N–C_α Bond Cleavage of the Peptide Backbone via Hydrogen Abstraction

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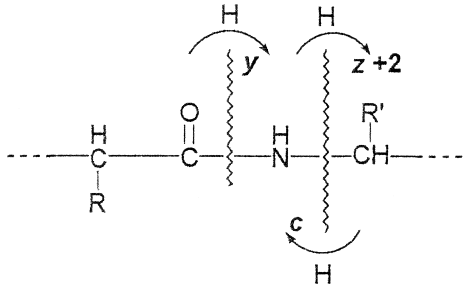
The specific cleavage of N–C_α bonds on the peptide backbone to form the so-called '***c***' and '***z*** + 2' products, which can be used for the rapid determination of protein amino-acid sequences, has been examined to clarify the mechanism(s) that occur during hydrogen abstraction induced by bombardment with 337-nm laser photons in matrix-assisted laser desorption/ionization (MALDI) method. Intramolecular hydrogen abstraction, which results from the hydrogen(s) on the C_α or C_β carbon, did not occur with a deuterium-labeled dodecapeptide. To confirm a proposition that intermolecular hydrogen abstraction occurs between the peptide and the MALDI matrix, a deuterium dodecapeptide embedded in a deuterium 2,5-dihydroxybenzoic acid matrix at a molar ratio of 1:7000 was analyzed. The resulting deuterium ***c*** product ions suggested that ***c*** ions form via intermolecular hydrogen abstraction, although the results obtained did not deny any other possibilities such as intramolecular transfer of labile hydrogen. A mechanism for the N–C_α bond cleavage has been proposed that the formation of hypervalent radical species and subsequent prompt bond cleavages occur. The proposed mechanism successfully rationalizes the formation of both the ***z*** + 2 and the ***c*** product ions. (J Am Soc Mass Spectrom 2001, 12, 1044–1049) © 2001 American Society for Mass Spectrometry

Two fascinating methods for analyzing the primary structure of intact proteins have been recently developed: in-source decay (ISD) [1], using matrix-assisted laser desorption/ionization (MALDI) [2] coupled with a time-of-flight (TOF) mass analyzer; and electron capture dissociation (ECD) [3], using electrospray ionization [4] with Fourier transform mass spectrometry (MS). Using ISD, it is possible to identify quickly genomic proteins by sequence comparison with protein databases [5, 6]. Both ISD and ECD processes are characterized by products formed through cleavage of the N–C_α bond on the peptide backbone. Cleavage of the N–C_α bond leads to the formation of ***c*** and ***z*** + 2 product ions in ISD [Eq. (1)], but to ***c*** and ***z*** product ions in ECD. The products in [Eq. (1)] are labeled using Biemann's nomenclature [7].



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Unusual cleavage of the N–C_α bond on peptide backbone was first reported by Williams and coworkers [8] in an outstanding display of the capability of fast atom bombardment MS [9]. These authors deduced the fragmentation of sequence-reflected products such as ***c***, ***y*** and ***z*** + 2 ions, after experiments in which they bombarded an analyte peptide dissolved into a glycerol matrix with a 4-6 keV beam of argon atoms. It is important to recognize that all the products ***c***, ***y*** and ***z*** + 2 ions are formed through the abstraction of a hydrogen atom [Eq. (1)]. It has been proposed that ECD of multiply protonated proteins proceeds through hypervalent species that are produced by the binding of hydrogen atoms, $H^\cdot$, to carbonyl and amino groups on the backbone or S–S bond(s) [8, 10]. The $H^\cdot$ atoms can be produced when protons on protein side chains of high proton affinity are neutralized by electron capture. This intramolecular hydrogen transfer plays an important role in ECD of multiply-protonated proteins.

Brown and Lennon first reported that MALDI-ISD can be used for the rapid sequencing of proteins and peptides [11]. Although they have examined several factors that affect the backbone cleavage of the insulin B chain following laser-induced dissociation of the protein from a MALDI matrix [12], the mechanism of N–C_α bond cleavage remains unknown. It should be noted that ISD leading to ***c*** and ***z*** + 2 products takes place accompanying hydrogen abstraction [Eq. (1)], therefore, the mechanism of N–C_α bond cleavage in ISD may be connected to that of ECD. It would be of interest to determine the origin(s) of the hydrogen atoms, $H^\cdot$, that

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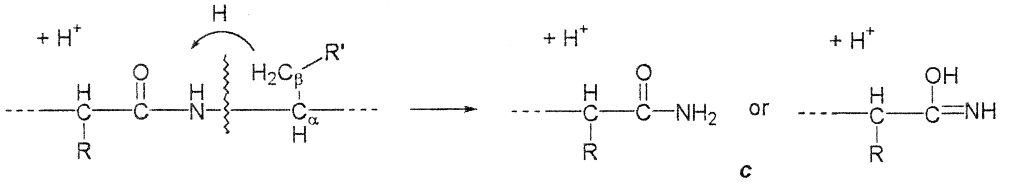
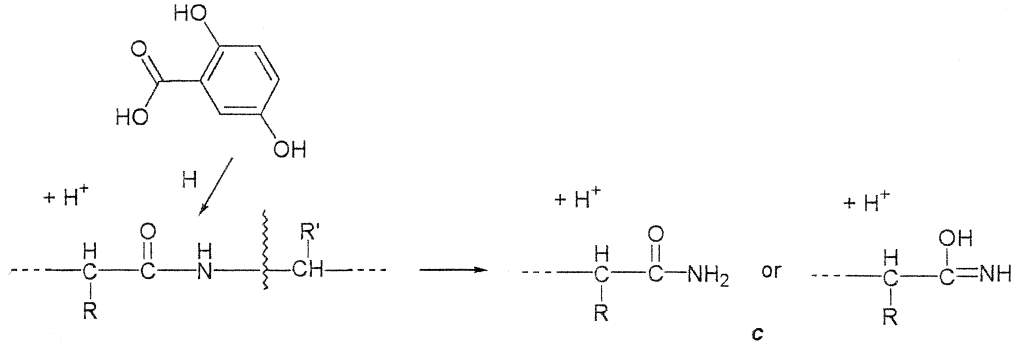
form the ***c*** product ions in MALDI-ISD experiments. It would be of interest to determine the origin(s) of the hydrogen atoms, $H^{\cdot}$, that form the ***c*** product ions in MALDI-ISD experiments. Although the $H^{\cdot}$ atoms must originate from either the peptide ions or the MALDI matrix such as 2,5-dihydroxybenzoic acid (2,5-DHB), there are at least four possible sources of this atom:

1. intramolecular transfer of labile hydrogen in the peptide ions [13, 14];
2. intramolecular transfer of non-labile hydrogen from e.g., C_α and C_β atoms [15];
3. intramolecular transfer of the hydrogen formed by electron capture in the multiply protonated peptides [3, 10];
4. intermolecular transfer of labile hydrogen from matrix [16].

It is generally difficult to determine the origin(s) of the hydrogen atoms in hydrogen transfer reactions, because the migration of active hydrogen takes place from a remote site on the peptide backbone of the same molecule [14]. Furthermore, some scrambling of hydrogen(s) complicates the origin(s) of the hydrogen atoms. Here I have limited the ISD experiments concerning hydrogen sources to two possible mechanisms: the ***c*** product is formed by abstracting an $H^{\cdot}$ atom intramolecularly from the hydrogen(s) on the C_α or C_β carbon [Eq. (2)] [15], and the hydrogen atom must originate intermolecularly from phenolic or carboxylic hydroxy group(s) in the matrix [Eq. (3)]. To rationalize the specific N–C_α bond cleavage under MALDI conditions, a deuterium-labeled peptide and a deuterium matrix 2,5-DHB-d_3 have been used.



(2)



(3)

Experimental

Materials

A peptide RLGNQWAVGDLAE (*M*r 1428.6) and a deuterium-labeled peptide RLGNQWA(d_3)VG(d_2)DLAE (*M*r 1433.6) were purchased from the Peptide Institute (Minoh, Osaka, Japan). Trifluoroacetic acid (TFA), acetic acid-d_1 (Ac-d_1), acetonitrile and a water-d_2 were purchased from Tokyo Kasei (Tokyo, Japan). The matrix material 2,5-dihydroxybenzoic acid (2,5-DHB) was purchased from Sigma (Milwaukee, WI, USA). All reagents were used without further purification.

MALDI-TOF Mass Spectra

All the mass spectra were acquired on a PerSeptive Biosystems Voyager-DE STR TOF reflectron mass spectrometer (PE Biosystems, Inc., Framingham, MA, USA) equipped with a N_2 laser (337 nm, 3 ns pulse length; Laser Science, Franklin, MA, USA) and a dual microchannel plate detector (Galileo, Sturbrige, MA, USA). The spectra were acquired in positive ion mode. The instrument was used in the delayed extraction reflector mode. The ion accelerating voltage and the delay time were 20 kV with the grid voltage set at 73.5% and 238 ns, respectively. The analyte peptide was dissolved in water or water-d_2 at a concentration of 200 pmol/μL, and 1 μL of the analyte solution was mixed with a 9 μL saturated matrix (2,5-DHB) of 0.1% TFA in water/acetonitrile (3:2 v/v) or a 9 μL saturated deuterium matrix (2,5-DHB-d_3) of 1.0% Ac-d_1 in water-d_2 /acetonitrile (3:2 v/v). A 0.5 μL of the solution was deposited onto a gold-coated target plate, and the solvents were removed by drying in air at room temperature.

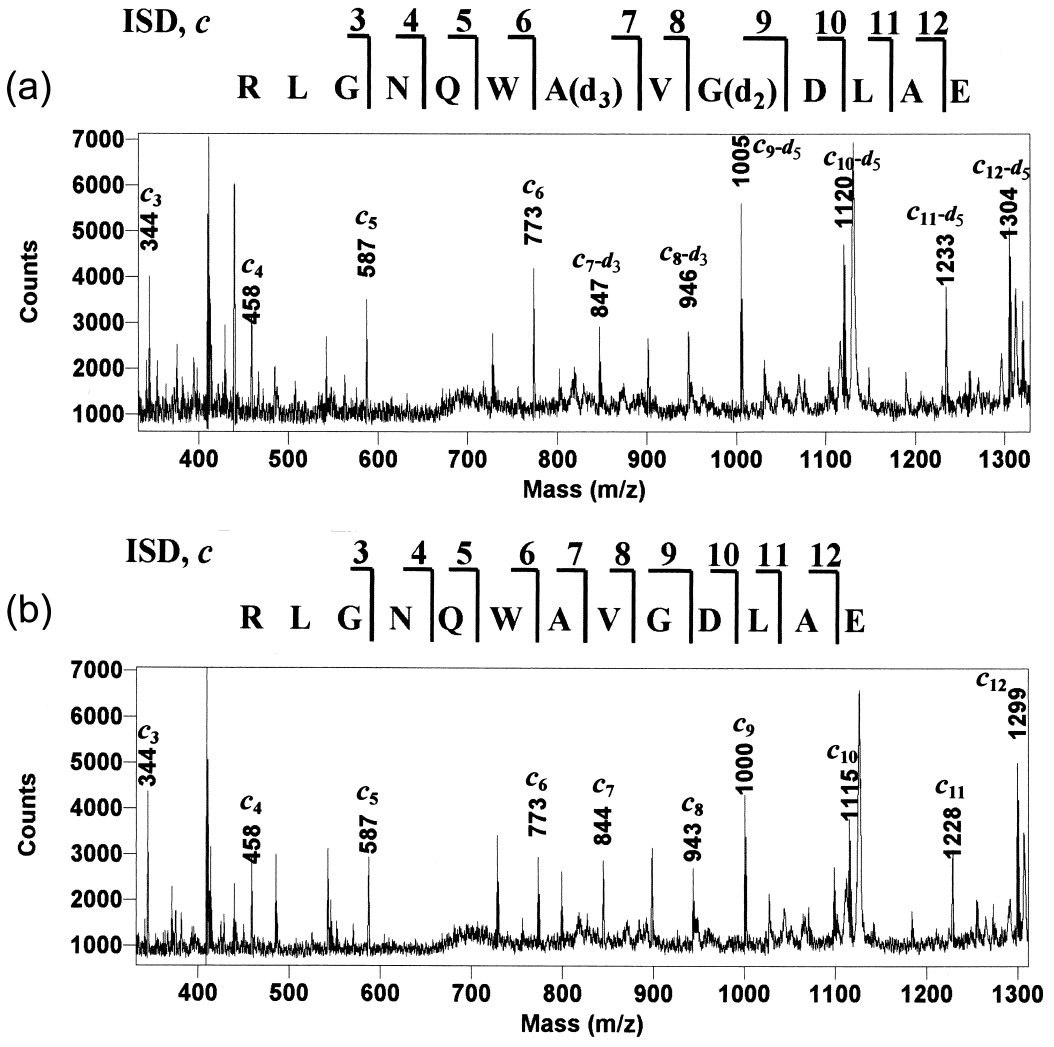


Figure 1. ISD spectra of **(a)** a deuterium-labeled dodecapeptide (**1**, *M*r 1433.6) and **(b)** a non-labeled dodecapeptide (**2**, *M*r 1428.6). Each ***c*** product ion accompanies a corresponding ***a*** product ion with the mass: ***c***–45-Da [25]. Several broadened peaks correspond to metastable peaks [18]. The mass difference between c_6 product ions in **1** and **2** indicates that the c_6 product ion at *m/z* 773 in **2** is not formed via intramolecular hydrogen abstraction from the Ala7(C_β) carbon. Likewise, the mass difference between the c_8-d_3 ion at *m/z* 946 in **1** and the c_8 ion at *m/z* 943 in **2** indicates that the c_8 product ion at *m/z* 943 in **2** is not formed via intramolecular hydrogen abstraction from the Gly8(C_α) carbon. Therefore, the hydrogen required to form ***c*** products does not originate from the hydrogen on either the C_α or C_β carbon.

Results and Discussion

Intramolecular Hydrogen Abstraction Does Not Occur in a Deuterium-labeled Peptide

It seems reasonable to assume that hydrogen atom leading to the formation of ***c*** products originates from the hydrogen(s) on the C_α or C_β carbon [Eq. (2)], because the ***c*** products can be observed in every amine-bond on the backbone, except for Xxx–Pro bonds. To examine the intramolecular hydrogen abstraction [Eq. (2)], it was attempted to rationalize the N–C_α bond cleavage that accompanies hydrogen abstraction during formation of the ***c*** product, by using pulsed-energy 337-nm photons impinging on the crystal sample of a deuterium-labeled peptide embedded in a 2,5-DHB MALDI matrix. An analyte to matrix molar ratio of 1:7000 was used. The ***c*** products resulting from the deuterium-labeled peptide (**1**) RLGNQWA(*d*3)VG(*d*2)DLAE (*M*r 1433.6), which contains Ala7 ($C_\beta D_3$) and Gly9 ($C_\alpha D_2$), were compared to those of a non-labeled peptide (**2**) RLGNQWAVGDLAE (*M*r 1428.6) (Figure 1). A series of the ***c*** product ions in **1** were observed at *m/z* 344 (c_3), 458 (c_4), 587 (c_5), 773 (c_6), 847 (c_7-d_3), 946 (c_8-d_3), 1005 (c_9-d_5), 1120 (c_{10}-d_5), 1233 (c_{11}-d_5) and 1304 (c_{12}-d_5) (Figure 1a), whereas **2** gave the ***c*** product ions at *m/z* 344 (c_3), 458 (c_4), 587 (c_5), 773 (c_6), 844 (c_7), 943 (c_8), 1000 (c_9), 1115 (c_{10}), 1228 (c_{11}) and 1299 (c_{12}) (Figure 1b). The mass shift was not observed in the c_6 products of **1** and **2**. The 3-Da mass shift between c_8 and c_8-d_3 is in agreement with the number of deuterium labels in the Ala7($C_\alpha D_3$)

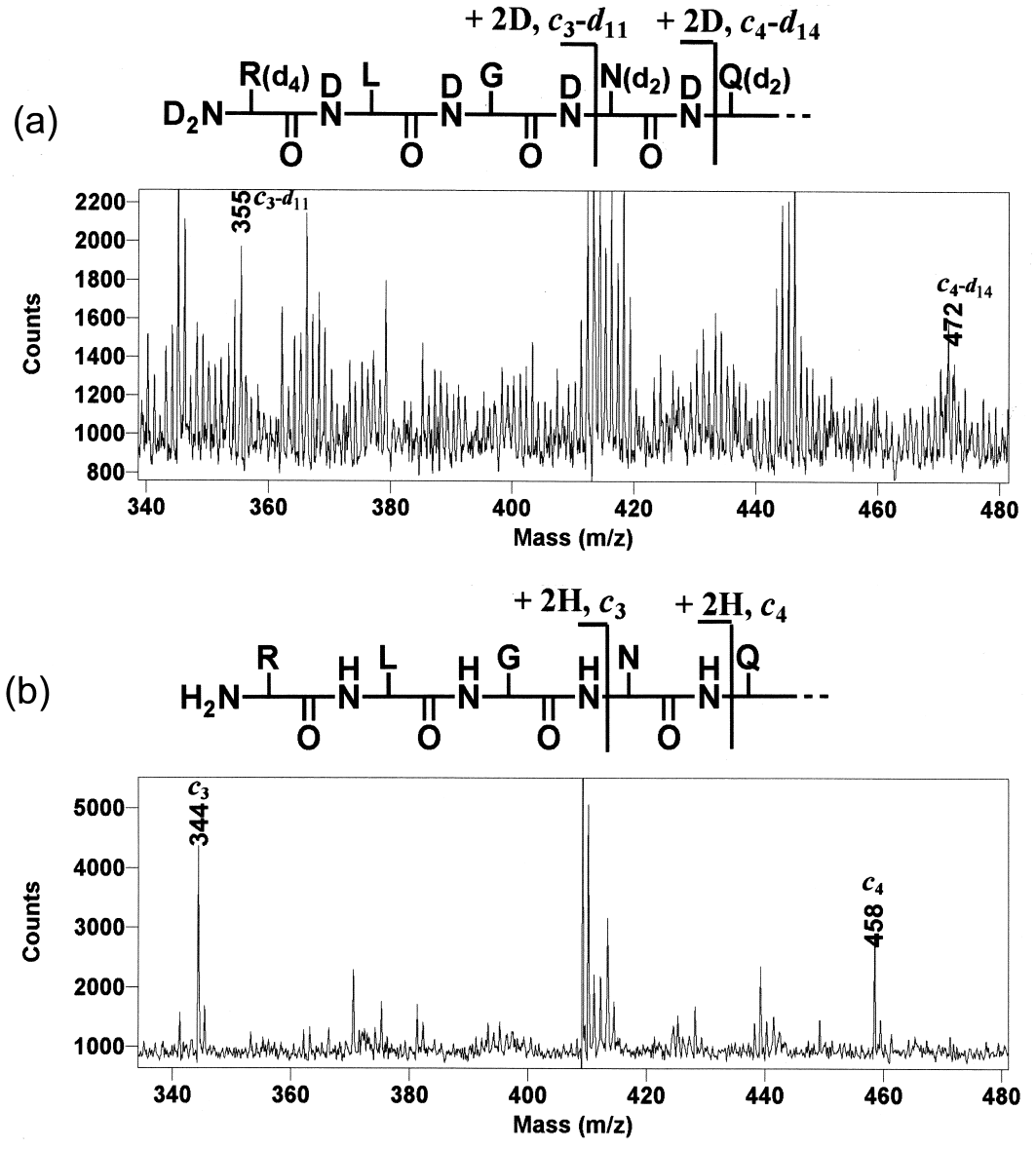


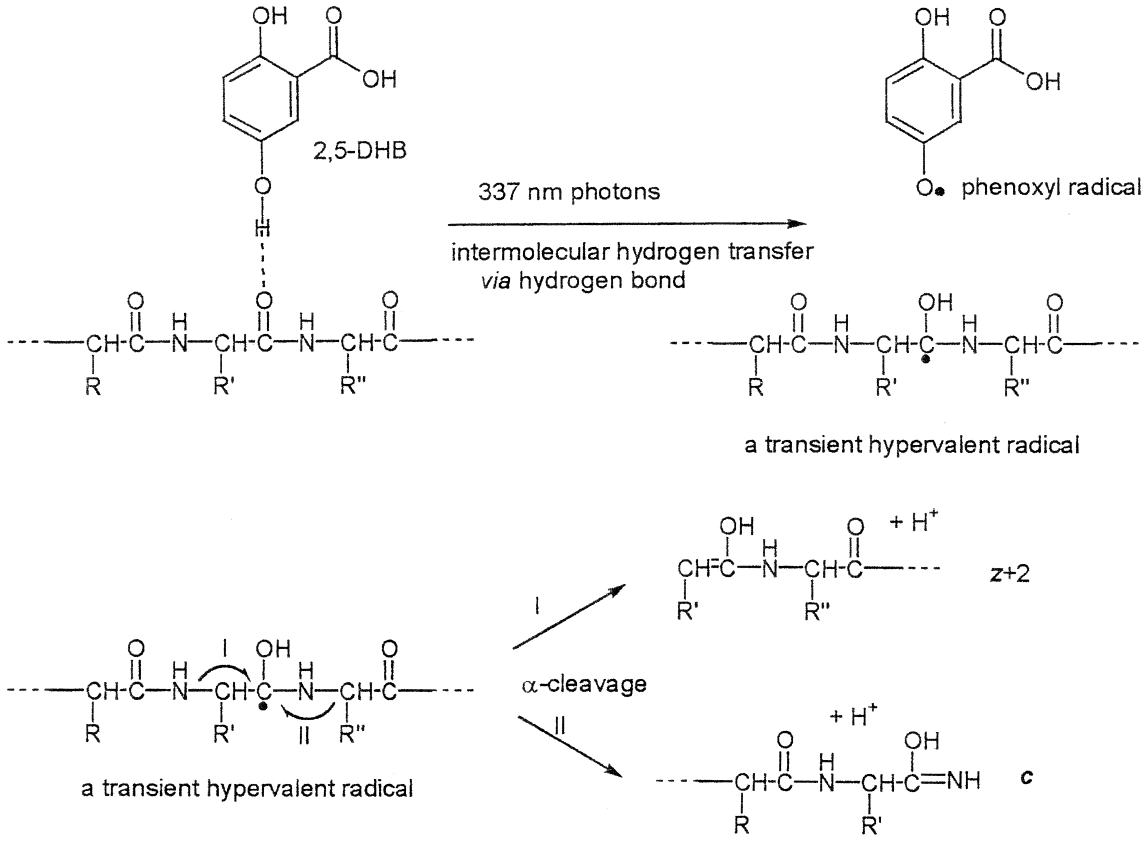
Figure 2. Partial ISD spectra of **(a)** a deuterium dodecapeptide (**2**-d_{26}, *M*r 1454.6) obtained with a deuterium matrix 2,5-DHB-d_3, and **(b)** a dodecapeptide (**2**, *M*r 1428.6) obtained with a 2,5-DHB matrix. In (a), the products c_3-d_{11} at *m/z* 355 and c_4-d_{14} at *m/z* 472 must be formed by deuterium abstraction and deuteronation. As the molar ratio of analyte to matrix is 1:7000, both deuterium and deuteron probably originate from the matrix 2,5-DHB-d_3, although a possibility of intramolecular deuteron transfer or hydrogen/deuteron scrambling never be denied.

residue of **1**. Consequently, the c_6 and c_8-$d_3$products in **1**, which originate from cleavage of the N–C_α bond at Trp6-Ala7($C_\beta D_3$) and Val8-Gly9($C_\alpha D_2$), respectively, are not formed through $D^\cdot$ abstraction from the Ala7(C_β) or Gly9(C_α) carbon, but instead through abstraction of an $H^\cdot$ atom from another hydrogen source. This means that the mechanism of intramolecular hydrogen abstraction proposed in Eq. (2) does not occur in formation of *c* product ions in ISD. Therefore another mechanism [Eq. (3)] must be considered, although the result obtained above does not deny the intramolecular hydrogen abstraction from any other side chain residues.

Intermolecular Hydrogen Abstraction May be Likely to Form c Product Ions

To obtain evidence that intermolecular hydrogen abstraction is responsible for formation of the *c* product ions [Eq. (3)], the ISD experiment was conducted by using a deuterium peptide (**2**-d_{26}, *M*r 1454.6) embedded in a deuterium matrix 2,5-DHB-d_3, which were obtained by dissolving peptide **2** or 2,5-DHB, respectively, into a water-d_2. The ISD spectrum mainly showed peaks corresponding to the deuteronated matrix [2,5-DHB-d_3 + D]$^+$ at *m/z* 139 and the deuteronated peptide [**2**-d_{26} +D]$^+$ at *m/z* 1456. Product ions c_3 and c_4, which originate from cleavage of the N–C_α bond at Gly3-Asn4

and Asn4-Gln5, were used to examine the deuterium abstraction. The spectrum showed the c_3-d_{11} at *m/z* 355 and c_4-d_{14} at *m/z* 472 (Figure 2a), though the spectrum was so noisy. This poor spectrum is due to imperfection of the rate of hydrogen/deuterium exchange. The observations of higher peaks at *m/z* 355 and at *m/z* 472 than isotope peaks suggest, however, that ten or thirteen active hydrogens plus one proton in the c_3 at *m/z* 344 or c_4 at *m/z* 458 of **2** (Figure 2b) changed to ten or thirteen deuteriums plus one deuteron in the corresponding products of **2**-d_{26}. The observation of c_3-d_{11} and c_4-d_{14} product ions does not rule out that intermolecular deuterium abstraction may take place between individual analyte molecules, but the low concentration of analyte peptide used compared with matrix (1:7000 molar ratio) suggests that the deuterium abstraction takes place from the 2,5-DHB-d_3 matrix to the deuterium peptide **2**-d_{26}.



Hypervalent Peptide Radical Explains the Formation of Both c and z + 2 Product Ions

From the results in Figure 2, it seems reasonable to assume that the H˙ atoms needed to form *c* product ions are abstracted intermolecularly from the matrix molecules [Eq. (3)]. In normal MALDI experiments, the matrix has essential functions, for example, as a photon absorbent, as a proton donor or acceptor for the formation of peptide/protein ions, and as a defensive material from the photo-induced degradation of analytes. Importantly, only a very few matrix materials produce peptide and protein ions, and the matrix 2,5-DHB [17] is unique in its ability to form ISD peptide product ions [18]. To understand the phenomenon of ISD, it is necessary to know what interactions occur between the matrix and the protein molecules in the crystal. An X-ray crystallographic study of the 2,5-DHB matrix with and without protein incorporation into the matrix crystal has been reported [19]. During crystal growth, the 2,5-DHB molecules form a hydrophobic (011) planar network owing to intermolecular hydrogen bonding, and the stacking of the hydrophobic (011) planes along the direction of crystal growth forms a (100) surface. This (100) surface is normally irradiated by the 337-nm laser photons in MALDI experiments, and is hydrophilic in nature because of phenolic or carboxylic hydroxy groups exposed on the matrix surface. Importantly, after protein incorporation this crystal structure does not change, although the (100) surface becomes smoother [19]. Here, using scanning probe microscopy, I also observed that the layered steps that form on the (100) surface of 2,5-DHB disappear after incorporating a peptide (data not shown). This indicates that the hydro-

philic (100) surface of the matrix crystal is covered with protein or peptide molecules, probably through a variety of different intermolecular interactions [19].

The importance of the intermolecular hydrogen bonds in condensed-phase proton transfer between the matrix material sinapinic acid and a Gly-Ser backbone has been pointed out by Vertes and coworkers [16]. Furthermore, certain phenolic compounds readily form phenoxyl radicals by donating an $H^{\cdot}$ atom to any hydrogen-accepting sites [20, 21]. Carbonyl oxygens have high $H^{\cdot}$ atom affinity [3], and $H^{\cdot}$ atom abstraction at a backbone carbonyl group results in the production of hypervalent species, as in the ECD mechanism [3, 10]. The results presented here suggest a model for the mechanism of ISD of the peptide backbone under MALDI conditions [Eq. (4)]. Intermolecular hydrogen bonding between a carbonyl oxygen on the peptide backbone and a phenolic hydrogen in the matrix may enable an $H^{\cdot}$ atom to move preferentially from the matrix to the backbone carbonyl oxygen [Eq. (4a)]. The excess energy gained by the hypervalent peptide radical on binding the $H^{\cdot}$ atom would then be used for α-cleavage I or II [Eq. (4b)]. In fact, a radical site drives the very fast process [22, 23] of α-cleavage to form ***c*** products. Furthermore, Eq. (4) also explains formation of the $z + 2$ product ion in ISD [α-cleavage I in Eq. (4b)], which is appropriate as the N–C_α bond cleavage that forms ***c*** product ions occurs independently of the protonation site(s) on the peptide backbone [24, 25].

Conclusions

Although the MALDI-ISD method has a significant limitation for the analysis of mixtures, it is of great advantage to obtain sequence information of intact proteins without enzymatic pre-digestion. Furthermore, the understanding of the MALDI-ISD process is of basically interests in connection with the prompt fragmentation of peptides in the other energy-sudden desorption methods such as FAB [7, 8, 24] and plasma desorption [26].

It is evident that the observed ***c*** product ions do not form *via* intramolecular hydrogen abstraction from the hydrogen(s) in C_α or C_β carbons on the peptide backbone, although the result obtained here does not deny any other intramolecular hydrogen abstraction from labile hydrogen in the acidic amino acids or C-terminal hydroxy group. The use of the deuterium peptide and the deuterium matrix suggests that intermolecular hydrogen abstraction from the matrix to the analyte peptide takes place to form ***c*** product ions. The assumption of hypervalent peptide radical, which may be formed by binding the $H^{\cdot}$ atom to the backbone carbonyl oxygen, successfully rationalizes the prompt fragmentation of the N–C_α bond on peptide backbone. The proposed α-cleavages [Eq. (4)] in the hypervalent peptide radical explain the formation of both ***c*** and $z + 2$ product ions.

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