Review

Study of Ion Dynamics by Electron Transfer Dissociation: Alkali Metals as Targets

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High energy collision processes for singly charged positive ions using an alkali metal target are confirmed, as a charge inversion mass spectrometry, to occur by electron transfers in successive collisions and the dissociation processes involve the formation of energy-selected neutral species from near-resonant neutralization with alkali metal targets. A doubly charged thermometer molecule was made to collide with alkali metal targets to give singly and doubly charged positive ions. The internal energy resulting from the electron transfer with the alkali metal target was very narrow and centered at a particular energy. This narrow internal energy distribution can be attributed to electron transfer by Landau–Zener potential crossing between the precursor ion and an alkali metal atom, and the coulombic repulsion between singly charged ions in the exit channel. A large cross section of more than $10^{-11}$ cm$^2$ was estimated for high-energy electron transfer dissociation (HE-ETD). Doubly protonated phosphorylated peptides obtained by electrospray ionization were collided with Xe and Cs targets to give singly and doubly charged positive ions. Whereas doubly charged fragment ions resulting from CAD were dominant in the case of the Xe target, singly charged fragment ions resulting from ETD were dominant with the Cs target. HE-ETD using the Cs target provided all of the z-type ions by N–C$_n$ bond cleavage without the loss of the phosphate groups. The results demonstrate that HE-ETD with an alkali metal target allowed the position of phosphorylation and the amino acid sequence of peptides with post translational modifications (PTM) to be determined.

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INTRODUCTION

Since the demonstration by J. J. Thomson that charged particles in the gas phase can be separated based on differences in mass-to-charge ratios, mass spectrometry has developed into an analytical method with an enormous range of applications in many areas of science. One of the most valuable techniques in mass spectrometry, tandem mass spectrometry (MS/MS), involves the excitation of mass-selected ions followed by the dissociation of the sample into product ions, the mass/charge (m/z) ratios of which can also be measured. There are a variety of scan modes in tandem mass spectrometry, which constitute powerful tools that provide structural information on ions and/or classes of ions in the gas phase. Tandem mass spectrometry experiments are also a rich source of information on energetics and mechanisms of fragmentation processes associated with internally excited ions.

At first glance, mass spectrometric methods appear to be limited to the analysis of charged particles, however, the development of MS/MS techniques such as neutralization-reionization mass spectrometry (NRMS) and charge inversion mass spectrometry have made it possible to characterize neutral species in the gas phase. NRMS involves the formation of a reactive neutral fragment by neutralization of the corresponding ion followed by a reionization step, after which, the generated ionic species are mass-analyzed and detected. Most NRMS experiments involve the detection of positive ions formed by the neutralization-reionization of primary positive ions. NRMS cannot be performed using a low-energy type mass spectrometer, such as FT-ICR-MS or ion trap, because the transient neutral fragments cannot be controlled by the electro-magnetic field. In high-energy mass spectrometry, most of the transient neutral fragments move along in the same direction as the precursor ions and enter the second mass spectrometer, where the fragment ions are mass-analyzed after reionization. In the case of charge inversion mass spectrometry, mass selected...
precursor ions are made to collide with a gaseous target (or less frequently with a target surface) and the resulting product ions, with a polarity opposite to the precursor ion formed upon two-electron transfer, are mass analyzed and detected. This type of MS/MS experiment is also known as charge permutation (CP) or charge reversal (CR) mass spectrometry. Most charge inversion reactions are performed in the keV energy range mainly because, at these energies, electron stripping and electron transfer reactions have effective cross sections. It should be noted that the charge inversion process from positive ions to form negative ions using alkali metal gaseous targets is the most effective process in various types of charge inversion mass spectrometry.

The mass analysis of proteins and peptides has become possible since the development of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) techniques. Dissociation processes for these ions have been studied in order to obtain information regarding the dissociation of molecular projectile ions, particularly amino acid sequences in peptides and proteins. Although the mass analysis of phosphorylated proteins and peptides is now possible, the determination of the position of the phosphorylation has not yet been achieved by the most popular dissociation method, namely, low-energy collisionally activated dissociation (LE-CAD) performed by using the tandem mass spectrometry (MS/MS). Electron capture dissociation (ECD) using a Fourier transform ion cyclotron resonant mass spectrometer (FT-ICR-MS) and electron transfer dissociation (ETD) using a linear trap mass spectrometer can provide information regarding c- and z-type ions that are produced via cleavage of the N–Cα backbone with no loss of labile PTM groups, such as phosphate and sulfate groups. N–Cα backbone cleavage is different from other fragmentations brought about by collisional activation or the infrared photodissociation of closed-shell cations. The mechanism of N–Cα bond cleavage reactions from charge reduced peptide cation radicals formed by ECD and ETD is known to provide dissociation at various positions via the low energy transition-state for bond cleavage. Differences between ECD and ETD have also been reported. N–Cα bond cleavage induced by electron transfer processes of multiply charged peptides on collision with neutral targets, such as alkali metals or metastable rare gases, have also been reported. Because the dissociation behavior of isolated molecules is controlled by the internal energy distribution of the molecular population, it is of fundamental importance to be able to characterize this distribution for charged ions whose charge is reduced by electron transfer. Current interest in the dissociation of multiply charged ions of large biomolecules also makes it important to develop methods for characterizing their internal energy distributions. Theoretical calculations for estimating internal energy and cross section are virtually impossible for electron transfer processes that involve the production of multiply charged ions derived from large biomolecules, because of the multitude of ion structures and dissociation pathways involved.

Wysocki et al. reported on the use of so-called thermometer molecules to measure the distribution of internal energy of gas-phase ions. Using the same method, Cooks and coworkers determined the internal energy distributions, for doubly charged ions following electron transfer in high-energy collisions by using a sector-type mass spectrometer and in low-energy collisions by using a triple quadrupole mass spectrometer with various atomic and molecular targets. Differences between the deposition of internal energy induced by collisional activation and electron transfer were investigated using W(CO)6 ions on collision with a rare gas and with an alkali metal target. The internal energy deposition in collisionally activated dissociation (CAD) evaluated with a rare gas target was broad and decreased with increasing internal energy. On the contrary, internal energy depositions evaluated with alkali metal targets were very narrow and centered at a particular energy. From these findings, we proposed that the term “collision-induced dissociation (CID)” taken literally, includes all dissociation processes induced by collisions, and therefore encompasses both CAD and ETD processes in the present work. Although the terms CID and CAD have been defined similarly, we propose that they should not be used interchangeably, based on the fact that there are differences in the observed ions and in their intensities with rare gas and alkali metal targets.

In this review article, the author present a concise review of charge inversion mass spectrometry by using a rare gas target, since two review articles were already appeared. The internal energy distribution of HE-ETD measured using a thermometer molecule is discussed. The usefulness of HE-ETD using an alkali metal target for the sequencing of peptides with PTM is discussed.

**CHARGE INVERSION MASS SPECTROMETRY**

In charge inversion mass spectrometry, one of the branches of the MS/MS method, the electric charge of the precursor ions is opposite that of the secondary product ions. Charge inversion mass spectrometry is classified into four types, depending on the electric charge and time scales of the collisions. In the case of a rare gas or a simple molecular target, a double electron transfer in one collision is effective for forming positive ions from negative ions, while, in the case of alkali metal targets, successive single electron transfers involving two collisions is effective for forming negative ions from positive ions. The author measured the internal energy distribution of neutral species formed through neutralization in charge-inversion mass spectrometry with alkali metal targets by using the thermometer molecule W(CO)6 and a partially deuterated molecule. The internal energy distributions in charge-inversion mass spectrometry indicated that dissociation occurs in the energy-selected neutral species that is formed by way of near-resonant neutralization. On the basis of the observed target-density dependence of the product ion intensity and thermochemical considerations for internal energy distribution using thermometer molecules, charge inversion processes using alkali metal targets were confirmed to occur by electron transfer in successive collisions and the dissociation processes were found to occur in energy-selected neutral species formed from near-resonant neutralization with alkali metal targets. While CAD is due to the dissociation of activated ions with broad internal energy distributions, the charge inversion processes using an alkali metal target is due to the dissociation of energy-selected neutral species with narrow internal energy distributions.
Charge inversion spectra using alkali metal targets provided clear differentiation of the isomeric cations of CH$_3$I, CH$_3$Br, and CH$_3$Cl isomers, along with the formation of a fragment pair in the ground internal states. From a comparison of the kinetic energy release (KER) value calculated from the trapezoidal shape of $\Gamma$ with the available energy of the near-resonant level on the CH$_3$I potential energy curve, as reported by ab initio calculations, the trapezoidal part can be attributed to the dissociation to CH$_3$I$^+$I$^-$ via the repulsive $^3$Q state of CH$_3$I, which is not dominant in the photo-dissociation of CH$_3$I. The trapezoid shape of the CH$_3$I$^+$ peak that was observed only with the Cs target shown in Fig. 1(g) indicates spontaneous dissociation via a repulsive potential from the $^3$R$_2$ Rydberg state, although the correlation between the $^3$R$_2$ Rydberg state and relevant repulsive states has not been reported by any theoretical calculations.

Charge inversion mass spectrometry using alkali metal targets for singly protonated peptides and amino acids provide information on radical traps in the dissociation mechanism of the charge reduced doublet state peptides, as evidenced by a computational study. For multiply charged peptides upon collision with alkali metals, N-C$_n$ bond cleavage induced by electron transfer processes have been reported by Hvelplund et al. A combined experimental and computational study of the effect of proline in model dipeptides, i.e., Pro–Gly and Gly–Pro, was performed. Gas-phase protonated peptide ions were discharged by collision with potassium or cesium atoms at 3 keV collision energies, and the peptide radical intermediates and their dissociation products were analyzed following collisional ionization to anions. As shown in Fig. 2, dramatic differences were observed in the charge inversion mass spectra of (Pro–Gly+H)$^+$ and (Gly–Pro+H)$^+$, depending on the precursor ions, which provided a sensitive probe of ion structure.

![Fig. 1. Charge inversion (high-energy electron transfer dissociation) spectra of CH$_3$X (X=Cl, Br and I) measured using alkali metal targets: (a) CH$_3$Cl$^+$, Cs; (b) CH$_3$Cl$^+$, K; (c) CH$_3$Cl$^+$, Na; (d) CH$_3$Br$^+$, Cs; (e) CH$_3$Br$^+$, K; (f) CH$_3$I$^+$, Na; (g) CH$_3$I$^+$, Cs; (h) CH$_3$I$^+$, K; (i) CH$_3$I$^+$, Na. Reproduced from S. Hayakawa, T. Tsujinaka, A. Fujihara, Dissociation mechanisms of excited CH$_3$X (X=Cl, Br, and I) formed via high-energy electron transfer using alkali metal targets, J. Chem. Phys. 137: 184308 2012, with the permission of AIP publishing.](image1)

![Fig. 2. Charge inversion mass spectra of (a) (Pro–Gly+H)$^+$ and (b) (Gly–Pro+H)$^+$ obtained with cesium as the collision target. (Insets) Kinetic energy peak profiles in the charge inversion mass spectra. Reprinted with permission from S. Hayakawa, M. Hashimoto, H. Matsubara, F. Turecek, J. Am. Chem. Soc. 129: 7936–7949, 2007. Copyright (2007) American Chemical Society.](image2)
Whereas (Pro–Gly+H)\(^+\) completely dissociated upon charge inversion, (Gly–Pro+H)\(^+\) gave a non-dissociated anion as the most abundant product. \textit{Ab initio} and density functional theory calculations with the 6-311++G(3df,2p) basis set provided structures and vertical recombination energies \(RE_{\text{v}}\) for (Pro–Gly+H)\(^+\) and (Gly–Pro+H)\(^+\). The latter energies, \(RE_{\text{v}}=3.06\) and 3.36 eV for (Pro–Gly+H)\(^+\) and (Gly–Pro+H)\(^+\), respectively, were lower than the values for the alkali metal ionization energies and indicated that the collisional electron transfer to the peptide ions was endoergic. A radical (Pro–Gly+H)\(^+\) species was found to exist in a very shallow local energy minimum, with transition state energies for the loss and migration of H indicating very facile dissociation. In contrast, calculations for the (Gly–Pro+H)\(^+\) radical species indicated that it spontaneously isomerized upon electron capture to a stable dihydroxycarbiny1 isomer which could then undergo further isomerization by proline ring opening and intramolecular hydrogen atom transfers, thus yielding other stable radical isomers. Calculations regarding the dihydroxycarbiny1 radical and its stable isomers indicated that it has substantial electron affinities and thus can form stable anions, which were observed in the charge inversion mass spectra. RRKM kinetic analyses of the dissociation of the dihydroxycarbiny1 isomer indicates large kinetic shifts for the main channels which are ring cleavages in competition with the loss of aminoketyl hydrogen atoms. Theoretical predictions for radicals formed by electron transfer to a protonated arginine residue indicate that the hydrogen atom from the \(C_{\alpha}\) position undergoes an inverse migration to the guanidine carbon atom. Experiments intended to confirm that a fraction of arginine and arginine amide radicals undergo such an inverse hydrogen migration have been reported.\(^{46}\) The rearranged arginine and arginine amide \(C_{\alpha}\) radicals are detected as stable anions after charge inversion by collisions with Cs atoms of precursor cations at kinetic energies of 3 keV. RRKM calculations on the B3-P3P2/aug-cc-pVTZ potential energy surface indicate that the side chains of arginine radicals undergo rapid rotation, to produce conformers suitable for \(C_{\alpha}\)-H transfer which were calculated to be fast (\(<10^8\) s\(^{-1}\)) in radicals formed by electron transfer. By contrast, H-atom transfer from the guanidine group to the carboxyl or amide C=O groups is >50 times slower than the \(C_{\alpha}\)-H migration. The guanidine group in an arginine radical is predicted to be a poor hydrogen atom donor but a good H-atom acceptor and thus can be viewed as a radical trap. This property can explain the frequent observations of non-dissociating peptide cation radicals in electron capture and electron transfer mass spectra.

**NEW INSTRUMENTS FOR HIGH-ENERGY ELECTRON TRANSFER DISSOCIATION (HE-ETD)**

In order to investigate HE-ETD processes in which alkali metal targets are used, a sector type tandem mass spectrometer (MS/MS) was developed in collaboration with the Toyoda group at Osaka University.\(^{46}\) Mass-selected doubly charged ions were made to collide with alkali metal targets, and the resulting singly and doubly charged ions that formed upon electron transfer and collisional activation were mass analyzed by using the MS/MS instrument, as shown schematically in Fig. 3. The thermometer molecules formed doubly charged ions by 70 eV electron ionization. Doubly protonated peptides were formed using an electrospray ionization source (JEOL MS-AP110/BU). The formed doubly charged ions were accelerated to a kinetic energy of 10 keV by an accelerating voltage of 5 kV. The precursor ions were mass selected by a JEOL JMS-HX110 double-focusing mass spectrometer (MS-I). The central radius of the electric sector was 381 mm, and the magnetic field had an ion-orbital radius of 720 mm. The mass-selected precursor ions entered a 3.7 cm long collision cell located at MS-II, after refocusing by a Q-lens at the exit of MS-I. The alkali metal target was introduced into the collision cell in the form of a vapor from a reservoir through a ball valve. The collision energy in the laboratory system was 10 keV, since the target atoms were in thermal equilibrium in the collision cell. The singly and doubly charged positive ions were mass analyzed by a spherical electrostatic analyzer (ESA) with a central radius of 216 mm (MS-II), of the same type as that found in the Hitachi M80B instrument. The mass-analyzed ions were detected by a 9 kV post-acceleration secondary-electron multiplier. The collision cell was mounted in a

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Fig. 3. Schematic for the MS/MS instrument. Doubly charged positive ions are mass selected by MS-I. Alkali metal targets are introduced into the collision cell. Collisionally induced dissociation (CID) spectra of singly and doubly charged ions are mass analyzed by using MS-II.
vacuum chamber consisting of a perpendicular cylinder with an inner diameter of 112 mm and a height of 118 mm. A wedge-shaped slit 300 μm wide and 4.3 mm high in front of the collision cell confined the ions that entered the collision cell. The entrance opening of the collision cell was 1 mm wide and 8 mm high and the exit opening was 2 mm wide and 10 mm high. The position of the collision cell, which could be moved in a direction perpendicular to the ion beam, was adjusted so that the incident ions did not collide with either the entrance hole or the exit hole. Both the vacuum chamber and MS-II were differentially evacuated by Seiko STP-300H turbo molecular pumps. The pressures, as measured by ionization gauges mounted on both the vacuum chamber and on MS-II, were less than $6 \times 10^{-5}$ Pa when the target gas was not supplied.

CID spectra were measured by mass analyzing the singly and doubly charged positive ions leaving the collision cell by scanning the electric field in MS-II from 0 to twice the electric field required to cause the primary doubly charged ions to pass through the MS-II.46 The temperatures of the collision cell, the ball valve, and the reservoir were adjusted appropriately so as to control the density of alkali metal in the collision cell. The temperature of the collision cell was set to be higher than those of both the ball valve and the reservoir so that the alkali metal vapor did not deposit in the collision cell. The total gas conductance of the entrance and the exit opening of the collision cell, through which the target gas was evacuated, was $3 \text{ dm}^3 \text{s}^{-1}$. When the supply of alkali metal vapor from the reservoir was stopped by closing the ball valve, the pressure of the alkali metal vapor in the collision cell decreased to one-hundredth of its original value within 1 s, as estimated from the conductance of the holes in the collision cell and assuming a molecular flow. On the other hand, because the pressure of the alkali metal target is markedly dependent on the temperature and gas conductance from the reservoir to the target chamber,46 a precise estimation of the pressure was difficult as a result of using the ball valve. By changing the collision cell, the rare gas target could be introduced in a collision cell of the same size as the alkali metal target. The density of the rare gas target was controlled by means of a variable-leak valve (Whitey SS-22RS2). The target pressure was directly measured by means of a capacitance manometer (MKS Baratron 127AA-00001B) attached by a pipe with a 26 mm inner diameter and a gas conductance of $11 \text{ dm}^3 \text{s}^{-1}$.

High resolution TOF/TOF, which can be applicable to HE-ETD using alkali metal targets, was developed in collaboration with the Toyoda group at Osaka University.46 A preliminary type of the TOF/TOF in which the precursor ions were not selected in high resolution has already been published.41,62 Multiply protonated precursor ions were continuously formed by the ESI source. When the voltage of the exit part of the triple quadrupole ion guides was switched to the ground potential, ions flowed into the orthogonal acceleration (OA) region. The ions in the OA region were accelerated by applying a pulsed voltage to the acceleration grid. After a two stage acceleration, the voltage of the potential lift was switched to the ground potential during which the ions were present in the potential lift tube. Using this method, high-energy pulsed ions were obtained without high-voltage floating for the ESI source and the TOF mass spectrometer. MULTUM (MULTUM-S II)63 consists of four electrostatic (toroidal) sectors and two additional sectors. After an ion is injected in MULTUM, the voltage of the injection sector is turned off. Ions cycle in the MULTUM, until the voltage of the ejection sector is turned on. Since the peaks in the spectra represent ion clusters derived from a doubly protonated molecule, $m/z$ differences in these peaks were about 0.5. As the number of turns is increased, different isotopic peaks were distanced from each other. The resolution in 200 turns, as evaluated from the full width at half maximum of the mono-isotopic ions, exceeded 40,000. In order to obtain precise sequence information, mono-isotopic ions must be selected to enter the collision cell. To select the mono-isotopic ions, an ion-gate was installed in front of the collision cell. When the plus minus $50 \text{ V}$ of the ion-gate was switched to the ground potential at the time that the mono-isotopic ions were present in the ion-gate, other ions were deflected and the isolation of mono-isotopic ions was achieved. After the collision cell was filled with an alkali metal vapor, HE-ETD spectra of doubly protonated ions can be obtained. Fragment ions of low $m/z$ were reflected in lower voltage region in the quadrupolar field ion mirror,62,64 and the TOF became shorter than that of the precursor ions. On the other hand, since a charge reduced ion was reflected in the higher voltage region, the TOF then became longer than that of the precursor ion. Original HE-ETD spectra were obtained as a function of time of flight. Since the TOF of the fragment in the mirror was roughly proportional to the square root of the $m/z$ value, it is necessary to convert the horizontal axis to an $m/z$ value in order to compare other MS/MS spectra, and to identify the $m/z$ value of the observed peaks. While the size of the sector type instrument is about $4.5 \times 2.0 \text{ m}$, the size of the TOF/TOF is $1.4 \times 0.7 \text{ m}$. The much smaller TOF/TOF provided much higher resolution, as outlined below.

REACTION PROCESS AND INTERNAL ENERGY DISTRIBUTION IN HIGH ENERGY COLLISION

The CID of multiplied charged ions at high-energy may involve CAD and ETD. Unlike charge inversion mass spectrometry, fragment ions can be detected in single event of the electron transfer process because the charge reduced species have a remaining electric charge. In order to clarify the difference of dissociation process between CAD and ETD, internal energy distributions of both the rare gas and alkali metal targets were measured using a thermometer molecule W(CO)$_6$.36,37 CID spectra of the doubly charged tungsten hexacarbonyl cation W(CO)$_6^{2+}$ with Ar, K and Cs as the targets are shown in Figs. 4(a), 4(b), and 4(c). A clear differences in the CID spectra were observed between the rare gas Ar target and the alkali metal K and targets. Singly charged fragment ions were formed by electron transfer followed by dissociation, and doubly charged fragment ions were formed by collisional activation (CA). In the CID spectrum with the Ar target [Fig. 4(a)], the non-dissociated W(CO)$_6^{2+}$ peak at $m/z=176$ is, by far, the strongest. The high intensity of this W(CO)$_6^{2+}$ peak can be attributed to a precursor ion that does not interact with the target. The main dissociative peaks at $m/z=120, 134, 148$, and 162 are associated with W(CO)$_6^{n+}$ $(m=2\sim5)$ ions resulting from cleavage of the W–CO bonds
of doubly charged W(CO)$_2$ ions. The peak intensity decreases with increasing number of CO ligands lost. Singly charged ions at $m/z=184, 212, 240, 268, 296,$ and 324 are also observed, although the intensities of these ions are much weaker than those of doubly charged ions. The intensity distribution of the fragment ions in Fig. 4(a) indicates that the major process in CID with an Ar target is the dissociation of collisionally activated doubly charged ions. CID spectra with an Ar target were obtained for target pressures between 0.6 and 2.7 Pa. Whereas the relative abundances of the fragment peaks to the precursor ion peak in these spectra were dependent on the target pressure, the ratio of the abundances of the each of the fragment peaks to the total abundance of the fragment peaks was not dependent on the target pressure within an uncertainty of ±5%. This constancy in the relative abundances of the fragment peaks and the transmissions indicate that the fragment ions arose from single collisions.

In the CID spectrum with both K [Fig. 4(b)] and Cs [Fig. 4(c)] targets, the predominant peaks at $m/z=240$ and 268 are associated to give singly charged W(CO)$_2^+$ and W(CO)$_3^+$ ions, respectively. The singly charged fragment W(CO)$_2^+$ and W(CO)$_3^+$ ions result from electron transfer followed a loss of four and three CO ligands, respectively. The relative abundances of these two peaks are much different between the K and Cs targets. The relative abundance of the peak associated with W(CO)$_3^+$ ions compared with that for W(CO)$_2^+$ ions was determined to be 1.0:1.66 with the K target in Fig. 4(b) and 1.0:0.49 with the Cs target in Fig. 4(c). The relative abundances associated with W(CO)$_2^+$ ($m/z=212$) and W(CO)$_3^+$ (m/z=240) ions in the Cs target [Fig. 4(c)] are higher than those for the K target [Fig. 4(b)]. The higher intensity of the lower mass ions with the Cs target compared to that with the K target indicates that the internal energy of the charge-reduced W(CO)$_2^+$ ions obtained on collision with the Cs target was higher than that with the K target.

Very weak peaks associated with doubly charged W(CO)$_3^{2+}$ ($m=4, 5$) ions resulting from cleavage of the W–CO bonds are observed at $m/z=148$ and 162 in both spectra of Figs. 4(b) and (c). The relative abundances were evaluated from the average of several CID spectra measured at different target pressures under single-collision conditions. Contrary to CID spectra obtained using an Ar target Fig. 4(a), the peak intensities of the doubly charged fragment ions are much lower than those associated with singly charged W(CO)$_4^+$ ($m=2, 3$) ions. The excessive intensity difference between the singly charged ions and doubly charged ions indicate that the electron transfer process was much more effective than CA in the case of collisions with alkali metal targets. This result can be explained by the fact that the difference in the cross section of the electron transfer is the doubly charged ions on collision with the alkali metal targets, which is of the order of $10^{-14}$ cm$^2$, is much larger than that for CA, which is of the order of $10^{-16}$ cm$^2$.

A method for evaluating internal energy distribution $P(z)$ was reported by Wysocki et al. by using the peak abundances in the spectra and thermochemical data. Thermochemical data for IE/AE values for singly charged ions and for doubly charged ions reported by Wysocki et al. and Cooks et al. are shown in Table I. This method assumes that all of the ions that have an internal energy to permit them to dissociate do so and that they undergo the most endothermic reaction available to them. All the charge reduced W(CO)$_2^+$ ions with energies between the
activation energies for the formation of W(CO)\textsubscript{m} ions and W(CO)\textsubscript{m} ions [\Delta E(m) and \Delta E(m-1), respectively] are assumed to undergo fragmentation to yield W(CO)\textsubscript{m} ions. The relative abundance of each fragment W(CO)\textsubscript{m} is taken as a measure of the number of charge reduced W(CO)\textsubscript{m} ions with internal energies between \Delta E(m) and \Delta E(m-1). The abundances of fragment W(CO)\textsubscript{m} ions divided by the energy range \Delta E(m-1)–\Delta E(m) gives the data height in \textit{P}(\delta) between \Delta E(m) and \Delta E(m-1), which allows a comparison to be made between the relative abundances in \textit{P}(\delta) and those in the CID spectra.

The energy values of the fragments were scaled from the ground state of the precursor W(CO)\textsubscript{m} and a neutral alkali metal, which was the entrance channel of the collision. The average energy level of W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+} ions for K and Cs targets, as estimated from the internal energy distribution for the electron transfer process, were determined to be \(-7.8\) and \(-6.6\) eV, respectively. The energy level of \(-7.8\) (or \(-6.6\) eV) corresponds to the energy level of the W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+} ions (or Cs\textsuperscript{+}) reaction. The energy level of the exit channel of W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+} (or Cs\textsuperscript{+}) in the reaction of the present work was higher than that of W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+} (or Cs\textsuperscript{+}) by an amount equal to the ionization energy of the respective target. Since the ionization energies of K and Cs are 4.34 and 3.89 eV, respectively, the energy differences of 2.7 and 3.5 eV were evaluated to be those between the entrance channel of W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+} (or Cs\textsuperscript{+}) and the exit channel of W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+}\textsuperscript{-}K\textsuperscript{+} (or Cs\textsuperscript{+}). The process most likely to be responsible for electron transfer is Landau–Zener potential crossing\textsuperscript{30,31} between a W(CO)\textsubscript{m}\textsuperscript{+}\textsuperscript{+} ion and an alkali metal atom, and the coulombic repulsion between singly charged ions in the exit channel. In this model, the doubly charged ion and the target gas approach each other along an ion-induced dipole curve; at some critical distance (R\textsubscript{c}), an electron transfer takes place and the two singly charged positive ions exit on a coulombic repulsion curve. The energy difference (\Delta E) for this process between the entrance and the exit channels can be calculated by using Eq. (1),

\[
\Delta E = e^2/[4\pi\varepsilon_0 R_c] + \alpha(2e)^2/[32\pi^2\varepsilon_0^2 R_c^4]
\]

where \(e\) is the electric charge, \(\varepsilon_0\) is the permittivity of a vacuum, and \(\alpha\) represents the dipole polarizability of the target. The energy levels of the entrance channel decrease with decreasing internuclear distance, because of the large polarizabilities for the K and Cs targets. Based on the energy difference between the entrance and exit channels evaluated from the maximum in the internal-energy distribution, the critical distances of the electron transfer of W(CO)\textsubscript{m}\textsuperscript{+} and K and Cs targets were evaluated to be 5.88\times10\textsuperscript{-8} and 7.11\times10\textsuperscript{-8} cm, respectively. This potential crossing at this large internuclear distance meets the criterion for electron transfer by Landau–Zener potential crossing. The decrease in energy as a result of the ion-induced dipole in the entrance channel at critical points from the infinite internuclear distance is 1.05 eV for the K target and 0.67 eV for the Cs target. If these decreases are neglected, the critical distances for the K and Cs targets become 4.11\times10\textsuperscript{-8} and 5.33\times10\textsuperscript{-8} cm, respectively. The more than 25% decrease in the energy and the difference in the critical distances indicate that the decrease in energy as a result of an ion-induced dipole cannot be neglected in evaluating the critical distance for electron transfer in cases in which alkali metal targets are used. Whereas the ratio of the estimated critical distances between K and Cs is 0.83, the ratio of the internuclear distances for diatomic molecules is 0.87. The larger values of the critical distance compared with the internuclear distances of the molecules and the similarity of their ratios suggests that electron transfer between doubly charged ions and an alkali metal target takes place from the outermost s orbital.

While the cross section estimated on the basis of an internuclear distance of 5.88\times10\textsuperscript{-8} cm for the K target is 1.09\times10\textsuperscript{-14} cm\textsuperscript{2}, that of 7.11\times10\textsuperscript{-8} cm for the Cs target is 1.59\times10\textsuperscript{-14} cm\textsuperscript{2}. The cross section of the order of 10\textsuperscript{-18} cm\textsuperscript{2} estimated in the present work is much larger than that for collisional activation, which was estimated to be in order of 10\textsuperscript{-20} cm\textsuperscript{2}. The electron transfer processes of multiply charged ions of large biomolecules that follow collision with alkali metal targets provide information regarding the dissociation of molecular projectile ions, particularly amino-acid sequences in peptides and proteins, in the same manner as do the isomeric differentiations in charge-inversion mass spectrometry\textsuperscript{31,12} as described below.

**HE-ETD OF PEPTIDES WITH POST-TRANSLATIONAL MODIFICATIONS**

Post-translational modifications (PTMs) of proteins are of great importance in the activation, localization, and regulation of protein functions in vivo. Reversible protein phosphorylation, principally localized on serine, threonine or tyrosine residues, is one of the most important and well-studied PTMs. Phosphorylation plays crucial roles in the regulation of cellular processes such as cell cycle, growth, apoptosis and signal transduction. In order to demonstrate that the sequence of a phosphopeptide and the position of phosphorylation can be determined by HE-ETD using an alkali metal target in conjunction with a sector type MS/MS instrument, the single synthetic peptide (YGGMRQET(p)VDC) was utilized as a representative of phosphopeptide.\textsuperscript{61} Doubly-protonated phosphopeptide (YGGMRQET(p)VDC) ions obtained by electrospray ionization were collided with Xe and Cs targets to give singly- and doubly-charged positive ions via CID. The resulting ions were analyzed and detected by using an electrostatic analyzer (ESA). Whereas doubly-charged fragment ions resulting from CAD were dominant in the CID spectrum with the Xe target, singly-charged fragment ions result-
ing from ETD were dominant in the CID spectrum with the Cs target. The most intense peak resulting from ETD was estimated to be associated with a charged reduced ion in which H₂ had been abstracted from the precursor. Five c-type fragment ions with amino acid residues consecutively detached from the C-terminal were clearly observed without a loss of the phosphate group, and four z-type ions formed by analogously detaching amino acids from the N-terminal were also observed. The observation of c-type and the z-type ions are presumed to result from the cleavage of the N–C₉ backbone, as is known for ECD and ETD from negative ions. The dissociation process took place on a time scale shorter than 4.5 µs which was the passage time between the collision cell and the entrance of the electric sector. This time scale is much shorter than that for a FT-ICR instrument¹⁶,¹⁷ or for ETD in a linear trap instrument.¹⁸,¹⁹

The efficiency of ETD for the doubly protonated peptide using the alkali metal target was approximately ten times larger than that of CAD, the same as the thermometer molecule.²⁰ It was demonstrated that high energy ETD using an alkali metal target can be useful for determining the position of phosphorylation and the amino acid sequence of peptides with PTM.

In order to demonstrate the usefulness of HE-ETD with the alkali metal target, HE-ETD was applied to a series of synthesized phosphopeptides that contain two phosphorylation sites TGFLTP(E)Y(p)VATR (I) and in which different amino acid residues are phosphorylated YGGMHRQEX(p)VDC (2: X=S, 3: X=T, 4: X=Y), and were compared with those obtained for LE-ETD.²¹ Experimental results for high-energy collision induced dissociation (HE-CID) using the Xe and Cs targets were obtained for doubly-phosphorylated peptides TGFLTP(E)Y(p)VATR (I). While the most abundant product ions in the case of the Xe target were immonium ions and doubly-charged fragment ions, charge-reduced fragment ions were produced in the case of the Cs target, whose relative abundances were about ten times larger than those of the immonium ions and the doubly-charged fragment ions. The difference between the Xe and Cs targets indicates that collisions with the Cs target allow a much more effective ETD than CAD. In the CID spectra with the Cs target, c-type and z-type ions produced via backbone cleavage were clearly observed for all the peptides. For I, in particular, z-type ions from z-1 to z-11 were clearly observed. These fragment ions provided information on the complete peptide sequence and the position of the phosphorylation. Although HE-CID using the Xe target did not provide information on the amino acid sequence as a result of HE-CAD process, HE-CID using the Cs target provided all of the z-type ions without any loss of phosphate groups as a result of HE-ETD process. LE-ETD were obtained using a fluoranthene anion with a LTQ-XL (ThermoFisher Scientific, Waltham, MA, USA). While LE-ETD gave only z-type ions from z₁ to z₁₁, no charge-reduced fragment ions were observed in the mass region lower than that of the precursor ion in the spectra of LE-ETD. HE-ETD provided peptide sequences in the full mass range without losing phosphate groups. The CID spectrum of I indicated that only HE-ETD could reveal the position of phosphorylation, irrespective of the number of residues that were phosphorylated. HE-ETD was also applied to three other phosphopeptides YGGMHRQEX(p)VDC (2: X=S, 3: X=T, 4: X=Y). The CID spectra of 2, 3 and 4 showed a very similar trend as that for I, which provided c-type ions from c₁ to c₉ and z-type ions from z₁ to z₁₁ formed via N–C₉ bond cleavage without the loss of the phosphate group. These results indicate that HE-ETD is capable of providing information on peptide sequences, regardless of the amino acid that is phosphorylated. The finding that only z-type ions were observed for I represents a large difference between 1 and peptides 2–4 and was explained by the position of the arginine residue, whose side chain has the largest proton affinity among the amino acids. The difference in the results for HE-CID between the Xe and Cs targets demonstrates that the HE-ETD process with a Cs target was much more dominant than collisional activation. The difference between HE-ETD using Cs targets and LE-ETD using an anion demonstrated that mass discrimination was much weaker in the case of a high-energy process.

In order to examine the differences in the ETD spectra depending on the MS/MS instrument, ETD spectra of a doubly protonated phosphopeptide were obtained by TOF/TOF, the sector type and the ion trap, where HE-ETD and LE-ETD spectra were identical, as reported in previous studies,²⁵,²⁶ respectively. Figure 5 shows enlarged CID spectra of the doubly protonated phosphopeptide.

Fig. 5. CID spectra of the doubly charged phosphopeptide (1: YGGMHRQET(p)VDC) (a) by TOF/TOF with Cs target, (b) by sector with Cs target, (c) by Thermo LTQ XL with fluoranthene monoanion.
(YGGMHRQET(p)VDC) ions, (a): measured using the TOF/TOF instrument with a Cs target and (b): by using a sector type MS/MS instrument with a Cs target. Figure 5(c) is an ETD spectrum of the phosophopeptide that had collided with a singly-charged anion, and obtained using a Finnigan LTQ XL mass spectrometer. In all spectra, cα-type fragment ions from n=7 to n=11, and zα-type fragment ions from n=7 to n=11 without the loss of the phosphate group were clearly observed. As seen from a comparison between Figs. 5(a) and (b), the TOF/TOF instrument provided highly mass-resolved peaks. Because of this higher resolving power in TOF/TOF instrument, peak identification of the fragment peaks became much more reliable and a-type and y-type fragment were also clearly observed. These aα-type fragment ions from n=8 to n=11 and yα-type from n=7 to n=11 were not clear in spectra Fig. 5(b) obtained using the sector type instrument and were not observed in LE-ETD spectra in Fig. 5(c). The formation of these ions observed in high-energy collisions were assumed to result from the cleavage of Cα–C bonds and peptide bonds, respectively, due to the influence of additional collisional activation. The mass difference between aα and aβ indicate that a-type ions also undergo fragmentation without the loss of the phosphate group. The observation of not only c- and z-type ions by N–Cα bond cleavage but also α-type ions by the cleavage of Cα–C bonds and y-type ions by the cleavage of peptide-bonds is thought to permit the sequences of various peptides whose sequence are unknown to be determined. The relative intensity of about five times for c-type ions to a-type ions indicated that N–Cα bond cleavage, as indicated by radical fragmentation, are about five times more effective than Cα–C bond cleavage by vibrational excitation. The similarity in the relative intensity of c-type ions to a-type ions between Fig. 5(a) by the TOF/TOF and Fig. 5(b) by the sector instrument indicate that HE-ETD is a reproducible dissociation method by a single collision, regardless of the type of instrument used.

In order to examine the applicability of the HE-ETD approach to various peptides with PTM, HE-CID spectra of doubly protonated peptides containing an intrachain disulfide bond were obtained by MS/MS of the TOF/TOF and the sector type. Disulfide linkages are post-translational modifications that play key roles in stabilizing the native structures of many proteins and the identification and localization of such linkages is often an important aspect in primary structure characterization.69) Figures 6 show CID spectra of doubly protonated vasopressin (CYFQNCPRG) which contains an S–S bond, (a) by TOF/TOF with a Cs target, (b) by the sector with a Cs target, (c) by the sector with a Xe target. Similar to phosphorylated-peptides,67) while the abundant product ions with the Xe target were immonium ions and doubly-charged fragment ions, as shown in Fig. 6(c), those with the Cs target were charge-reduced fragment ions, whose relative abundances were about ten times larger than those of the immonium ions and doubly-charged fragment ions, as shown in Figs. 6(a) and (b). The difference between the Xe and the Cs target indicates that collisions with the Cs target allow for a much more effective ETD than CAD. In the CID spectra with the Cs target, c-type and z-type ions produced via backbone cleavages for the ETD process were clearly observed for the peptides, although the peptide contains an intramolecular ring structure. In particular, the presence of zα-type ions from n=4 to n=8 and cα-type ions from n=1 to n=5 indicate cleavages both at the disulfide bond and the N–Cα bond from a single electron transfer event. The mechanism responsible for the cleavage of the two bonds by the formation of a single radical in the ETD process was proposed by Xia and coworkers.70,71) The zα-S ions observed in Figs. 6(a) and (b) indicate C-S bond cleavage of the intramolecular ring. These fragment ions provide information for the complete peptide sequencing, and the position of the intramolecular ring. The similarity of the relative intensity of almost each fragment ion between Fig. 6(a) by the TOF/TOF and Fig. 6(b) by the sector instrument indicate that HE-ETD is a reproducible dissociation method that is applicable to different types of PTMs.

CONCLUSION

Charge inversion mass spectrometry using collisions with alkali metal targets in the keV energy collision range have provided insights into the structures and reactions of ions and neutral molecules. In addition, charge inversion mass spectrometry permits many isomeric cations whose CAD spectra of the isomeric cations are similar, to be
clearly differentiated. For charge inversion spectra of CH$_2$X (X=Cl, Br, I), a trapezoid shape for the I$^-$ ion was attributed to the direct dissociation from a triplet repulsive state by a comparison of peak width with a potential curve derived by theoretical calculations. In a study of charge inversion spectra combined with a computational study, the proline effect in charge reduced peptides was dissected by using the model dipeptides Pro–Gly and Gly–Pro. An inverse hydrogen migration in the arginine radical was also elucidated.

Two types of instruments applicable to HE-ETD for multiply charged ions were developed by sector type MS/MS and TOF/TOF. From the investigation of the nascent internal energy distribution of charge reduced ions using a thermometer molecule, potential crossing points between the initial channel of a doubly charged state and a neutral target and exit channel of coulombic repulsion state were evaluated. The evaluated potential crossing provides a very large cross section for electron transfer. The cross section of the order of 10$^{-16}$ cm$^2$ estimated in the present work is much larger than that for collisional activation, which was estimated to be in order of 10$^{-18}$cm$^2$.

The experimental results using HE-ETD for doubly protonated phosphorylated peptides indicated that HE-ETD can provide information on the peptide sequence, regardless of the type of phosphorylated amino acid residues. The difference in the results for HE-CID between the Xe and Cs targets demonstrated that the HE-ETD process with the Cs target took place much more dominantly than collisional activation. The difference between HE-ETD using a Cs target and LE-ETD using an anion demonstrated that mass discrimination was much weaker in the high-energy process. HE-CID spectra of a doubly protonated peptide containing an intrachain disulfide bond were obtained by MS/MS of the TOF/TOF and the sector type. The presence of z-type ions and c-type ions that were observed only in HE-ETD spectra indicate that both the disulfide bond and the N–C$_3$ bond are cleaved in a single electron transfer event. These fragment ions provided information on the complete peptide sequence, and the position of the intramolecular ring. It was demonstrated that high energy ETD using an alkali metal target can be useful in determining the position of modification and the amino acid sequence of a peptide that had been post-translationally modified. The similarity in the relative intensity of most fragment ions between by the TOF/TOF and by the sector instrument indicated that HE-ETD is a reproducible dissociation method regardless the types of MS/MS instruments or format being used.

In this article, the physicochemical basis of HE-ETD using alkali metal target and applications thereof were presented. The complete amino acid sequencing of phosphorylated and disulfide bond-bearing peptides suggests that HE-ETD is amenable for use in identifying other types of post-translational modifications. Considering its highly reproducibility from a single collision in both HE-CAD and HE-ETD, a broad utility of HE-CID can be expected as in the case of electron ionization. Hopefully, HE-ETD will be incorporated into commercial MS/MS instruments and will be widely used in the near future.

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