Insights on mechanisms for the generation of gas-phase peptide ions and their dissociation in matrix-assisted laser desorption ionization (MALDI) gained from the kinetic and ion yield studies are presented. Even though the time-resolved photodissociation technique was initially used to determine the dissociation kinetics of peptide ions and their effective temperature, it was replaced by a simpler method utilizing dissociation yields from in-source decay (ISD) and post-source decay (PSD). The ion yields for a matrix and a peptide were measured by repeatedly irradiating a region on a sample and collecting ion signals until the sample in the region was completely depleted. Matrix- and peptide-derived gas-phase cations were found to be generated by pre-formed ion emission or by ion-pair emission followed by anion loss, but not by laser-induced ionization. The total number of ions, that is, matrix plus peptide, was found to be equal to the number of ions emitted from a pure matrix. A matrix plume was found to cool as it expanded, from around 800–1,000 K to 400–500 K. Dissociation of peptide ions along b/y channels was found to occur statistically, that is, following RRKM behavior. Small critical energy ($E_0 = 0.6–0.7$ eV) and highly negative critical entropy ($S^\text{c} = -30$ to $-25$ eu) suggested that the transition structure was stabilized by multiple intramolecular interactions. © 2014 Wiley Periodicals, Inc. Mass Spec Rev 34:94–115, 2015

**Keywords:** MALDI; peptide ion formation; peptide ion dissociation; expansion cooling; thermal model; ion yield

**I. INTRODUCTION**

Matrix-assisted laser desorption ionization (MALDI) (Hillenkamp & Karas, 2007) has been widely used for the analysis of biomolecules. Inspired by its spectacular success in generating gas-phase ions from condensed-phase biomolecules, there have been considerable efforts to elucidate the characteristics of the technique. At the moment, an expert in the field can figure out what to do to get a decent MALDI spectrum from his sample. However, it is fair to say that he will devise an analytical approach not based on firm knowledge on what is going on in MALDI but based on phenomenological rules compiled over the years.

Investigation of MALDI in this laboratory started from curiosity about how large molecules such as peptide ions would react (dissociate). The research evolved into the study of the dissociation kinetics and dynamics of peptide ions and eventually into the investigation of how gas-phase peptide ions were produced in MALDI. Excellent review articles on the formation and fragmentation of gas-phase peptide ions in MALDI are available (Zenobi & Knochenmuss, 1998; Knochenmuss & Zenobi, 2003; Dreisewerd, 2003; Knochenmuss, 2006). Instead of re-reviewing the materials that have been reviewed already, we will focus on the insights on the same subjects gained from the ion yield (IY) and kinetic measurements. Measurement of the ionization efficiency (or ion yield) and determination of the dissociation kinetics for molecular ions are standard ways of learning about an ionization technique. Even though thousands of papers on MALDI have been published, it is surprising to find that only several of them dealt with the ion yield and the dissociation kinetics (Laskin, 2006; Moon et al., 2009). For convenience, we will briefly review some general concepts on the formation and fragmentation of peptide ions in MALDI in this chapter. The papers that dealt with the main subjects of the present review article will be reviewed in subsequent chapters.

In our own research, we dealt with the kinetics and dynamics of peptide ion fragmentation first. The results forced us to investigate the processes occurring in the MALDI plume and eventually the processes involved in the gas-phase ion formation. Here, we will follow the same bottom-up approach for ease of explanation. This review will be limited to UV-MALDI performed in the vacuum.

**A. Peptide Ion Formation**

An excellent review on the ion formation process in MALDI is available (Knochenmuss, 2006). The review is still useful and valid because further studies reported since 2006 have been made mostly to elaborate or extend the models described in the review. Even though a general consensus was not reached on the ion formation mechanism in MALDI, Knochenmuss noted that there was a consensus in one important aspect, that is, the ions appearing in a spectrum are generated via two steps. There are different opinions on the formation of primary ions in the first step. However, it is increasingly accepted that the secondary ions are formed from the primary ions in the second
step via ion-molecule reactions occurring in the MALDI plume and that conventional kinetic and thermodynamic treatments are applicable to the second step.

Generation of the primary ions is the most controversial part of the process. The models are divided into two groups, depending on whether the excited electronic state of the matrix is crucially involved in the matrix ion generation or not. In the models belonging to the second group, the electronic excitation of matrix molecules by laser is simply a way of supplying thermal energy needed for the ablation of a sample. This will be the case when the electronic-to-thermal energy conversion occurs efficiently. The lucky survivor (LS) model, or the cluster ion model, developed by Karas (Karas, Glückmann, & Schäfer, 2000; Karas & Krüger, 2003; Jaskolla & Karas, 2011) belongs to this group. Even though the details of the LS model have changed somewhat over the years, its main assumption remains the same. That is, a peptide is present in its protonated form, or a pre-formed ion form, inside a solid matrix-analyte mixture (sample) and desorbs in the form AH⁺ or as clusters consisting of positive ions (AH⁺+), matrix neutrals, and negative counter-ions. In addition to the intact AH⁺ ions, positively charged clusters contribute to the analyte ion (AH⁺⁻) signal through neutral loss after proton-transfer neutralization of counter-ions. The singly charged analyte ions surviving this process are called the LSs of the neutralization.

The models belonging to the first group must have been inspired by the fact that the irradiation of a molecule with a UV laser pulse can induce multitudes of excited state chemistry. In particular, rapid increase in ion yield with the laser fluence was small (only around 1 J per pulse according to our own estimation, Bae et al., 2012). It was found that certain complexes of peptides and matrices had reduced ionization energies and could be ionized via two-photon absorption (Zenobi & Knochenmuss, 1998). However, this is not important because the matrix-to-analyte ratio is around 1,000 or larger in typical MALDI studies. Hence, the possibility of direct multi-photon ionization of matrices used in MALDI (Zenobi & Knochenmuss, 1998). The probability for such a process would be very low especially because the laser energy used in MALDI was small (only around 1 µJ per pulse according to our own estimation, Bae et al., 2012). It was found that certain complexes of peptides and matrices had reduced ionization energies and could be ionized via two-photon absorption (Zenobi & Knochenmuss, 1998). However, this is not important because the matrix-to-analyte ratio is around 1,000 or larger in typical MALDI studies. Hence, the possibility of direct multi-photon ionization of matrices has been discarded. Two alternative models have been proposed, that is, the excited state proton transfer (ESPT) model and the exciton pooling model. ESPT is attractive because it is a one-photon event. In general, however, ESPT molecules were found unsuccessful as MALDI matrices possibly because the environment needed for efficient ESPT did not necessarily match that for MALDI (Zenobi & Knochenmuss, 1998). Pooling is a phenomenon in which the electronic excitation energies of nearby molecules are redistributed (Knochenmuss, 2006). Mobile excitations can be treated as pseudo-particles called excitons. Pooling of three or more excitons in a matrix molecule will induce its ionization. Exciton migration and pooling was demonstrated for the matrix 2,5-dihydroxybenzoic acid (DHB). In the gas-phase proton transfer (GPPT) model by Knochenmuss and coworkers (Zenobi & Knochenmuss, 1998; Knochenmuss et al., 2000; Breuker et al., 2003; Knochenmuss & Zenobi, 2003; Knochenmuss, 2006), three assumptions were adopted. First, a peptide was emitted from the sample as a neutral molecule. Second, a matrix was ionized via exciton pooling. Third, an analyte ion (AH⁺⁻) was formed via proton transfer from a matrix-derived ion to the neutral analyte (peptide). One thing that is unclear about this model is what exactly is the identity of this proton-donating matrix-derived ion. One might expect the matrix molecular ion (M⁺) as the candidate because it will be the product of the exciton pooling ionization. Intuitively, however, the protonated matrix MH⁺ generally observed in MALDI spectra would be a better candidate for the proton donor. In recent papers by Knochenmuss and Zhigilei (2010, 2012), some details of the ion formation in MALDI were investigated with molecular dynamics simulation. In all these works, DHB was adopted as the matrix. Extension to other matrix molecules is needed to check the general validity of the model. Recently, Jaskolla and Karas (2011) investigated MALDI of some molecules using deuterated and undeuterated tert-butyl ester of α-cyano-4-hydroxycinnamic acids as matrices. Even though protonated or deuterated analytes would dominate under the LS and GPPT models, respectively, both were observed. This was interpreted as evidence that both models contribute to the ion formation, with the LS model becoming more important with higher analyte basicity, lower solvent pH, and weaker laser fluence.

B. Peptide Ion Dissociation

Tandem mass spectrometry (McLafferty, 1983) has been widely used for the identification and sequence determination of peptides and proteins (Mann, Hendrickson, & Pandey, 2001; Kinter & Sherman, 2005; Hernandez, Muller, & Appel, 2006). Tandem mass spectrometry for peptide ions was practiced even before the invention of MALDI, that is, by using fast atom bombardment (FAB) as the ionization technique. Product ions generated by collisionally activated dissociation (CAD) (Cook, 1978; McLafferty, 1983; Martin & Biemann, 1990; Laskin & Futrell, 2003; Medzihradszky, 2005) were recorded. Subsequently, MALDI and electrospray ionization (ESI) (Kebarle & Ho, 1997) replaced FAB as the ionization techniques. Investigation of tandem mass spectra for singly protonated peptides—they will be called peptide ions in this work—recorded by utilizing CAD and other activation techniques revealed that the energy regime and the presence/absence of an arginine residue in a peptide ion were the most important factors determining the types and the relative abundances of product ions appearing in tandem mass spectra. Depending on the kinetic energy of a precursor ion, CAD is divided into two categories, low (around 100 eV) and high (higher than 1 keV) energy regimes. In the low energy CAD (Medzihradszky, 2005), a peptide ion gains rather small amount of internal energy through vibrational excitation. In this low energy regime, b and y type ions—see Roepstorff and Fohlman (1984) and Johnson, Martin, and Biemann (1988) for the product ion notation—formed via amide bond (CO–NH) cleavages are the major product ions regardless of the presence of arginine residue. a Type ions formed by the loss of CO from b type ions are another important sequence ions in these spectra.

Collisionally activated dissociation performed with keV kinetic energy belongs to the high-energy regime because the
accessibility of electronic excitations allows large kinetic-to-
internal energy conversion. Regardless, product ions formed are
similar to those in the low energy regime when the arginine
residue is absent. In addition to arginine (R), aspartic acid (D),
glutamic acid (E), and proline (P) on the fragmentation of peptide ions.

Other tandem mass spectrometric techniques include elec-
tron capture dissociation (ECD) (Zubarev, Kelleher, &
McLafferty, 1998; Cooper, Häkansson, & Marshall, 2005),
electron transfer dissociation (ETD) (Syka et al., 2004), surface-
induced dissociation (SID) (Mabud, Dekrey, & Cooks, 1985;
Laskin & Furtrell, 2003), and photodissociation (PD) (Bowers
et al., 1984; Martin et al., 1990; Williams, Furlong, &
McLafferty, 1990; Barbacci & Russell, 1999; Oh, Moon, &
Kim, 2004; Thompson, Cui, & Reilly, 2004; Choi et al., 2006).
ECD and ETD deal with the reactions of radical cations and will
not be included in this review. In SID, collision of a precursor
ion with a surface induces its vibrational excitation. Vibrational
excitation is also utilized in infrared multi-photon dissociation
(IRMPD) (Little et al., 1994; Laskin & Furtrell, 2005). Hence,
the spectral features in SID and IRMPD of peptide ions resemble
those in low energy CAD. PD by ultraviolet (UV) or vacuum
ultraviolet (VUV) light, or UV-PD, has been widely used to
study the structure and dissociation dynamics of small poly-
amomic ions (Dunbar, 1979; Choe & Kim, 1991; Dunbar, 2000;
Park, Kim, & Kim, 2002). UV-PD of peptide ions (Barbacci &
Russell, 1999; Thompson, Cui, & Reilly, 2004; Cui, Thompson,
& Reilly, 2005; Moon, Yoon, & Kim, 2005a) has been
investigated recently. Since the technique involves an electronic excitation(s), the tandem mass spectra thus obtained resemble
those of high energy CAD. This will be dealt with in the next
chapter.

Since the present review deals with MALDI of peptides, a
brief description of the tandem mass spectrometric techniques in
MALDI-tandem TOF will be given. One of the first tandem
mass spectrometric techniques used in MALDI-tandem TOF
was the post-source decay (PSD) (Kaufmann, Kirsch, &
Spengler, 1994). Here a precursor ion is selected by the first
stage TOF and its spontaneous dissociation products are
analyzed by the second stage TOF that is equipped with a
electroreflector. Since the driving force for PSD is the internal energy
retained by the precursor when it emerges from the ion source,
PSD is essentially the same as the metastable ion decomposition
(MID) (Cooks et al., 1973). By introducing collision gas to a cell
located at the first time focus, high energy CAD can be
performed. Also, PD can be performed by irradiating the ion
packet with a laser at the same position. As in other techniques
such as electron ionization mass spectrometry (EI-MS), a
precursor ion can dissociate inside the ion source (in-source
decay, ISD)—strictly speaking, ISD is not a tandem mass spectrometric technique. The fact that ISD is not a prompt
process but involves metastable ion decay was established by
Brown, Carr, and Lennon (1996). Until recently, ISD has not received much attention possibly because a MALDI spectrum
shows not only a precursor ion and its ISD products but also the
ions derived from the matrix. Recently, ISD is receiving further
attention because ISD of large peptides with basic residue(s)
generates “c” and “z” ions as in ECD (Brown & Lennon, 1995;
Takayama, 2001a,b; Köcher, Engström, & Zubarev, 2005). In
ISD of small peptide ions investigated in our laboratory, however, mostly b, y, and their consecutive reaction products
were observed and “c” and “z” type products were weak. That is,
the product ions formed by ISD and PSD were virtually the
same in such cases (Bae, Moon, & Kim, 2011; Yoon, Moon, &
Kim, 2011). Then, the widely different time scales for the occurrence of ISD and PSD—(100 ns or less for ISD vs. around
10 μs for PSD)—make them useful techniques to study the
dissociation kinetics of peptide ions. In most of the fundamental
studies on MALDI of peptides carried out so far, the decrease of
a precursor ion signal by its ISD and PSD and PSD of ISD
products have not been taken into account. Ignoring them often
resulted in serious errors such as in the determination of ion
yield and dissociation rate constant.

There have been extensive investigations on the formation of
b and y type ions. Oxazolone pathways (Polce, Ren, &
Wesdemiotis, 2000; Paizs & Suhai, 2002, 2005; Aribi et al.,
2003) have been proposed to explain their formation. In this
model, the ionizing proton migrates to an amide nitrogen, the
rate-determining cleavage of the protonated amide bond
occurs via a 5-membered ring transition structure, and a
proton-bound dimer of an oxazolone derivative and a smaller
peptide is formed. Upon the break-up of this dimer, b or y
type ion is formed depending on whether the charge is
retained in the oxazolone derivative or in the smaller peptide,
respectively. Additional reactions occurring in the high-energy
regime for peptide ions with arginine were investigated by
Biemann and coworkers (Johnson, Martin, & Biemann, 1988)
and by Reilly and coworkers (Cui, Thompson, & Reilly,
2005). Here, the homolytic cleavage of a Cα—CO bond is
thought to generate a + 1 or x + 1 radical cation depending on
whether the charge is retained by N- or C-terminal moiety,
respectively. The a + 1 further dissociates to a and d ions
while x + 1 to v, w, and x.

II. PHOTODISSOCIATION OF PEPTIDE IONS

As mentioned in the section B of the previous chapter,
photodissociation in ultraviolet (UV-PD or simply PD) has been
utilized mostly as a method to study the structure and dissociation
dynamics of small polyatomic ions. Kim and coworkers
(Oh, Moon, & Kim, 2004) noted several advantages of PD over
CAD in MALDI-tandem TOF mass spectrometry. The most
obvious is that collision gas does not have to be introduced to a
mass spectrometer. The second is that photo-excitation would
not affect the dynamical properties of an ion beam, which can be
useful for high-resolution tandem TOF. The third is the monoisotopic selection of a precursor ion through synchroniza-
tion of the PD laser and ion beam pulses. For fundamental
studies, the most important advantage is that a monochromatic
laser allows chromophore-specific, or site-specific, excitation. This can be useful for the study of the dynamics of dissociation such as whether the dissociation of a peptide ion would proceed site-specifically or statistically.

The fact that peptide ions could be photodissociated with pulsed UV lasers was demonstrated by several laboratories. This includes the 193 nm PD study by McIver and coworkers (Lebrilla et al., 1989) and by Biemann and coworkers (Martin et al., 1990) for peptide ions generated by FAB. Similar study was made by McLafferty and coworkers (Williams, Furlong, & McLafferty, 1990) for ions generated by laser desorption. Then, PD of peptide ions generated by MALDI followed. Russell and coworkers (Gimon-Kinsel et al., 1995) reported 193 nm PD of protein ions generated by MALDI using a two-stage linear TOF instrument. Reilly and coworkers (Thompson, Cui, & Reilly, 2003, 2004) reported tandem mass spectra for some peptide ions obtained by PD at 157 and 193 nm using a homebuilt MALDI-tandem TOF with a lift cell. They reported that the quality of the PD tandem mass spectra was far better at 157 nm than at 193 nm. In subsequent studies by the same group, 157 nm PD in ion trap (Kim, Thompson, & Reilly, 2005) and linear ion-trap TOF (Kim, Schwartz, & Reilly, 2009) were demonstrated. Kim and coworkers (Oh, Moon, & Kim, 2004; Moon, Yoon, & Kim, 2005b) designed and constructed MALDI-tandem TOF for the photodissociation study. They tested several instrumental configurations and succeeded to obtain high quality tandem mass spectra by PD at 266 and 193 nm for monoisotopic peptide ions. Their MALDI-tandem TOF instruments are equipped with linear plus quadratic potential (LPQ) reflectrons, that are especially useful for the study of ion yield and dissociation kinetics.

A. MALDI-Tandem TOF Equipped with an LPQ Reflectron

A typical MALDI-tandem TOF mass spectrometer consists of a MALDI source with delayed extraction, the first stage TOF to time-separate ions with different \( m/z \), an ion gate to select a precursor ion, and the second stage TOF to analyze the product ions generated from the precursor ion. The first stage TOF is usually a linear type even though reflectron analyzers were also used (Cornish & Cotter, 1993; Beussman et al., 1995). An ion gate (LaiHing et al., 1989; Rinnen et al., 1989) is located near the first time focal point. Even though the design of the ion gate has improved over the years, selecting a monoisotopic ion remains a difficult task. The second stage TOF is always a reflectron. The most important factor affecting the ion-optical performance of a reflectron is the electrostatic potential \( V(x) \) inside the reflectron. Here \( x \) is the distance from the reflectron entrance that is grounded. A high voltage is applied to the final electrode. Neighboring electrodes are connected by resistors such that a desired potential is obtained. The most popular has been the linear potential type, \( V = c_1x \), that provides a constant electric field along the reflectron axis (Mamyrin et al., 1973). This will be called the linear potential or linear (L) reflectron. One of the requirements for a successful reflectron is that a prompt ion time-focused at the first time focal point must be time-focused again at the detector position, that is the second time focal point. This requirement can be met even when the potential has nonlinear components (Rockwood, 1986; Cornish & Cotter, 1994; Woods et al., 1998). An example is the LPQ reflectron that has both linear and quadratic components (Yoshida, 1984), that is, \( V = c_1x + c_2x^2 \). Without the linear component, the reflectron becomes a quadratic (Q) one (Doroshenko & Cotter, 1998).

When an L reflectron is optimized for the ions generated inside the source, the second time focusing condition is not met for their products formed outside the source. Techniques such as the reflectron voltage stepping and the use of a lift cell are efforts to improve the time resolution for tandem TOF equipped with L reflectron. For Q reflectron, the second time focusing condition is met whether the ions are formed inside or outside the source. The main problem here is that the first and second time focal points lie at the reflectron entrance/exit, a requirement that forbids an optimum design of PD tandem TOF. For Q reflectron, Kim and coworkers (Bae et al., 2010) also found a significant mass discrimination.

The LPQ reflectron (Oh, Moon, & Kim, 2004; Moon, Yoon, & Kim, 2005b; Bae et al., 2010) is a compromise between the L and Q reflectrons. The linear component of the potential allows a sufficient reaction time for the dissociation of a photo-excited precursor ion while the quadratic component improves the time resolution for low mass product ions. In fact, better than unit mass resolution was achieved for MALDI-tandem TOF with LPQ reflectron without using techniques such as voltage stepping. Kim and coworkers (Oh, Moon, & Kim, 2004; Moon, Yoon, & Kim, 2005b; Bae et al., 2010) also found that the mass discrimination, that was significant in Q reflectron, disappeared for properly designed LPQ reflectron. These are important advantages when the absolute (ion yield) measurement of an ion abundance is needed. In the section B of the previous chapter, we mentioned that not only the peptide ion but also its ISD product ions and matrix-derived ions appear in an ordinary MALDI-TOF spectrum of a peptide. PSD product ions also arrive at the detector. In a spectrum recorded with an L instrument, a PSD product ion with \( m/z \) quite smaller than that of its precursor forms a broad background. In contrast, all the PSD product ions form well-resolved peaks when the spectrum is recorded with an LPQ instrument (Oh et al., 2004; Moon, Yoon, & Kim, 2005b). Not only the peptide ion and its ISD products but also the PSD products of the peptide ion and PSD of its ISD products form well-resolved peaks and hence can be identified. That is, all the PSD product ions appear in a MALDI-TOF spectrum. Then, activating the ion gate is simply to sort these ions into groups belonging to each precursor. In terms of efficiency and reproducibility, the capability to measure the abundances of all these ions from one mass spectrum is an important advantage. ISD, PSD, and PSD of ISD product ions are marked in the MALDI spectrum of \( Y_0 \) shown in Figure 1.

A schematic drawing of a MALDI-PD tandem TOF apparatus equipped with an LPQ reflectron built by Kim and coworkers (Moon, Yoon, & Kim, 2005b; Yoon & Kim, 2007) is shown in Figure 2. As in other MALDI-tandem TOF instruments, the ion gate is located near the first time focus. To eliminate PSD product ions generated inside the first stage TOF, a deflection system (Yoon et al., 2006) consisting of four bipolar deflectors is installed between the ion source and the ion gate. A PD cell consisting of four electrodes (E1–E4) is installed near the first time focus. A mesh grid with 90% transmission is attached to each electrode. E1–E2, E2–E3, and E3–E4 distances
are 4, 11, and 4 mm, respectively. Performance of the PD cell in time-resolved PD study will be presented later.

B. PD Spectra of Peptide Ions

Photodissociation tandem mass spectra for peptide ions reported so far were obtained via photo-excitation at 266, 193, and 157 nm. According to Kim and coworkers (Choi et al., 2006), the $\pi_A^* \rightarrow \pi_A$ transition localized at an aromatic amino acid residue, that is, phenylalanine (F), tryptophan (W), and tyrosine (Y), is responsible for PD at 266 nm while PD at 193 nm occurs via the $\pi_P^* \rightarrow \pi_P$ transitions in peptide bonds. Reilly and coworkers (Cui, Thompson, & Reilly, 2005) suggested that an excited electronic state(s) with the $n_C^* \pi_P$ character was accessed at 157 nm.

For PD of peptide ions at 157 nm, Reilly and coworkers (Thompson, Cui, & Reilly, 2004; Cui, Thompson, & Reilly, 2005) established the spectral correlation that $v$, $w$, and $x$ type product ions were dominant for peptide ions with arginine (R) at the C-terminus while $a$ and $d$ type ions were dominant with the same residue at the N-terminus. They also suggested that these product ions were formed from $x_n + 1$ and $a_n + 1$, respectively, which, in turn, were generated by $C_n-CO$ cleavages in a dissociative electronic state. Kim and coworkers (Choi et al., 2006) observed the same types of product ions in PD at 193 and 266 nm. It is highly unlikely that the $\pi_A^* \pi_A^*$, $\pi_A^* \pi_P^*$, and $n_C^* \pi_P^*$ excited electronic states accessed at 266, 193, and 157 nm, respectively, are repulsive, especially along the $C_n-CO$ coordinates in all the cases. In particular, the $\pi_A^* \pi_A^*$ accessed at 266 nm is localized in an aromatic residue(s). It is unlikely that this state is repulsive even along the $C_n-CO$ coordinates located near the N-terminus. Hence, even when an arginine residue is present, UV-PD of a peptide ion must occur in a common electronic state, that is probably the ground electronic state.
C. Time-Resolved PD

Suppose that a precursor ion \((m^+_1)\) with the kinetic energy \(K_0\) is introduced into the PD cell floated at a high voltage \(V\), is excited by a PD laser, and undergoes dissociation to a product ion \((m^+_2)\). Let us call the same \(m^+_1\) formed inside and outside the cell as its in-cell (I) and post-cell (P) components, respectively. Since their kinetic energies after the cell are different as shown below, they appear at different positions in the TOF spectrum.

\[
K_{2,\text{post-cell}} = \frac{m_2}{m_1} K_0
\]

\[
K_{2,\text{in-cell}} = \frac{m_2}{m_1} (K_0 - eV) + eV
\]

The product ion \(m^+_2\) can also be formed consecutively via an intermediate ion \(m^+_1\).

\[
m^+_1 \rightarrow m^+_i \rightarrow m^+_2
\]

\(m^+_2\) generated by this reaction will form a peak separated from the in- and post-cell components when the first step of the reaction occurs in the field-free region inside the cell and the second step outside. The kinetic energy of such a product ion \((m^+_2)\) is as follows.

\[
K_{2,\text{consec}} = \frac{m_2}{m_1} (K_0 - eV) + \frac{m_2}{m_1} eV
\]

The peak splitting patterns of some PD product ions of \([Y_6 + H]^+\) (Moon, Yoon, & Kim, 2009) are shown in Figure 3. Kim and coworkers (Shin, Moon, & Kim, 2008) developed an algorithm to determine \(m/z\) of the intermediate ion for each consecutive (C) component.

D. Evidence for the Statistical Dissociation of Peptide Ions

It is established that the tandem mass spectral patterns for most of small polyatomic ions is explained by the microcanonical transition state theory for unimolecular reaction, viz. Rice–Ramsperger–Kassel–Marcus theory (RRKM) (Lifshitz, 1989; Holbrook, Pilling, & Robertson, 1996; Baer & Mayer, 1997). Here, rapid electronic and vibrational relaxations are assumed to assure quasi-equilibrium of the internal energy prior to the dissociation of a precursor ion. Even though it is highly likely that the statistical model is an adequate description for the dissociation of large molecules such as peptide ions, there has been no hard evidence to support it. Morgan and Russell (2006) studied the effects of the charge site and the fragmentation time scale in 193 nm PD for cationized bradykinin and some of its analogs and concluded that photo-excited peptide ions dissociated via the statistical scheme. In contrast, Futrell and coworkers (Laskin, Bailey, & Futrell, 2003, 2004) postulated a shattering mechanism for the rapid formation of some product ions in SID of peptide ions, which is a nonstatistical model. As noted previously, Reilly and coworkers (Thompson, Cui, & Reilly, 2004) suggested that PD at 157 nm of the peptide ions with arginine occurred in dissociative excited electronic states. We mentioned that the similarity of PD spectra at 157, 193, and 266 nm was evidence against the nonstatistical model.

RRKM rate constant \((k(E))\) increases with the internal energy of the precursor. One way of changing the internal energy of peptide ions in MALDI is to use different matrices. Let us take \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA), DHB, and sinapinic acid (SA) as examples. It is usually thought (Gabelica, Schulz, & Karas, 2004) that the internal energies of peptide ions generated with these matrices are in the order CHCA \(>\) SA \(>\) DHB. For example, in PSD of \([Y_6 + H]^+\) generated using these matrices, Kim and coworkers (Moon, Yoon, & Kim, 2009) observed the product ion intensity order of CHCA \(>\) SA \(>\) DHB. More definite evidence for the statistical dissociation of peptide ions was found in the time-resolved PD splitting pattern. It is to be recalled that I and C components are due to the dissociation of the precursor ion inside the cell while P components are due to those outside. That is, smaller P components relative to I + C mean more rapid dissociation. The peak splitting patterns for 193 nm PD of \([Y_6 + H]^+\) generated with the three matrices are shown in Figure 3. The order of the relative abundance of P components (CHCA \(>\) SA \(>\) DHB) is in agreement with statistical kinetics. The peak splitting patterns for the same product ions obtained by 266 nm PD are shown Figure 3(d). Here again, as the photon energy increases, the relative abundances of P components decrease.

Finally, let us consider a case in which a precursor ion (M\(^+\)) dissociates to two product ions \(m^+_1\) and \(m^+_2\) with the rate constant \(k_1\) and \(k_2\), respectively. When the two reactions are in statistical competition, the time-evolutions of \(m^+_1\) and \(m^+_2\) are governed by the total rate constant \(k_{\text{total}} = k_1 + k_2\) and their branching ratio is determined by the rate constant ratio \((k_1/k_2)\). It is to be noted that the time evolutions, that is, the branching ratios of the three product ion peaks \((b_2, y_2, \text{and } b_3)\) look similar in Figure 3, which
is another indication for the statistical dissociation of the peptide ion.

III. DISSOCIATION KINETICS OF PEPTIDE IONS IN GAS PHASE

As mentioned in the previous chapter, it has been found that most of small polyatomic ions dissociate statistically and that RRKM theory provides good fits to the experimental data. A RRKM rate constant, \( k(E) \), is a function of the internal energy of the precursor, or increases with \( E \). Hence, the first step to study the dissociation kinetics and dynamics of a particular system is to measure the rate constant as a function of internal energy and to attempt a RRKM fit for the results. One of the major problems here has been in generating energy-selected precursor ions. Techniques such as photoelectron-photoion coincidence spectrometry (Baer, 1979) have been useful in this regard. By employing mass-analyzed threshold ionization (Lee & Kim, 2007), it is also possible to carry out the study for vibrationally selected systems. Preparing energy-selected precursor ions is also one of the main problems in the study of gas-phase peptide ions. Even the effective temperature is unknown for peptide ions generated by popular ionization techniques such as ESI and MALDI.

So far, three techniques for ion activation have been used for the study of the dissociation kinetics and dynamics of peptide ions. Williams and coworkers (Price et al., 1996; Schnier et al., 1996, 1997) developed a kinetic method based on blackbody infrared radiative dissociation (BIRD). Even though the general scheme for data analysis utilizing RRKM rate constants and vibrational transition probabilities in the master equation approach looks rather complicated, the method becomes rather straightforward under the “rapid energy exchange (REX) limit” as summarized by Dunbar (2004). Here peptide ions were introduced to an ion cyclotron resonance (ICR) cell. Keeping the wall temperature of the cell constant, time-evolutions of the precursor and product ion signals were measured. The slope of the semi-log plot of the precursor ion abundance versus time was taken as the total rate constant, \( k_{tot}(T) \), where \( T \) was the wall temperature. The \( k_{tot}(T) \)s were measured at different \( T \) and the activation energy \( (E_a) \) and the \( A \) factor were determined through the Arrhenius plot. Finally, the microcanonical rate parameters, that is, the critical energy \( (E_0) \) and the critical entropy \( (\Delta S^\circ) \) were calculated from \( E_a \) and \( A \). Simply speaking, the method treated the internal temperature of a peptide ion as equivalent to the wall temperature in the REX limit. It was suggested that this limit would be rapidly reached for sufficiently large peptides and proteins because their large vibrational degrees of freedom would allow rapid absorption and emission of infrared radiation. Williams and coworkers (Price et al., 1996) demonstrated the validity of the model via calculations using vibrational transition moments obtained at the semi-empirical (AM1) level. However, the transition moments thus calculated were multiplied by three, or nine times larger transition probabilities than calculated, had to be used. Williams and coworkers interpreted it as due to the inaccuracy in the calculation at the AM1 level. It is to be noted that the heat capacity also increases with molecular size because of the increase in the vibrational degrees of freedom. For example, the heat capacities at the constant pressure for \( \text{C}_8\text{H}_{12} \) and \( \text{C}_{10}\text{H}_{22} \) are around 37 and 71 cal K\(^{-1}\)mol\(^{-1}\), respectively, at 400 K. That is, not only the infrared energy exchange rates but also the heat capacity scales with mass. Then, there is no reason to believe that the temperature of a larger ion increases more rapidly than that of a smaller ion inside the ICR cell. This suggests that the temperatures used in the analysis of BIRD data were overestimated, which, in turn, means that the rate constant thus estimated are smaller than correct values.

Time- and energy-resolved SID has been developed by Laskin and coworkers (Laskin, Bailey, & Futrell, 2004) for the kinetic study of peptide ion dissociation. A precursor ion was accelerated to a desired kinetic energy and collided with a coated metal surface. The time evolutions of the precursor and product ion signals were measured in FTMS. \( E_0 \) and \( \Delta S^\circ \), and hence \( k(E) \), for the overall and individual dissociation reactions were determined by fitting the data. Specifically, the internal energy distribution of a precursor ion after surface collision was treated by using five adjustable parameters. In addition to the usual RRKM process, an instantaneous dissociation called “shattering” was assumed to occur at higher energy. Accordingly, the rate constant for each dissociation reaction was taken as the sum of those for fast (shattering) and slow (RRKM) channels. The former was treated as a step-function originating from an assumed threshold energy while \( E_0 \) and \( \Delta S^\circ \) were taken as adjustable parameters for the latter. The fact that quite a few parameters must be adjusted in data fitting is one of the problems in the SID kinetics. Probably more troublesome is the assumption and treatment of shattering. For the dissociation of \( [\text{RVYIHPF} + \text{H}]^+ \), 12.0 eV was reported as the shattering threshold where the RRKM rate constant was smaller than \( 10^7 \text{ s}^{-1} \). This indicates that the nonstatistical process was assumed to dominate in the time range shorter than could be studied by FTMS. In an SID study of peptide ions using a MALDI tandem TOF, Wysocki and coworkers (Gamage et al., 2004) observed dissociations occurring on the time scale of \( 10^{-7} \text{ s} \). In PD of peptide ions, Kim and coworkers (Moon, Yoon, & Kim, 2007; Yoon, Chung, & Kim, 2008; Yoon, Moon, & Kim, 2010b) routinely observe the dissociation of peptide ions occurring on the similar time scale. These suggest that treating a reaction occurring on the time scale of \( 10^{-3} \text{ s} \) or shorter as nonstatistical might be unrealistic. Treating the fast component as nonstatistical and eliminating it from the RRKM analysis would result in a smaller RRKM rate constant.

By utilizing the time-resolved PD technique, Kim and coworkers (Moon, Yoon, & Kim, 2009) developed a method to determine RRKM rate constants for the dissociation of peptide ions generated by MALDI. Their method and the results will be presented after a brief description of the RRKM algorithm used in the data analysis.

A. RRKM Rate Constant for Peptide Ion Dissociation

The RRKM rate constant, \( k(E) \), for a unimolecular reaction is given as follows.

\[
k(E) = \frac{\rho(E) N(E - E_0)}{\hbar \rho(E)}
\]

Here \( \rho(E) \) is the density of vibrational states at the internal energy \( E \), \( E_0 \) is the critical energy of the reaction, \( N(E - E_0) \) is the sum of the vibrational states from \( 0 \) to \( E - E_0 \) at the transition
state (TS), \( h \) is Planck’s constant, and \( \sigma \) is the reaction path degeneracy which is 1 for complex molecules.

To calculate \( k(E) \), \( E_0 \) and the vibrational frequencies at the reactant equilibrium geometry and at the transition state are needed. The latter is the main difficulty in the calculation of \( k(E) \). Even though the approximate Kassel form may be used to avoid the difficulty (Schlag & Levine, 1989), \( k(E) \) thus evaluated was found to be erroneous by many orders of magnitude (Derrick, Loyd, & Christie, 1995). Quantum chemical calculation is not practical for large molecules such as peptides. In this regard, it is fortunate to note (Lifshitz, 1989; Baer & Mayer, 1997) that the rate constants calculated with substantially different sets of frequencies are similar as far as the critical entropy (\( S^c \)) calculated at 1,000 K is kept the same. Hence, one starts with a reasonable set of vibrational frequencies for the reactant and select and adjust some of them to prepare a set for TS with a designated value of \( S^c \). In this sense, RRKM fitting becomes a two-parameter problem.

Even though accurate vibrational frequencies are not needed, it is still a formidable task to prepare a frequency set that is roughly consistent with the molecular structure. Griffin and McAdoo (1993) prepared the frequency set of a peptide ion by collecting the frequencies of all individual stretching and bending modes associated with each structural unit. Avoiding redundant selection of bending frequencies was one of the problems there. Derrick and coworkers (Derrick, Loyd, & Christie, 1995) proposed to utilize the frequency distribution for model peptide molecules. The frequency set thus prepared is not sequence-specific.

By far the most systematic method that can automatically generate a sequence-specific frequency set for a peptide ion was developed by Kim and coworkers (Moon, Oh, & Kim, 2006). In this method, the vibrational frequencies for 20 amino acids were obtained by density functional theory calculations at the B3LYP/6-31G** level. Then, subtracting some frequencies due to the motions of the \(-\text{OH}\) and \(-\text{NH}_2\) groups, the frequency sets for an amino acid when it is located inside a peptide or at the N- or C-terminus were prepared. When the sequence of a peptide ion was designated, these residue frequency sets were called in. Then, additional frequencies associated with the motions of the \(-\text{NH}\)--\(-\text{CO}\)—group and the ionizing proton were added to prepare the complete set of frequencies for the reactant. From this set, 30 frequencies in the range 0–1,500 cm\(^{-1}\) were chosen and multiplied by a factor to prepare the set for TS such that a designated value of \( S^c \) was obtained. In addition to the direct counting of the state density and sum, an improved Whitten–Rabinovitch approximation (Sun, Moon, & Kim, 2007) and a grouped frequency method (Moon, Sun, & Kim, 2007) were developed to improve the efficiency of calculation.

**B. Time-Resolved Photodissociation Kinetics**

As mentioned earlier, \( b \) and \( y \) type ions are the major product ions from a peptide ion without arginine over a wide internal energy range. It is thought that the formation of a complementary pair of \( b \) and \( y \) ions shares a common reaction path until after the rate-determining TS (Polce, Ren, & Wesdemiotis, 2000; Paizs & Suhai, 2002, 2005). In addition, the peak splitting patterns for various \( b \) and \( y \) ions obtained by the time-resolved PD are similar, indicating that such dissociation channels are in statistical competition. Then, the total dissociation rate constant, \( k_{\text{tot}}(E) \), for such peptide ions will be the sum of the rate constants for individual channels, that is, \( k_{\text{tot}}(E) = \sum_k k_k(E) \), where \( n \) is the number of the amide bonds. Taking \( k_0(E) \) as the typical rate constant for a \( b/y \) channel, \( k_{\text{tot}}(E) \) can be further simplified to \( nk_0(E) \). Then, \( k_{\text{tot}}(E) \) will be specified by two parameters, the critical energy (\( E_0 \)) and entropy (\( S^c \)) for a typical \( b/y \) channel. This was one of the assumptions adopted by Kim and coworkers (Moon, Yoon, & Kim, 2009).

In addition to \( E_0 \) and \( S^c \), the internal energy of a precursor ion affects the dissociation rate constant. A peptide ion generated by MALDI suffers many collisions before coming out of the source. Hence, a thermal distribution of the internal energy is generally assumed (Knochenmuss, 2006) even though there has been no consensus on the actual temperature. Under the thermal assumption—this was the second assumption adopted by Kim and coworkers—the internal energy distribution for peptide ions coming out of the source, \( P_d(E) \), can be calculated once the effective temperature is designated. Then, the energy distribution (\( P(E) \)) of the precursor ions that survive at any time \( t \) after it comes out of the source becomes \( P_d(E) \exp(-k_0(E) t) \). With \( P_d(E) \) normalized, the area under \( P(E) \) becomes the survival probability of a peptide ion at time \( t \). That is, the survival probability of a precursor ion at any location can be calculated once the effective temperature, \( E_0 \), and \( S^c \) are designated. The survival probability at any time after photodissociation can be calculated similarly.

Experimentally, the relative PSD yield (\( Y_{\text{PSD}} \equiv \text{[total PSD]/[precursor]} \)) and the intensity ratio of the post-cell to in-cell components \( (C_D \equiv \sum \text{[P]}/(\sum \text{[P]} + C)) \) were measured. YPSD and CPD were related to the survival probabilities at various locations through inequality relations, which took into account the maximum possible errors. Data analysis were done as follows. Using a set of \( (E_0, S^c, T) \), \( k_{\text{tot}}(E) \) and \( P_d(E) \) were calculated, from which the survival probabilities at various locations were calculated. These were inserted into the inequality relations for YPSD and CPD. The \( (E_0, S^c, T) \) set was taken when both inequality relations were satisfied. Calculation was done with nearly one hundred million sets of \( (E_0, S^c, T) \) with \( E_0 \) at 0.001 eV interval, \( S^c \) at 0.1 eV interval, and \( T \) at 10 K interval.

\([Y_0 + H]^+\) was the first system investigated by the PD kinetic method. The peak splitting patterns in 193 and 266 nm PD of some product ions of \([Y_0 + H]^+\) generated by MALDI with CHCA, DHB, and SA as the matrices are shown in Figure 3. Experimental data obtained with the above three matrices were analyzed together to minimize the errors in the determination of \( E_0 \), \( S^c \), and \( T \). Use of a different matrix would change the peptide ion temperature, but not \( E_0 \) and \( S^c \). \( (E_0, S^c, T) \) sets consistent with the experimental data obtained with each matrix are shown in Figure 4. Among these sets, those showing the same \( E_0 \) and \( S^c \) regardless of the matrices were chosen. For the \( (E_0, S^c, T) \) sets thus chosen, the averages of \( E_0 \), \( S^c \), and \( T \) were calculated. The results are listed in Table 1 together with those for other peptide ions determined by ISD–PSD kinetics (to be explained). The matrix-dependence of the peptide ion temperature was in the order DHB (396 ± 22 K) < SA (445 ± 29 K) < CHCA (469 ± 22 K) in agreement with the general consensus (Spengler, Kirsch, & Kaufmann, 1992; Karas, Bahr, & Strupat, 1995; Gabelica, Schulz, & Karas, 2004). According to previous quantum chemical calculations (Paizs & Suhai, 2002, 2005), \( E_0 \) for the dissociation of \([G_3 + H]^+\) to \( b_2 \) product ion was 1.14 eV and \( S^c \) estimated for the reported
transition state was $-2.65$ eV ($1 \text{eu} = 4.184 \text{J mol}^{-1} \text{K}^{-1}$). Both of them are much larger than $E_0$ and $\Delta S^i$ of $0.60 \pm 0.03$ eV and $-28.4 \pm 2.1$ eV, respectively, determined for $[Y_6 + H]^+$ by PD kinetics. The experimental results suggest that the intramolecular interaction in the transition structure for a $b/y$ channel is more important than predicted by quantum chemical calculations for small peptide ions.

**C. Dissociation Kinetics for $[YGGFL + H]^+$**

Singly protonated leucine enkephalin, $[YGGFL + H]^+$, is a benchmark peptide ion studied previously by BIRD and by SID. Williams and coworkers (Schnier et al., 1997) performed the BIRD study of this peptide ion, suggested that its dissociation occurred in the “rapid energy exchange (REX)” limit, and reported $E_0$ of $1.10 \text{eV}$ and the $A$ factor of $10^{10.5} \text{s}^{-1}$. By conversion of this $A$ factor in the REX limit (Dunbar, 2004), Kim and coworkers (Moon et al., 2010) obtained $\Delta S^i$ at $1.00 \text{K}$ of $-14.9$ eV. Laskin (2006) performed the SID kinetic study of $[YGGFL + H]^+$ and reported $E_0$ of $1.13 \text{eV}$ and $\Delta S^i$ of $-10.3$ eV. Kim and coworkers (Moon et al., 2010) carried out the PD kinetic study of the same peptide ion generated by MALDI and reported $E_0$ of $0.67 \pm 0.08 \text{eV}$ and $\Delta S^i$ of $-24.4 \pm 3.2$ eV. The results from the three laboratories are listed in Table 2. The total rate constants, $k_{\text{rel}}(E)$, calculated using the kinetic parameters reported by the three laboratories are shown in Figure 5 (Moon et al., 2010). It is to be noted that the rate-energy relation determined by the PD kinetics, $k_{\text{rel}}(E)_{\text{PD}}$, is significantly different from those determined by BIRD and SID, $k_{\text{rel}}(E)_{\text{BIRD}}$ and $k_{\text{rel}}(E)_{\text{SID}}$, respectively, while the latter two are rather similar. The least one can say about RRKM modeling is that it is a good way of fitting experimental kinetic data. For example, a significant difference between $k_{\text{rel}}(E)_{\text{PD}}$ and $k_{\text{rel}}(E)_{\text{SID}}$, and $k_{\text{rel}}(E)_{\text{BIRD}}$ also, means that MALDI-PD tandem TOF data are incompatible with $k_{\text{rel}}(E)_{\text{SID}}$. This was demonstrated by Kim and coworkers (Moon et al., 2010) as follows. They converted their CPD data to the average PD rate constant and obtained $1.0 \times 10^7 \text{s}^{-1}$ for PD at $266 \text{nm}$ for $[YGGFL + H]^+$ generated

![FIGURE 4. ($E_o$, $\Delta S^i$, $T$) sets simultaneously satisfying the inequality relations for PSD and those for 193 and 266 nm PD of $[Y_6 + H]^+$ generated by MALDI using DHB (green), SA (red), and CHCA (blue) as matrices. For each set, the subset which shares common $E_0$ and $\Delta S^i$ values in the three sets, but different $T$, is represented by spheres with deeper color. Corresponding ($E_o$, $\Delta S^i$) region is shown as a shadow at the bottom plane. The data reported by Kim and coworkers (Moon, Yoon, & Kim, 2009) were retreated due to a minor software error in the original work.](image)

**TABLE 1. Product ion abundances, survival probabilities, $E_0$, and $\Delta S^i$**

<table>
<thead>
<tr>
<th></th>
<th>$Y_{\text{total}}$ ISD</th>
<th>$Y_{\text{total}}$ PSD</th>
<th>$S_{\text{in}}$</th>
<th>$S_{\text{post}}$</th>
<th>$E_0$ (eV)</th>
<th>$\Delta S^i$ (eu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[Y_6 + H]^+$</td>
<td>4.05</td>
<td>5.06</td>
<td>0.198</td>
<td>0.165</td>
<td>0.600</td>
<td>-28.4</td>
</tr>
<tr>
<td>$[Y_5H + H]^+$</td>
<td>0.50 ± 0.06</td>
<td>1.73 ± 0.22</td>
<td>0.67 ± 0.03</td>
<td>0.37 ± 0.03</td>
<td>0.621 ± 0.003</td>
<td>-28.0 ± 0.2</td>
</tr>
<tr>
<td>$[HY_5 + H]^+$</td>
<td>0.37 ± 0.06</td>
<td>2.59 ± 0.63</td>
<td>0.73 ± 0.03</td>
<td>0.28 ± 0.05</td>
<td>0.609 ± 0.008</td>
<td>-28.8 ± 0.4</td>
</tr>
<tr>
<td>$[HY_5Y + H]^+$</td>
<td>0.65 ± 0.07</td>
<td>1.97 ± 0.05</td>
<td>0.61 ± 0.03</td>
<td>0.34 ± 0.01</td>
<td>0.617 ± 0.001</td>
<td>-28.1 ± 0.1</td>
</tr>
<tr>
<td>$[HY_5Y + H]^+$</td>
<td>0.47 ± 0.08</td>
<td>1.17 ± 0.13</td>
<td>0.68 ± 0.04</td>
<td>0.46 ± 0.03</td>
<td>0.632 ± 0.004</td>
<td>-27.5 ± 0.2</td>
</tr>
<tr>
<td>$[Y_5K + H]^+$</td>
<td>0.42 ± 0.04</td>
<td>1.29 ± 0.10</td>
<td>0.71 ± 0.02</td>
<td>0.44 ± 0.02</td>
<td>0.630 ± 0.003</td>
<td>-27.6 ± 0.2</td>
</tr>
<tr>
<td>$[KY_5 + H]^+$</td>
<td>0.82 ± 0.14</td>
<td>1.86 ± 0.13</td>
<td>0.55 ± 0.05</td>
<td>0.35 ± 0.02</td>
<td>0.623 ± 0.005</td>
<td>-27.8 ± 0.1</td>
</tr>
<tr>
<td>$[HY_5K + H]^+$</td>
<td>0.51 ± 0.07</td>
<td>1.92 ± 0.45</td>
<td>0.66 ± 0.03</td>
<td>0.34 ± 0.05</td>
<td>0.618 ± 0.007</td>
<td>-28.1 ± 0.4</td>
</tr>
<tr>
<td>$[KY_5R + H]^+$</td>
<td>0.49 ± 0.06</td>
<td>0.94 ± 0.07</td>
<td>0.67 ± 0.03</td>
<td>0.51 ± 0.02</td>
<td>0.639 ± 0.003</td>
<td>-27.1 ± 0.2</td>
</tr>
<tr>
<td>$[KY_5R + H]^+$</td>
<td>0.47 ± 0.04</td>
<td>0.45 ± 0.11</td>
<td>0.96 ± 0.01</td>
<td>0.69 ± 0.05</td>
<td>0.660 ± 0.007</td>
<td>-27.2 ± 0.3</td>
</tr>
<tr>
<td>$[RY_5 + H]^+$</td>
<td>0.063 ± 0.042</td>
<td>0.47 ± 0.23</td>
<td>0.94 ± 0.04</td>
<td>0.69 ± 0.12</td>
<td>0.661 ± 0.017</td>
<td>-27.6 ± 0.6</td>
</tr>
<tr>
<td>$[Y_5RY_5 + H]^+$</td>
<td>0.024 ± 0.005</td>
<td>0.23 ± 0.06</td>
<td>0.98 ± 0.01</td>
<td>0.81 ± 0.04</td>
<td>0.678 ± 0.008</td>
<td>-26.6 ± 0.3</td>
</tr>
<tr>
<td>$[RY_5R + H]^+$</td>
<td>0.10 ± 0.03</td>
<td>0.52 ± 0.13</td>
<td>0.91 ± 0.02</td>
<td>0.66 ± 0.06</td>
<td>0.658 ± 0.006</td>
<td>-27.1 ± 0.4</td>
</tr>
<tr>
<td>$[HY_5RY_5 + H]^+$</td>
<td>0.079 ± 0.026</td>
<td>0.44 ± 0.10</td>
<td>0.93 ± 0.02</td>
<td>0.69 ± 0.03</td>
<td>0.658 ± 0.007</td>
<td>-26.9 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$E$_0$ and $\Delta S^i$ for $[Y_6 + H]^+$ were determined by PD kinetics. They were used as benchmarks to determine E$_0$ and $\Delta S^i$ for the dissociation of other peptide ions by ISD-PD kinetics.

$^b$1 eu = 4.184 J K$^{-1}$ mol$^{-1}$.
by MALDI (DHB). The internal energy corresponding to this rate constant was 11.85 eV according to \( k_{\text{tot}}(E)_{\text{SID}} \). Subtracting the photon energy at 266 nm (4.66 eV), the average internal energy before the photo-excitation became 7.19 eV, which corresponded to the peptide ion temperature of 1,090 K at the source exit according to \( k_{\text{tot}}(E)_{\text{SID}} \). Then, the same kinetics predicted 0.01% survival of \([\text{YGGFL} + \text{H}]^+\) at the detector when photo-excitation was not done. That is, if the rate-energy relation determined by SID or BIRD had been correct, it should have been virtually impossible to record PD spectra for peptide ions. Or, even recording ordinary MALDI spectra for peptides have been virtually impossible to record PD spectra for peptide ions. Alternatively, ordinary MALDI spectra for peptides would have been a struggle.

As an explanation for the significant difference in dissociation kinetics between PD and SID (BIRD also) studies, Kim and coworkers (Moon et al., 2010) suggested that a transition state switching (Chesnavich et al., 1981) might occur at relatively low internal energy. That is, dissociation reactions occurring on millisecond time range in BIRD and SID are limited by energy bottlenecks while those occurring on nanosecond to microsecond time scale in PSD and PD are limited by entropy bottlenecks. In our opinion, the kinetic analyses of both BIRD and SID data might have underestimated the rate constants as suggested at the beginning of this chapter.

### IV. Peptide Ion Temperature and ISD–PSD Kinetics

#### A. A Brief Review on the Temperature of Peptide Ions Generated by MALDI

The internal energy content for a peptide ion is an important factor that affects its dissociation rate constant and hence its mass spectral pattern. In the previous chapter, it was mentioned that the thermal distribution of the internal energy was generally accepted in MALDI because many collisions would occur in the high density early plume (Spengler, 1997). Then, knowledge on the effective temperature would allow the estimation of the internal energy distribution. Even with the thermal assumption, a problem still remained, that is, there was no reliable method to determine the effective temperature of the matrix plume. The effective temperatures reported in literature vary widely, from 400 to 1,000 K or higher.

Mowry and Johnston (1994) carried out the photoionization (PI) of gas-phase \( n \)-alkylamine neutrals generated by laser-induced desorption from matrix-analyte mixtures, viz. matrix-assisted laser desorption (MALDI). By comparing PI mass spectral patterns with those in the temperature-dependent gas-phase PI mass spectra, the effective temperatures of 400–500 K were estimated for the desorbed neutrals. Zenobi and coworkers (Koubenakis et al., 2004) measured blackbody radiation from DHB excited at 355 nm and found the maximum surface temperature of 850 K or higher. Yergey and coworkers (Campbell et al., 2007) measured the extent of ISD for \([\text{YGGFL} + \text{H}]^+\) and obtained the effective temperature of 736–960 K through kinetic analysis. Finally, Kim and coworkers (Moon, Yoon, & Kim, 2009; Moon et al., 2010) estimated the effective temperatures of some peptide ions through the kinetic analysis of the PSD and time-resolved PD results and obtained 400–470 K. The widely different values of the effective temperature reported so far may look quite disappointing. However, one can see a clear trend in the values measured by different groups with different techniques. In terms of the measurement time, the data reported by Zenobi and coworkers and by Yergey and coworkers correspond to those for the molecules well within 100 ns after the irradiation of MALDI laser, which is shorter than the typical delay time used in delayed-extraction MALDI-TOF. On the other hand, Mowry and Johnston’s measurement of PI mass spectra was made 4 \( \mu \)s after MALD. Similarly, the measurements of PSD and time-resolved PD were made around 10–16 \( \mu \)s after MALDI in the PD kinetics of Kim and coworkers. In other words, 700–1,000 K seems to be the temperature for peptide ions in the early stage of MALDI, or in the “early” plume, while 400–500 K seems to be in the “late” plume, or after the completion of plume expansion.

#### B. In-Source Decay (ISD) and the Time-Evolution of the Plume Temperature

As in 70 eV EI spectra, the fragment ions generated by ISD form well-resolved peaks in ordinary MALDI-TOF spectra. Also

### Table 2. E\(_0\) and \(\Delta S^i\) for \([\text{YGGFL} + \text{H}]^+\) and \([\text{Y}_6 + \text{H}]^+\) determined by PD, BIRD, and SID and the effective temperature (\(T\)) of the peptide ions formed by MALDI (DHB)

<table>
<thead>
<tr>
<th>(E_0), eV</th>
<th>(\Delta S^i), eu</th>
<th>(T), K</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{YGGFL} + \text{H}]^+)</td>
<td>0.71±0.03</td>
<td>−22.9±4.9</td>
<td>478±77</td>
</tr>
<tr>
<td>([\text{Y}_6 + \text{H}]^+)</td>
<td>0.67±0.08</td>
<td>−24.4±3.2</td>
<td>454±47</td>
</tr>
<tr>
<td>1.10</td>
<td>−14.9</td>
<td>BIRD Schnier(1997)</td>
<td></td>
</tr>
<tr>
<td>1.13</td>
<td>−10.3</td>
<td>SID Laskin(2006)</td>
<td></td>
</tr>
</tbody>
</table>

\(\Delta S^i\) refers to the entropy change of the system. Ref. indicates the reference for the effective temperature. pd refers to the plasma density.

### Figure 5. \(k_{\text{tot}}(E)\) curves for \([\text{YGGFL} + \text{H}]^+\) calculated with \(E_0\) and \(\Delta S^i\) determined by PD/PSD (—), BIRD (— —), and SID (— — —). The estimated rate constant range covered in each experiment is marked.

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*1 eu = 4.184 J K\(^{-1}\) mol\(^{-1}\).*
known is that ISD is not a prompt process but has a finite lifetime (Takayama, 2001a). Recently, ISD of relatively large peptide ions has attracted much attention because of the formation of c and z type product ions. Preferential generation of b and y type product ions in ISD of relatively small peptide ions without arginine has not received as much attention, even though it provides an opportunity to study dissociation reactions occurring on a time scale much shorter than that for PSD—$10^{-7}$ s for ISD versus $10^{-5}$ s for PSD. Inspired by the higher temperature for peptide ions undergoing ISD (Campbell et al., 2007) than those investigated by PD kinetics, Kim and coworkers (Yoon, Moon, & Kim, 2010b; Bae, Moon, & Kim, 2011) performed a rather extensive study on the formation of b and y ions by ISD of [Y$_6$ + H]$^+$. An ion formed inside a MALDI source, whether it is a peptide ion or its ISD product, may undergo dissociation after it comes out of the source. That is, to evaluate the abundance of a peptide ion at the source exit, and that of an ISD product also, ion loss due to post-source dissociation must be taken into account. In this regard, a MALDI-tandem TOF instrument equipped with an LPQ reflectron that can detect ISD, PSD, and PSD of ISD simultaneously is useful.

Let us suppose that the effective temperature of a peptide ion—and hence its dissociation rate constant—remains constant throughout the plume expansion. Then, the product ion yield in ISD would be far smaller than that in PSD because the reaction time in PSD is much longer. ISD and PSD fragment ions generated from [Y$_6$ + H]$^+$ in MALDI(CHCA) are shown in Figure 1—“c” and “z” type product ions did not appear in this spectrum. It is obvious that ISD is far more efficient than PSD, and ISD lifetime of 50 ns means that only 2 $\times$ 10$^{-9}$ of peptide ions survive at the source exit. As in 70 eV EI, a very broad internal energy distribution can explain the survival of molecular ions even when very fast dissociations occur inside the source. However, the internal energy distribution under thermal conditions was found to be too narrow to warrant such an explanation (Yoon, Moon, & Kim, 2011). Then, the only explanation for the delay time independence of $Y_{\text{total ISD}}$ was that the effective temperature of the peptide ion decreased as it moved from the early to late plume and that ISD occurred in the former. In the analysis of the time-resolved PD data, we assumed that the effective temperature of the peptide ion that had come out of the source did not change. This will be explained in the next section.

With the late plume colder than the early one established, Kim and coworkers (Yoon, Moon, & Kim, 2011) attempted to estimate the effective temperature of the early plume by analyzing the total ISD yield ($Y_{\text{total ISD}}$) with the rate-energy relation, $k_{\text{iso}}(E)$, determined by PD kinetics. A brief summary of the method is given below with [Y$_6$ + H]$^+$ as an example. The intensities of product ions appearing in the PSD spectrum of [Y$_6$ + H]$^+$ were added and normalized to the intensity of [Y$_6$ + H]$^+$ to get the PSD yield, $Y_{\text{PSD}}$. This was multiplied by a correction factor of six to estimate the total post-source dissociation yield, $Y_{\text{total PSD}}$. For each ISD product ion, a similar correction was made by utilizing the intensities of its PSD products. The corrected intensities of ISD products were summed and normalized to get $Y_{\text{total ISD}}$.

![FIGURE 6. $Y_{\text{total ISD}}$ versus delay time (±20 nsec accuracy) in MALDI(CHCA) of Y$_6$. Laser pulse energy was 1.5 µJ/pulse. The data reported by Kim and coworkers (Yoon, Moon, & Kim, 2010b) were corrected for PSD of ISD.](image_url)

Then, the survival probability of peptide ions undergoing ISD ($S_{\text{post}}$) was estimated as 1/(1 + $Y_{\text{total ISD}}$) and 1/(1 + $Y_{\text{total PSD}}$), respectively. Based on the assumption of 50 ns life time—$1.4 \times 10^7$ s$^{-1}$ in rate constant—for ISD, the threshold internal energy of 11.585 eV was estimated from $k_{\text{iso}}(E)$ for [Y$_6$ + H]$^+$. That is, [Y$_6$ + H]$^+$ with the internal energy smaller than 11.585 eV was assumed to survive ISD. Finally, the temperature of the peptide ion was adjusted such that the area under the energy distribution function below 11.585 eV became the same as $S_{\text{post}}$. The effective temperature in the early plume of CHCA thus determined was 881 K. This was in good agreement with 22 K determined by PD kinetics. Moreover, the temperature of the peptide ion that had come out of the source did not change. This will be explained in the next section.

C. Expansion Cooling in Matrix Plume

There have been attempts to explain the occurrence of ISD and PSD by invoking different ion activation processes. The majority view (Spengler, 1997; Hillenkamp & Karas, 2007; Demeure, Gabelica, & De Pauw, 2010) has been that the exothermicity in the GPPT between the protonated matrix and the neutral peptide provides the internal energy needed for ISD and that low energy collisional activation is responsible for PSD. Recently, Kim and coworkers (Bae, Moon, & Kim, 2011) evaluated the above claims and suggested that the expansion cooling of the matrix plume could explain the peptide ion dissociation on substantially different time scales. A brief summary is as follows.
Kim and coworkers measured UV-MALDI spectra at 337 nm of Y₆ using CHCA, DHB, and SA as the matrices and IR-MALDI at 2.94 μm of the same peptide using glycerol as the matrix. PSD spectra for [Y₆+H]+ was also recorded for the four cases. It is known (Kampmeier et al., 1997; Hillenkamp & Karas, 2007) that the amount of material ablated in IR-MALDI is ten times more than in UV-MALDI because IR penetrates deeper. This suggests stronger PSD signals in IR-MALDI than in UV-MALDI if multiple collisions at low energy were responsible for PSD. In IR-MALDI-PSD, peaks due to CAD were identified, that had longer flight times than usual PSD peaks. However, their intensities were around 1% of the corresponding peaks in MALDI(CHCA)-PSD. More importantly, such peaks were not observed in UV-MALDI-PSD, strong evidence against the CAD model for PSD.

Kim and coworkers also determined the early and late plume temperatures. The temperatures in the early plumes were glycerol (≤721 K) < DHB (788 K) < SA (790 K) < CHCA (874 K), while those in the late plume were glycerol (≤347 K) < DHB (423 K) < SA (443 K) < CHCA (461 K). ΔH in the GPPT reaction between the matrix cation (MH+) and the analyte neutral (A), MH+ + A → M + AH+, is given by the difference in the proton affinity between the matrix and the analyte, PA(M) - PA(A). If the exothermicity (∆H) in this reaction is the driving force for ISD, use of a matrix with larger PA will results in lower early plume temperature. Ignoring CHCA whose PA is uncertain, the PA order for the remaining three matrices predicts the early plume temperature order of SA < glycerol < DHB, in disagreement with the experimental order.

With the exothermicity model for ISD and the low energy CAD model for PSD invalidated, Kim and coworkers suggested the thermal energy gained during photo-ablation in MALDI as the driving force for ion fragmentation. Then, the large difference in temperature between the early and late plumes dictates that the temperature of a peptide ion drops spontaneously as the plume expands. In fact, formation of a high density material pulse and its expansion to the vacuum in MALDI led many investigators to suggest that expansion cooling would occur in the plume. Vertes and coworkers (Vertes, Iriany, & Gijbels, 1993) carried out hydrodynamic analysis for MALD of large molecules and concluded that a dramatic expansion cooling could occur. Experimentally, the occurrence of expansion cooling was inferred from supersonic “initial” ion speeds (Berkenkamp et al., 2002) and was proposed to stabilize labile analyte ions (Dreisewerd, 2003; Hillenkamp & Karas, 2007). A significant temperature difference between the peptide ions contributing to ISD and PSD observed by Kim and coworkers (Bae, Moon, & Kim, 2011) is strong evidence for the expansion cooling.

Even though the occurrence of the expansion cooling is highly likely, the factors determining the actual temperature have not been established yet. Hillenkamp and Karas (2007) proposed to classify matrices as “hard” or “soft” with a connation that a hard matrix will require more energy for ablation and hence generate hotter plume. Kim and coworkers (Bae, Moon, & Kim, 2011) suggested that both the mechanical hardness of a matrix and the absorption coefficient might determine the early plume temperature and its time evolution.

Kim and coworkers noted that the occurrence of expansion cooling in MALDI also provides an explanation for the fact that labile species are difficult to detect when a matrix is not used such as in laser desorption. In this regard, expansion cooling in the matrix plume is one of the key characteristics of MALDI in the mass spectrometry of labile molecules.

### D. ISD-PSD Kinetics and the Influence of Basic Residues on the Dissociation Rate

Even though time-resolved PD is a powerful technique for the study of dissociation kinetics, large errors in the kinetic parameters thus determined was a handicap in the investigation of delicate effects on the rate constant such as the influence of basic residues (Yoon et al., 2009a; Yoon, Moon, & Kim, 2009b). A better approach may be to take a system as a benchmark, study other systems under the condition established for the benchmark, and evaluate the difference. Kim and coworkers (Yoon, Moon, & Kim, 2011) noted that the process devised to estimate the early and late plume temperatures (Yoon, Moon, & Kim, 2010b; Bae, Moon, & Kim, 2011), when taken in reverse, would lead to a new technique for the study of dissociation kinetics. Specifically, [Y₆+H]+ was taken as the benchmark and its Y₅ total ISD and Y₅ total PSD were measured with the laser pulse energy of 2 times the threshold (see Yoon, Moon, & Kim, 2010b for the definition of the threshold). Analysis of the data by utilizing kₒ(E) calculated with E₀ of 0.600 eV and ΔSᵣ of -28.4 eu resulted in Tₑ and Tₑₑ of 881 and 463 K, respectively. For other peptide ions, Y₅ total ISD and Y₅ total PSD were measured at the same experimental condition as for [Y₆+H]+. The threshold rate constant for ISD was set at 1.4 × 10⁸ s⁻¹ as explained earlier. Then, the energy corresponding to this rate constant was determined such that the area below this energy in the energy distribution at 881 K became Sₑₑ. This produced one point on the rate-energy plane. Similar analysis of Y₅ total PSD produced another point. Then, E₀ and ΔSᵣ were adjusted such that the calculated kₒ(E) passed these two points. Various tests showed the utility of this ISD–PSD kinetics. Finally, it is to be emphasized that the ISD–PSD kinetics can be used only when the product ions of ISD are mostly b, y, and their consecutive reaction products.

MALDI(CHCA) and PSD spectra for Y₆X (X = Y, H, K, and R) reported by Kim and coworkers (Yoon, Moon, & Kim, 2011) are shown in Figures 7 and 8, respectively. Product ions appearing in MALDI and PSD spectra were mainly b, y, and their dissociation products. “c” and “z” type product ions are hardly observable. Y₅ total ISD, Y₅ total PSD, Sₑₑ, Sₑₒ, E₀, and ΔSᵣ obtained for some model peptide ions containing basic residues are listed in Table 1. Y₅ total ISD for [Y₆+H]+ is larger than those with histidine (H) or lysine (K) by an order of magnitude, which, in turn, are larger than those with arginine by an order of magnitude. Similar trends were observed for PSD spectra, with smaller difference in intensity. In terms of the survival probability, influence of the basic residues became less spectacular. Hence, the influence of the basic residue on kₒ(E) was not quite significant as shown in Figure 9 and in E₀ and ΔSᵣ in Table 1.

Kim and coworkers noted that E₀ values for [Y₆+H]+, [Y₆,K+H]+, and [Y₆,R+H]+ were larger than that for [Y₅+H]+ by 0.021, 0.030, and 0.060 eV, respectively, while the proton affinities (Harrison, 1997) for H, K, and R were larger than that for Y by 0.46, 0.64, and 1.04 eV, respectively. That is, even though E₀ gets larger in the presence of a more basic residue, changes in E₀ are much smaller than those in PA, indicating that the ionizing proton does not completely migrate.
to an amide backbone, but keeps interacting with a basic site even in the TS. This is compatible with highly negative $\Delta S^{\ddagger}$ found for peptide ions. By modifying the quantum chemical TS for the oxazolone pathway (Paizs & Suhai, 2002, 2005; Aribi et al., 2003), Kim and coworkers devised a TS in which the ionizing proton interacted both with a basic site and with an amide backbone (Fig. 10). As the basicity of the site interacting with the proton in TS increases, it will interact more with the proton and hence make the amide backbone less labile. This explains larger $E_0$ in the presence of a more basic residue.

**V. ION YIELDS FOR PEPTIDE IONS AND THE THERMAL MODELS FOR THEIR FORMATION**

Information on the ionization efficiency, or IY, is essential for the systematic development of an ionization technique. Almost all the measurements of IY in MALDI were made in 1990s when the phenomenon was poorly understood. In a recent monograph on MALDI, Hillenkamp and Karas (2007) acknowledged that "no accurate numbers of IY had been determined." In the kinetic study of peptide ions, Kim and coworkers identified various factors affecting IY measurement and devised the methods to calibrate such influences. We will start this chapter by reviewing IY measurements made in 1990s. Then, the method developed by Kim and coworkers (Moon et al., 2012) will be reviewed. Finally, the insights on the peptide ion formation in MALDI gained from the IY data will be presented.

**A. A Brief Review on the Measurement of Ion Yields in MALDI**

Even though the ion yield (IY = number of gas-phase ions formed ÷ number of molecules loaded) in MALDI must be important for elucidating the ion formation mechanism, it has not received much attention because of various experimental difficulties. Standing and coworkers (Ens et al., 1991) counted...
the number of insulin ions emitted from focused spots of a 308 nm laser in MALDI with SA. From the laser spot size and the disappearance of the insulin ion signal after 100 laser shots, $4 \times 10^7$ insulin molecules were estimated to have been removed per laser shot. Then, taking $10^4$ as the number of insulin ions ejected per shot, IY of $3 \times 10^{-4}$ was estimated. Mowry and Johnston (1993) carried out MALDI for some alkylamines and estimated the total number of co-desorbed neutrals by photoionization. The laser spot size was estimated by visual inspection with a calibrated microscope to determine the region damaged (to be called “burn mark” from now on) when the sample was irradiated with a higher energy laser pulse. IY thus estimated was $10^{-5}$–$10^{-4}$ for the analytes. According to Beavis (1992), the effective size of focused laser spot in MALDI depends not only on the focusing optics but also on the laser fluence. That is, the size of the burn mark made at higher fluence would be an overestimation that would underestimate IY. In addition, the matrix-to-analyte ratio of 1–10:1 adopted by Mowry and Johnston was lower than in usual MALDI experiments by orders of magnitude. IYs for pure matrices were measured by two groups. Sundqvist and coworkers (Quist, Huth-Fehre, & Sundqvist, 1994) studied the laser desorption of ferulic acid at 355 nm. The numbers of charged particles, both cations and anions, were measured by extracting them with an electric field. The total amount of desorbed neutrals was measured by depositing them on a quartz crystal microbalance. IY for the cations thus determined was $10^{-7}$ near the threshold fluence and increased to $10^{-6}$ at higher fluence. Puretzky and Geohegan (1998) studied the laser desorption of 3-hydroxypicolinic acid (3-HPA). The gas-phase density of desorbed 3-HPA was measured by absorption spectroscopy while the ion density was measured by an ion probe. $10^{-5}$ was reported as IY for the matrix. In MALDI of a sample with the matrix-to-peptide ratio of $10^3$–$10^4$, the abundance of peptide-derived ion signals is comparable to that of the matrix-derived ions, suggesting that IYs for analytes are larger than those for matrices by the same ratio. Then, IY values of around $10^{-4}$ for analytes reported by Standing and coworkers and by Mowry and Johnston indicate IY values of $10^{-8}$–$10^{-7}$ for matrices. That is, $10^{-5}$ for 3-HPA estimated by Puretzky and Geohegan must be an overestimation.
As mentioned earlier, Hillenkamp and Karas acknowledged that no accurate data were available for IY, but speculated that IYs for analytes and matrices would be around 10^{-3}–10^{-1} and 10^{-5}–10^{-3}, respectively. Both of them are much higher than the experimental results. More troublesome is that the above numbers were often quoted indiscriminately in literature, that is, without any information on whether the number quoted was that for the matrix or for the analyte.

B. Determination of Ion Yields by Repeated Irradiation of Extended Regions

Determination of IY requires the data for the number of ions formed and the number of neutrals consumed, for the matrix and the analyte. One way of getting such data is to measure both of them, as was done by Mowry and Johnston (1993). Guaranteeing the same quantum efficiency for the measurement of ions and neutrals is a problem in such an approach. An alternative is to deplete all the materials in part of a sample—depleting the whole sample is extremely time-consuming—and to measure the number of ions. Then, IY can be determined once the number of neutrals in that part can be estimated. This was the approach taken by Standing and coworkers (Ens et al., 1991). A problem in their work was that they did not take the sum of ion signals collected until the complete depletion of the sample in a spot. Another problem, which was probably more serious, was in the estimation of the spot size. The area of the region was measured for the burn mark formed by the laser with a CCD camera. Instead of two times the threshold laser pulse energy used in typical MALDI measurements, four times the threshold or higher was used because the burn mark did not form clearly at lower pulse energy. The analyte-derived peaks in the integrated spectrum were assigned and the number of ions in each peak was calculated using the method reported by Kim and coworkers (Yoon, Moon, & Kim, 2010b). Unlike in the earlier studies, Kim and coworkers (Moon et al., 2012) also included the abundances of fragment ions generated by the dissociation of a precursor ion occurring inside and outside the source. The total number of the analyte-derived ions was evaluated, which was multiplied by the area ratio (sample area/extended region area) to estimate the total number of the analyte-derived ions from the entire sample. Finally, this was divided by the number of the analyte molecules loaded on the target to get IY for the analyte. The same process was used to obtain IY for the matrix. One of the factors that would affect the accuracy of the method is the sample homogeneity. Samples prepared by air-drying showed small crystallites distributed mostly at the sample boundary. Material distribution was more uniform for vacuum-dried samples prepared with CHCA as the matrix. Figure 11 shows the total numbers of the matrix (CHCA)- and analyte (Y5R)-derived ions emitted from different locations on a sample. Location-dependent fluctuation of the ion signals was ±20% or less.

C. Ion Yields for Some Salts and Gas-Phase Ion Formation from Pre-formed Ions

Before investigating peptides, Kim and coworkers (Moon et al., 2012) studied some salts expecting that the process would

FIGURE 9. (a) $k_{\text{tot}}(E)$s for $[Y_{6} + H]^{+}$ (——), $[Y_{5}K + H]^{+}$ (-----), and $[Y_{5}R + H]^{+}$ (——). Two threshold rate constants are marked. (b) Internal energy distributions for $[Y_{6} + H]^{+}$ at 463 and 881 K.

FIGURE 10. A proton-bound tricyclic transition structure in which a proton interacts both (a) with the side chain of lysine at the C-terminus and (b) with an amide backbone.
simply involve the release of pre-formed ions in the solid into
the gas phase. IYs for benzyltriphenylphosphonium chloride
(BTPP-Cl) and two room temperature ionic liquids, 1-butyl-3-
methylimidazolium hexafluorophosphate (BMI-PF6) and trihex-
yl tetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphi-
nate (THTDP-BTMPP) were measured using CHCA as the
matrix. Since the IY data for the three salts were similar, we will
focus on BTPP-Cl.

The total numbers of BTPP-Cl- and CHCA-derived positive
ions and their IYs versus the analyte amount are listed in Table 3.
IY for pure CHCA was 1.2 \times 10^{-7}, in excellent agreement with
10^{-8}–10^{-7} speculated in the first section of this chapter. IYs of
less than 10^{-3} for BTPP-Cl looked surprisingly
small because one would expect higher emission efficiency for
pre-formed ions in a solid sample. IYs for the salts were close to
IY for insulin reported by Standing and coworkers (Ens
et al., 1991) and IYs for alkylamines reported by Mowry and
Johnston (1993). However, they were smaller than 10^{-3}–10^{-1}
speculated for peptides and proteins by Hillenkamp and Karas
(2007).

In the LS model (Karas, Glückmann, & Scha¨fer, 2000;
Karas & Krüger, 2003; Jaskolla & Karas, 2011), positive cluster
ions consisting of pre-formed cations, matrix neutrals, and
counter anions are released into the gas phase, lose ion pairs,
and form positive analyte ions. For example, detachment of
BTPP^+Cl^- from BTPP[H]^+Cl^- would release BTPP^+ into the
gas phase. An exhaustive search in MALDI and PSD spectra
found no trace of such cluster ions (Moon et al., 2012), which
invalidated the cluster ion part of the model.

A plausible explanation for the small IY for BTPP-Cl is that
a neutral ion pair of BTPP^+ and a counter-anion is bound by a
strong Coulombic force and is not easily detached during
ablation. In fact, the equilibrium constants at 1,000 K estimated
for a neutralization reaction, A^+ + B^- \rightarrow AB, forming an ion
pair with the charge separation of 5 and 6 Å are 3 \times 10^{14} and
1 \times 10^{12}, respectively. Then, IYs for the room-temperature ionic
liquids might be larger than that for BTPP-Cl due to larger

### TABLE 3.
The total numbers of BTPP-Cl- and CHCA-derived positive ions and their IYs in MALDI(CHCA) of BTPP-Cl (further abbreviated as B–C) as a function of the analyte amount in 25 nmol CHCA

<table>
<thead>
<tr>
<th>Number of ions from sample</th>
<th>B–C, pmol</th>
<th>B–C, 10^{-8} CHCA, 10^{3}</th>
<th>B–C, 10^{-8} CHCA, 10^{3}</th>
<th>B–C, 10^{-8} CHCA, 10^{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td></td>
<td>0.52 ± 0.03</td>
<td>1.8 ± 0.2</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td>1.5 ± 0.2</td>
<td>2.1 ± 0.4</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>4.5 ± 0.8</td>
<td>1.8 ± 0.2</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>11 ± 2</td>
<td>1.4 ± 0.2</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>23 ± 1</td>
<td>0.81 ± 0.11</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>28 ± 4</td>
<td>0.29 ± 0.11</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>
charge separation. From similar IYs for the three salts, Kim and coworkers concluded that IYs for salts were not governed by the equilibrium thermodynamics for the neutralization reaction.

From the data in Table 3, IYs for BTPP-Cl and CHCA, which are nearly constant at low analyte concentration, decrease steadily as the analyte concentration increases. In MALDI of peptides, this is called the “matrix suppression effect” (Knochenmuss & Zenobi, 2003; Knochenmuss, 2006) and is taken as evidence for the GPPT. Since proton transfer is not needed in the formation of gas-phase BTPP, the above matrix suppression must occur through the formation of neutral ion pairs, which is more prevalent at higher analyte concentration. Assuming that an ion pair is difficult to break up, Kim and coworkers (Moon et al., 2012) proposed two models to explain very small IY values for salts. In Model 1, the gas-phase cations released by MALDI originate from free cations in the solid sample, which constitute a tiny fraction of pre-formed cations. Following Zenobi and Knochenmuss (1998), Kim and coworkers further elaborated that the dielectric screening by residual solvent, matrix, or non-ionic parts of large molecules would reduce the Coulombic potential for an ion and allow its easy release. Hence, Model 1 was called “the dielectrically screened pre-formed ion emission” model. In Model 2, a pre-formed cation is released as part of an ion pair. Then, anion loss occurs in the gas phase via reaction with a matrix-derived cation. Kim and coworkers (Moon et al., 2012) estimated that a molecule in MALDI plume would suffer around 10^3 collisions. IY of 10^{-7} for CHCA meant that a neutral ion pair containing BTPP^+ would encounter a matrix-derived cation once in every 10^7 collisions. Then, the probability for the ion pair to encounter a cation would be 10^{-4} (=10^3 x 10^{-7}), in agreement with Model 2. This model was named “the ion pair emission/matrix-assisted charge separation” model.

### D. Ion Yields for Peptides and the Thermal Models for Their Formation in MALDI

Kim and coworkers (Bae et al., 2012) carried out similar studies for several peptides by MALDI(CHCA). Since the results were similar for all the peptides studied, we will focus on Y_5R. From MALDI and PSD spectra, they searched for the positive cluster ions that might be the precursors to peptide ions (Jaskolla & Karas, 2011) and concluded that the cluster ion model was invalid, as has also found in MALDI of salts.

The concentration dependence of IY for Y_5R in CHCA is listed in Table 4. The trend in IY of this peptide versus concentration was similar to those of the salts, even though IYs for peptides were a little smaller; for 1 pmol of an analyte in 25 nmol of CHCA, IY for Y_5R was (2.3 ± 0.6) x 10^{-4} versus (7.5 ± 1.4) x 10^{-4} for BTPP-Cl. IYs for peptides were in good agreement with 3 x 10^{-4} for insulin in SA (Ens et al., 1991). However, they were smaller than 10^{-3} to 10^{-4} speculated by Hillenkamp and Karas (2007). IYs for CHCA were virtually the same regardless of the analyte, that is, around 10^{-7} for the samples containing peptides and salts. For example, IYs for CHCA with 1 pmol Y_5R and BTPP were (0.95 ± 0.13) x 10^{-7} and (1.2 ± 0.2) x 10^{-7}, respectively. IY for CHCA agrees with 10^{-2} to 10^{-6} estimated from the data reported by Sundqvist and coworkers (Quist, Huth-Fehre, & Sundqvist, 1994), but much smaller than 10^{-5} reported by Puretzky and Geoffeean (1998), and even more smaller than 10^{-5} to 10^{-3} speculated by Hillenkamp and Karas (2007).

As observed for salts, IYs for peptides and CHCA decreased as the analyte concentration increased. This suppression effect in peptide MALDI had been attributed to the competition for protons between matrix and peptide molecules. It is to be noted that the total number of ions from a sample, that is, the sum of the peptide- and matrix-derived ions, remains constant regardless of the peptide concentration, or is the same as that from a pure matrix (Bae et al., 2012).

The most interesting observation (Bae et al., 2012; Moon et al., 2012) was that IYs for salts and peptides were unaffected by the laser pulse energy. As an example, IYs for Y_5R- and CHCA-derived ions from 1 pmol Y_5R in 25 nmol CHCA measured as a function of laser pulse energy, 4–10 times the threshold, are shown in Figure 12. Independence of IY from the laser pulse energy is surprising because it is known that an ion signal in MALDI increases with the laser fluence (Dreisewerd, 2003), by orders of magnitude near the threshold. A close look at the ion intensity versus the laser fluence data in the literature (Dreisewerd et al., 1995; Westmacott et al., 2002; Dreisewerd, 2003) shows that the ion intensity saturates, or becomes constant, at high fluence. This was usually attributed to extensive ion dissociation and/or detector saturation at high fluence (Westmacott et al., 2002; Dreisewerd, 2003). Peptide ion dissociation was included in the measurement by Kim and coworkers. A thorough test showed that the detector saturation did not occur. Hence, they concluded that the independence of IY from the laser pulse energy was genuine.

In the GPPT model, peptide ions are generated by GPPT occurring in the plume between matrix-derived proton donors and neutral peptide molecules. In addition, laser-induced ionization of matrix molecules, presumably via exciton pooling, is assumed to be responsible for the generation of matrix-derived proton donors. In that case, IY for the matrix, and analyte also, must increase rapidly with the laser fluence, in complete disagreement with the above data. However, the key assumption of the model, that is, the ionization of a peptide by proton transfer from a matrix-derived proton donor, is in agreement with the concentration dependence of IY.

What one observes in a typical MALDI experiment is basically a single shot spectrum obtained at one laser focal spot. In contrast, Kim and coworkers measured IY by integrating all the spectra recorded until the complete depletion of the sample in an extended region. In a single shot MALDI spectrum, more ions will be detected when the material emission efficiency improves or when the material emission efficiency improves. In the laser ablation of molecular solids (Zhigilei et al., 2003), two processes compete near the threshold, thermal desorption and phase
explosion (or ablation). Even though the thermal desorption is the main process at low fluence, the ablation suddenly becomes dominant above a certain critical fluence and saturates. Kim and coworkers suggested that the sudden increase and saturation of ion signals with the fluence in single shot MALDI occur due to the change in the emission process, that is, from desorption to ablation.

Kim and coworkers (Moon et al., 2012) found that the cluster ion part of the LS model is invalid, just as in MALDI of salts. Then, two pathways remain for the LS model, just as for salts. In Model 1, gas-phase peptide ions are formed directly from dielectrically screened peptide ions (pre-formed) in the solid sample. In contrast, Model 2 postulates the release of peptide ions into the gas phase as part of neutral ion pairs, which further undergo a reaction with matrix-derived cations and release the peptide ions. \[\text{CHCA} - \text{H}^- \text{formed by the following condensed-phase reactions will be the counter anion in the ion pair.}\]

\[
\begin{align*}
\text{CHCA} + \text{CHCA} & \leftrightarrow [\text{CHCA} + \text{H}]^+ + [\text{CHCA} - \text{H}]^- \\
Y_5\text{R} + \text{CHCA} & \leftrightarrow [Y_5\text{R} + \text{H}]^+ + [\text{CHCA} - \text{H}]^- 
\end{align*}
\] (6) (7)

In the Knochenmuss’s version of the GPPT model, the assumption of the primary ion formation via laser-induced ionization of matrix must be discarded. Retaining the assumption that a peptide is emitted in its neutral form, the GPPT model becomes an assertion that a peptide ion is generated from the corresponding neutral via transfer of a proton from a matrix-derived species. Based on the appearance of the concentration effect, this proton-donating matrix-derived species must be a CHCA-derived cation(s). Unlike in the original GPPT model, such ions are not formed via laser-induced ionization of a matrix molecule. Rather, such ions may have been formed via thermal reactions such as auto-ionization in the gas phase or in the condensed phase (reaction (6)). This was called Model 3 (Bae et al., 2012).

It is to be noted that Models 1 and 2 deal with the mechanism for the primary ion formation while Model 3 deals with that for the secondary ion formation. Primary peptide ions generated via Models 1 and 2 will suffer many collisions. Then, it is likely that the IY of analyte-to-IY of matrix ratio, or the concentration effect, is determined by the GPPT. Finally, the overall pattern of a MALDI spectrum will be governed by the effective temperatures in the early and late plumes, which, in turn, will be determined by the matrix and the laser fluence used.

Kim and coworkers (Bae et al., 2012; Moon et al., 2012) observed that the maximum number of peptide-derived ions is limited to the number of ions produced from a pure matrix, which is presumably determined by processes such as auto-ionization. Then, the driving force for MALDI is the thermal energy gained by the sample through the electronic excitation of matrix molecules followed by intramolecular relaxation processes such as the internal conversion. In this sense, the models for the gas-phase peptide ion generation in MALDI derived from the study of the ion yield may be called “thermal” (Bae & Kim, 2013).

VI. CONCLUSION

The following thermal explanations for the generation of gas-phase peptide ions and their dissociation in MALDI have emerged from the studies of ion yield and dissociation kinetics.

1. Matrix- and peptide-derived gas-phase primary ions are generated by one or both of the two processes, “the dielectrically screened pre-formed ion emission” and “the ion pair emission/matrix-assisted charge separation.” In particular, the matrix-derived primary ions may be formed by thermal processes such as auto-ionization, not by laser-induced ionization.

2. The total number of ions, that is, matrix plus peptide, generated by MALDI is equal to those from a pure matrix sample. The number of each type of ions, that is, matrix- or peptide-derived, is determined by GPPT occurring in the early plume.

3. A matrix plume cools as it expands. For example, the effective temperatures of CHCA in the early and late plumes are roughly 900 and 450 K, respectively. Rapid dissociation in the early plume contributes to ISD via b/y channels. For peptide ions that have undergone the expansion cooling, some may dissociate slowly (PSD) while others survive until they arrive at the detector. The
exothermicity in the GPPT is not the driving force for ISD via b/y channels. Also, PSD is not driven by low energy collisional activation.

4. Dissociation of peptide ions along b/y channels occurs statistically (RRKM theory). Even though basic residues tremendously affect the dissociation efficiency, the effect arises from small differences in $E_0$ and $\Delta S^\circ$ with the former lying in the range 0.6–0.7 eV and the latter in the range –30 to –25 eu. The rate-determining cleavages of amide bonds occur via transition structures which are stabilized by multiple intramolecular interactions and hence are entropy bottlenecks.

**ACKNOWLEDGMENTS**

This work was financially supported by the National Research Foundation, Republic of Korea. Y.J. Bae thanks the Ministry of Education, Science and Technology, Republic of Korea, for Brain Korea 21 Fellowship.

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