other terms were added to the potential such as the square root and cubic components through SIMION calculations. The situation hardly improved, however, suggesting that LPQ may be close to the optimum.

Instrument Design

The schematics of the instrument optimized as of now is shown in Figure 2. A center-holed MCP (40 mm o.d., Burle, Lancaster, PA) is installed at 10 mm from the reflectron entrance. A stainless steel tube with 5 mm o.d. (4 mm i.d.) and 23 mm in length is inserted into the MCP center hole almost all the way to the reflectron entrance. Both the tube and the reflectron entrance are grounded such that the voltage applied to the MCP does not affect the incoming ions. d_1 and d_r were determined from the kinetic consideration. 530 and 264 mm were chosen for d_1 and d_r , respectively. When the parent ion kinetic energy is 14.4 keV and 17 kV is applied to the last electrode of the reflectron, which is the typical operating condition, an ion with m/z 1000 spends 10.069 μ s between the first focus and the reflectron entrance and 16.962 μ s inside the reflectron. Inserting d_1 , d_2 , d_r , V_r and K into eqs 9 and 14 and solving, 13.56 and 3.44 kV are obtained for V_1 and V_2 , respectively. Then c_1 and c_2 are determined by eqs 10 and 11, respectively, which specifies the electric field inside the

Figure 3. Entrance electrode of the reflectron (a) before and (b) after the modification. Grids with 90 and 95% transmissions are shown as dashed and dotted lines, respectively. Figures in the bottom are the top views of the electrode.

reflectron through eq 8. The reflectron consists of 24 ring electrodes (50 mm i.d., 100 mm o.d., 1 mm thickness, separated by 10 mm). Two or three resistors with accurately known resistances connect adjacent electrodes such as to produce the desired potential on the reflectron axis.

The MALDI source consists of acceleration electrode assembly, einzel lens, and deflection plates. The acceleration electrode assembly consists of the sample plate (S), an intermediate ring electrode (E_1), and the ground plate (G). The distance between the source ground plate (G) and the first time focus is 640 mm. An ion gate consisting of two deflection plates (40 mm $\times$ 10 mm) separated by 5 mm and two ground ring electrodes is installed immediately before the first focus. An MCP (18 mm o.d., Burle, Lancaster, PA) mounted on a linear drive is installed immediately after the first focus.

The first LPQ reflectron we built had a circular grid mounted on the entrance electrode to ensure the ground potential at this position and the symmetry axis of the reflectron coincided with the instrumental axis. We found through SIMION calculation that many ions reflected by the reflectron passed through the center hole of the second MCP and were not detected. We avoided this problem by tilting the reflectron 0.9° with respect to the instrumental axis. We also found, through experiments, that scattering and sputtering by ions and neutrals hitting the grid generated significant chemical noise. Hence, the entrance electrode was redesigned as shown in Figure 3. Grid is not present on the incoming ion trajectory in this design while it is still present on the reflected ion trajectory. The latter is needed to block the MCP field such that the incoming ion is not affected by it. We had also installed a grid with 90% transmission at the final electrode of the reflectron. It was found that scattering and sputtering at this grid generated some chemical noise also. We tested with a grid with 95% transmission and with a completely gridless de-

sign. Since the results were comparable, we chose the former. Then, V_1 and V_2 were reoptimized through SIMION calculations. Even though exact time focusing was not possible with the above modifications, a peak width less than 1 ns could be achieved for parent ions in the m/z 500–2000 range. V_1 and V_2 thus determined were 13.74 and 3.26 kV, respectively. Hence, the potential to be applied on the last electrode of the reflectron was 17.00 kV and the calculated c_1 and c_2 were 52,040 V m⁻¹ and 46,770 V m⁻², respectively. These results could not be directly used to determine the resistances between the adjacent electrodes because the potential at the center of each electrode was different from that on the electrode. Iterative SIMION calculation was performed to determine the resistances which resulted in the desired potential in the reflectron axis.

We also calculated the resolving power of the instrument for the analysis of the daughter ions generated from the parent ions excited at the first focus. When the kinetic energy release in the dissociation was ignored, the resolving power for the daughter ion with an m/z half that of the parent, say $1000^+ \rightarrow 500^+$ case, was 800. With the kinetic energy release of 0.1 eV, this decreased to 500, barely enough for a unit mass resolution.

Experimental

A pulsed nitrogen laser (VSL-337ND-S, Laser Science, Franklin, MA) with 300 μ J per pulse at 337.1 nm and <4 ns pulse width is used for MALDI. Its incident angle is 60° to the sample surface normal. The laser is focused to the spot size of ~ 20 μ m. Laser power is adjusted with a neutral density filter at 10% above the MALDI threshold. Sample is loaded on a 8 mm $\times$ 46 mm stainless steel plate and introduced to the source via a homemade vacuum lock. A 20 kV high voltage power supply (603C-200P, Bertan, Hicksville, NY) provides DC voltage (~ 13 kV) to the S and E₁ electrodes. The same type of the power supply is used for the reflectron (17 kV). A high voltage pulser (PVX-4140, Directed Energy, Fort Collins, CO) switches the high voltage from a supply (PS350, SRS, Sunnyvale, CA) and provides an extraction pulse (~ 1.4 kV) to the source plate (S) via AC-coupling. The same types of power supplies and switches are used throughout except for the MCPs and the final deflector located before the second MCP. Power supplies with lower ripple (PMT30C-N, Bertan, Hicksville, NY) are used for the latter. The MCP output is amplified by 100 with a preamplifier (9306, EG&G ORTEC, Oak Ridge, TN) and is recorded by a digital storage oscilloscope (LT372, LeCroy, Chestnut Ridge, NY) with 1 G sample/s sampling and 500 MHz bandwidth. The fourth harmonic, 266 nm, of an Nd:YAG laser (Surelite III-10, Continuum, Santa Clara, CA) with 5 ns pulse width was used as the photodissociation laser. Its maximum output is 100 mJ/pulse and the pulse-to-pulse jitter is ± 0.5 ns with respect to an external trigger. The laser was focused with a cylindrical lens to 15 μ m width at the first time focusing position. Its height of 7

Figure 4. Pulsing chronograph for the PD experiment. ΔT_1 is the time delay for the delayed extraction. The parent ion is selected by its time of flight to the ion gate, ΔT_2 . The PD laser is pulsed immediately after the ion gate is opened.

mm perpendicular to the ion beam direction allowed complete overlap between the laser and ion beams.

The overall pulsing chronograph is shown in Figure 4. N₂ laser is for MALDI, source pulsing is for delayed extraction, and the ion gate is for the selection of a parent ion to be photodissociated. The PD laser was fired a short time after the ion gate was opened. This timing sequence was controlled by three delay generators (DG535, SRS, Sunnyvale, CA) as shown in Figure 4. A delay generator triggers the N₂ laser, the extraction pulse, and the digital storage oscilloscope. Another DG535 controls triggering of the deflection voltage in the ion gate for the parent ion selection. A third DG535 is needed to trigger the photodissociation laser. Spectra were recorded with and without the PD laser alternately and averaged for 10–25 PD laser shots. The results were transferred to a personal computer via GPIB and processed further. A spectrum recorded without the PD laser consists of the parent ions transmitted by the ion gate and their PSD products. Its variation by the PD laser appears in the laser-on spectrum. Subtraction of the former from the latter will result in the laser-induced spectral change only. Alternate acquisition was to minimize the influence of the spot dependence in MALDI on the subtracted spectrum.

Samples

Peptide samples, angiotensin I and II, bradykinin and its fragments, and the matrix, α -cyano-4-hydroxy cinnamic acid (CHCA), were purchased from Sigma (St. Louis, MO) and used without further purification. Matrix solution was prepared daily using acetonitrile and 0.1% trifluoroacetic acid, which was mixed with the peptide solution. The final peptide concentration prepared for the PD experiments was ~ 100 pmol/ μ l. 1 μ l of the solution was loaded at the center of the sample plate.

Figure 5. MALDI spectrum of a mixture of angiotensin I and II, bradykinin, and its fragments. $[M + H]^+$ from angiotensin II and $[M - OH]^+$ from CHCA were used for mass calibration.

Results and Discussion

Figure 5 shows the ordinary MALDI spectrum of a peptide mixture obtained with the present instrument averaged over 100 N_2 laser shots. The instrument was tuned with the $[M + H]^+$ ion from angiotensin II. The optimum tuning condition depended on the m/z chosen, even though only slightly. The peak widths were 4.5 ~ 5 ns at the optimum regardless of m/z . These correspond to the resolving power ($m/\Delta m \sim t/2\Delta t$) of ~5000 for $[M + H]^+$ from angiotensin II.

The following well-known equation has been found to be adequate for the mass calibration of the ordinary MALDI spectra recorded with the present instrument.

$$T = am^{1/2} + b \quad (18)$$

$[M + H]^+$ from angiotensin II and $[M - OH]^+$ from CHCA were used to mass-calibrate the spectrum in Figure 5. The simple scheme adopted provided mass accuracy better than 0.1 Da.

The PSD spectrum of $[M + H]^+$ from angiotensin II selected by the ion gate is shown in Figure 6. The spectrum is in excellent agreement with that in a previous report [37]. The homemade ion gate had a resolution of 10 ~ 20 Da. Since isotope selection was not possible, isotopic clusters appeared for all the daughter ions. It can be seen that the resolution gets poorer as the daughter ion m/z decreases. In particular, unit mass resolution has not been achieved at half the m/z of the parent ion. Poorer resolution than expected may be due to the fact that metastable ion decomposition occurs

over the entire field-free region, between the source exit plate (G) and the reflectron entrance, and that the kinetic energy release affects lighter daughter ions more. It is to be mentioned that light daughter ions can be better resolved once the reflectron voltage is lowered. We also recorded PSD spectra with a parabolic reflectron and obtained excellent resolution for light daughter ions. We do not favor this design, however, for reasons explained already. Namely, even though lengthening of d_1 results in poor resolution for light daughter ions, it is unavoidable for a PD mass spectrometer of this design.

Since the kinetic energy of a daughter ion depends on its m/z , the simple relation in eq 18 can not be used to mass calibrate a PSD spectrum. Hence, we had to use eq 8 or only its second term, because the time of flight in the field-free region is constant. For daughter ions, this term can be written as

$$t_r = (2m_2/ec_2)^{1/2}[\pi/2 - \sin^{-1}(1 + 4c_2K_1m_2/ec_1^2m_1)^{-1/2}]. \quad (19)$$

Even though c_1 and c_2 are instrumental parameters determined by the resistances used in the reflectron, we took them as variables and adjusted them to fit some known PSD peaks from reference compounds. For example, three prominent peaks in the PSD spectrum of $[M + H]^+$ from angiotensin I were used to obtain the parameters, which were used to mass-calibrate the PSD spectrum of $[M + H]^+$ from angiotensin II. The results

Figure 6. PSD spectrum of $[M + H]^+$ from angiotensin II.

for some prominent PSD peaks are listed in Table 1. Mass accuracy better than 0.4 Da could be achieved with this simple scheme.

Parent Ion Selection in Photodissociation

We mentioned that the ion gate installed in the instrument had the parent ion mass selectivity of around ± 10 Da. Namely, a monoisotopic selection of a parent ion is not possible with the ion gate. We will first show that this can be achieved in PD, even though for relatively small parent ions only. The molecular ion region of the ordinary MALDI spectrum of angiotensin II recorded using the first microchannel plate (MCP) detector is shown in Figure 7. Assuming that the time of flight t_0 to the first MCP is proportional to $m^{1/2}$, the difference in the time of flight between m^+ and $(m + 1)^+$ is $\Delta t = (1/2 m)t_0 \propto m^{-1/2}$. Such a calculation for $[M + H]^+$ from angiotensin II, m/z 1046.54, results in 7.0 ns which is rather close to the 6.8 ns isotopic difference observed in

Figure 7. As far as the isotopic peaks are time-separated as in this case, it is not the width of each peak but that of the PD laser that is important for the laser selection of a single isotope. Then, the 5 ns pulse width of the Nd:YAG laser used in this work allows isotope-selective excitation of $[M + H]^+$ from angiotensin II. Let us assume that the laser isotope selection is possible until

Figure 7. Mass spectrum in the molecular ion region of angiotensin II detected by the 1st MCP.

Table 1. Mass calibration for the PSD spectrum of $[M + H]^+$ from angiotensin II

Ions	Calculated mass ^a (Da)	Theoretical mass (Da)	Calibration error (Da)
$[M + H]^+$	—	1046.54	—
y_3	931.67	931.52	0.15
b_7+18	899.65	899.47	0.18
b_6	784.74	784.41	0.33
a_6	756.68	756.42	0.26
b_5	647.54	647.35	0.19

^aBy eq 19.

Figure 8. Photodepletion of $[M + H]^+$ from angiotensin II obtained by synchronizing the PD laser pulse with (a) A and (b) A + 1 peaks. The PD laser in (b) was pulsed 7 ns after (a). Dotted and solid curves mean laser-off and laser-on spectra, respectively.

the isotopic time-of-flight difference narrows down to the laser pulse width, 5 ns in this case. Then the fact that Δt is proportional to $m^{-1/2}$ suggests that the isotope selection is possible up to 2000 Da. Lengthening the first field-free region by a factor of two, from 64 to 128 cm, would increase the laser mass selection limit by a factor of 4, to 8000 Da.

Photodissociation Spectrum

Since the ion beam-laser beam intersection was made to occur at the position of the first MCP, the time of flight in Figure 7 could be used to set the PD laser irradiation time. The molecular ion region of the MALDI spectrum of angiotensin II recorded by using the second MCP is shown in Figure 8. Here, dotted and solid curves show the spectra recorded without and with the PD laser, respectively. The top PD trace was recorded with the PD laser synchronized with $[M + H]^+$ consisting of the lowest mass isotopes, the A peak according to the McLafferty's terminology [38], while the bottom trace synchronized with the (A + 1) peak. Laser isotope

selection is amply demonstrated in these spectra. These spectra, which show 23% depletion of each parent ion, were recorded with the PD laser energy of 5 mJ/pulse. Nearly complete depletion of the parent ion was possible when the laser pulse energy was increased, say to over 50 mJ/pulse.

Since the velocity of a daughter ion produced in the field-free region is the same as that of the parent ion it is generated from, the former can not be separated from the latter by the ion gate. In addition, some of the parent ions may dissociate after passing the ion gate even in the absence of the PD laser. The PSD spectrum of the $[M + H]^+$ ion from angiotensin II in Figure 6 is reproduced in Figure 9a for ease of explanation. A few peaks in the spectrum magnified along the mass coordinate are shown as insets in the figure to demonstrate that the peaks appear as isotopic clusters. The tandem mass spectrum of the same parent ion obtained with 5 mJ/pulse of the PD laser is shown in Figure 9b. The laser was synchronized with the A peak of the parent ion in this case. Laser-induced changes from the PSD spectrum can be noticed in this spectrum. For example, the peak at m/z 931.5 is weaker than in the PSD spectrum while some new peaks such as at m/z 910.4 appear. A better way to observe the photo-induced changes is to subtract the PSD spectrum from the present tandem mass spectrum. The result, which will be called the PD spectrum, is shown in Figure 9c. The spectrum consists of both positive-going, or positive, peaks and negative peaks. A negative peak is formed when the daughter ion generated by PSD is depleted more than is formed by PD of the parent ion or other PSD ions with higher m/z . Similarly, a positive peak will result when the situation is the other way around. Even though a photoproduct ion can be generated either by PD of the parent ion or by that of higher mass PSD ions, the former is much more likely because the intensity of the parent ion is much higher than those of PSD ions. Hence, one may loosely say that a negative peak is due to PD of a PSD ion and that a positive peak corresponds to a daughter ion generated by PD of the parent ion. It is apparent from the spectra that the intensities of PD signals can be made almost comparable to those of PSD signals with an appropriate instrumental design and by selection of a good experimental condition. Namely, PD does not have to be considered as an exotic technique useful for some fundamental research but not for analytical application. We could generate PD spectra with the quality comparable to Figure 9c for other prototype peptides such as angiotensin I, bradykinin and its fragments, etc.

Since the ion beam-laser beam synchronization allows isotopic selection of the parent ion, it is possible to record the isotope-selected PD spectra. The most useful in this regard is to record the PD spectrum of the A type parent ion because the A + 1, A + 2, ... parent ions will generate daughter ions consisting of more than one isotopic peak. It is to be noted that each peak in Figure 9c looks sharper than the corresponding peak in Figure

Figure 9. Daughter ion spectra for the A peak of $[M + H]^+$ from angiotensin II. (a) PSD. (b) PSD with PD laser on. (c) Pure PD spectrum obtained by subtracting (a) from (b). The PSD spectrum consists of isotopic clusters while the PD spectrum consists of monoisotopic peaks as shown in the insets.

9a. For example, the resolving power for the peak at m/z 600 in the former is 600. Monoisotopic nature of PD peaks can be better seen in the x-magnified insets shown in the figure. Not only the positive peaks but also the negative ones display the monoisotopic characteristics as they should.

We also checked the influence of the PD laser power on the PD spectrum of $[M + H]^+$ from angiotensin II.

The PD spectrum improved monotonically up to the laser pulse energy of ~ 5 mJ. Increase of the pulse energy above this value began to show changes in the spectral pattern. The PD spectra recorded with the pulse energy of 5, 20, and 40 mJ are compared in Figure 10. It is readily seen that the intensities of the negative signals in the high m/z range increase with the laser pulse energy while those of the positive signals de-

Figure 10. The PD spectra obtained with 5, 20, and 40 mJ/pulse of the PD laser.

crease. At the highest pulse energy used, 40 mJ/pulse, the positive signals at high mass range become very small or even become negative such as the peak at m/z 910.4 while those at the very low mass range increase even though not shown here. Namely, as was mentioned in the theoretical section, pumping too many photons into the parent ion leads to its extensive fragmentation, resulting in less informative spectral data.

Due to some instrumental deficiencies, we have not attempted to photodissociate very high mass, such as m/z 5000 Da, biopolymer ions yet. Based on the theoretical considerations presented above, we have reasons to believe that sufficient number of photons can be supplied with commercial pulsed lasers to enable their dissociation. Taking polypeptides as examples, there are many labile bonds with similar critical energy for dissociation. As the molecular weight increases, the number of the competing channels increases, leading to poor signal intensity for each channel. This may be one of the difficulties in the tandem mass spectrometry of very large biopolymer ions relying on the statistical reaction dynamics. We mentioned earlier that one of our aims in studying photodissociation was to search for the possibility to induce site-specific or chromophore-specific reactions. Reduction in the number of channels in such cases may also help to improve the signal quality in the high mass tandem mass spectrometry.

Elimination of Some Background Noises

The most time-consuming step in our effort to build a MALDI-TOF-PD-TOF instrument that can generate decent PD spectra was to eliminate various noises and hence enhance the spectral quality. In our initial attempt, we installed a parabolic reflectron a few cm after the second MCP and an ion gate immediately before it. The PD laser was introduced between the MCP and the reflectron entrance. A PD tandem mass spectrum recorded with this instrument is shown in Figure 11. The strong background in the left is due to the ion gate pulsing. The strong signals in the far right are the parent ions passing the ion gate. Most of the signals in-between are due to PSD except for the strong daughter ion signal in the center-left. This was due to a very small m/z daughter ion generated with excessive PD laser intensity. The broad background spanning the full spectral range, it turned out, was due to the ions generated from the residual gas through multiphoton ionization. The ion beam–laser beam intersection point had to be moved far behind the MCP surface to eliminate this background. This was one of the main reasons why we abandoned the parabolic reflectron. Employing the LPQ reflectron instead and eliminating this main noise, a decent PD signal began to emerge, which improved as the distance between the first time focus point and reflectron entrance was systematically increased and the PD laser power was lowered. Finally,

Figure 11. Tandem mass spectra of $[M + H]^+$ from des-arg-bradykinin in the presence of the PD laser pulse recorded with (a) parabolic and (b) LPQ reflectrons. Very intense noise in the far left is due to the ion gate pulsing and laser discharge. The broad background appearing over the entire spectral range in (a) is due to the multiphoton ionization of the residual gas.

we found that a grid installed at the entrance of the reflectron generated a significant amount of noise throughout the spectrum. We eliminated this noise by using a gridless reflectron and partially blocked MCP.

Conclusions

PD spectra of peptide ions obtained with TOF mass spectrometers were reported from Reilly's and Russell's laboratories [17, 18]. The PD results from the former were preliminary [18] and details have not been presented yet. The best results by Russell and coworkers [17] were obtained by suppressing PSD signals almost entirely with a proper choice of the matrix material and the MALDI condition. In comparison, PD spectra with decent quality were obtained in this work without taking such a painstaking measure, which was possible through instrumental optimization and suppression of various noises. Finally, monoisotopic selection of the parent ion has been achieved for the first time in this work.

So far, description of the present work has been focused on the instrumental aspect and acquisition of high quality PD spectra. Characteristics of the PD spectra and their utility as an analytical tool have not been presented yet. These are the ongoing research subjects in this laboratory.

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