

Ultraviolet Laser Induced Hydrogen Transfer Reaction: Study of the First Step of MALDI In-Source Decay Mass Spectrometry

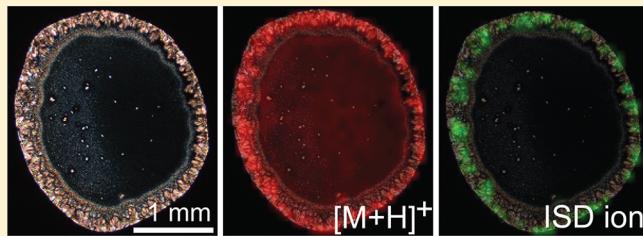
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Supporting Information

ABSTRACT: The early mechanisms of matrix-assisted laser desorption/ionization in-source decay (MALDI-ISM) are described herein. MALDI-ISM is initiated by the hydrogen transfer from excited matrix molecules to the carbonyl oxygen of the peptide backbone, which is followed by a radical-induced cleavage, producing the $c'/z\bullet$ fragment pair. As expected, the use of 2,5-DHB or 1,5-DAN was efficient to induce MALDI-ISM, and the strongest intensity of MALDI-ISM fragments was observed when laser shots were performed on matrix crystals. In contrast, the hydrogen radical transfer reaction was suppressed by using ionic liquid and amorphous structure of 2,5-DHB and 1,5-DAN mixture as a matrix. Our results suggest that the hydrogen transfer occurs on the matrix crystal during the dissipation of the laser energy and before desorption, following ISD fragments formed in the MALDI plume.



INTRODUCTION

Matrix-assisted laser desorption/ionization in-source decay (MALDI-ISM) has been used to determine the sequence of proteins without any enzyme digestion.^{1–5} MALDI-ISM is initiated by the hydrogen transfer from excited matrix molecules to the carbonyl oxygen atoms of peptide backbone.⁶ Subsequently, $c'/z\bullet$ fragment pairs are formed by the radical-induced cleavage at the N–C_α bonds.⁷ Radical $z\bullet$ fragments undergo either gain of a hydrogen atom or loss of side chain, leading to z' or w fragments, respectively.^{7–9} Those reactions occur competitively and depend on the collision rate in the MALDI plume: collisions with matrix molecules in the MALDI plume conduce to hydrogen attachment to give a z' fragment, while unimolecular dissociation leads to side chain loss from $z\bullet$ radical fragments, leading to w fragments.^{8,9}

Recently, it was found that the use of oxidizing matrixes, e.g., 5-formylsalicylic acid and 5-nitrosalicylic acid for MALDI-ISM, resulted in the generation of a and x ions by cleavage of the C_α–C bond.¹⁰ The use of oxidizing matrix leads to the formation of oxidized peptide molecules [M–H] \bullet containing a radical site on the amide nitrogen. Subsequently, the [M–H] \bullet generates the $a\bullet/x$ fragment pair and the a fragment was formed by further hydrogen abstraction from the $a\bullet$ radical fragment.¹¹ The hydrogen-deficient peptide radical [M–H] \bullet also leads to the formation of [M–2H] by further hydrogen abstraction. The hydrogen abstraction probably occurs in the crystal after the laser shots, but the subsequent reactions that lead to the $a\bullet/x$ fragment pair or to the oxidizing product [M–2H] take place in the plume, thus explaining the influence of initial velocities for the formation of these products.^{9,12}

In this study, we focused our attention on the first step of MALDI-ISM, i.e., hydrogen transfer reaction between peptides

and matrix molecules, leading to the production of peptide radicals. Previously, the importance of the intermolecular hydrogen bonds in condensed-phase leading to the hydrogen transfer from 2,5-dihydroxybenzoic acid (2,5-DHB) matrix to peptide backbone has been reported by Takayama.⁶ In contrast, hydrogen radicals have been detected in the MALDI plume¹³ and hydrogen attachment to the peptide may occur. In the present study, we investigated the initial step of the ISD mechanism and found that ISD fragmentation and reduction of the disulfide bond essentially occur when laser irradiations are performed on matrix crystals. It suggests that the first step of MALDI-ISM mainly occurs in matrix crystals, before desorption.

EXPERIMENTAL SECTION

Materials. 2,5-DHB, 1,5-diaminonaphthalene (1,5-DAN), and *p*-methoxybenzyl chloride were purchased from Sigma-Aldrich (Steinheim, Germany). The peptides, substance P, fibrinopeptide A, and calcitonin (salmon I) were purchased from Bachem (Weil am Rhein, Germany). All of the solvents used were HPLC grade quality. All reagents were used without further purification except for water, which was purified through a Milli-Q water purification system (Millipore; Billerica, MA, USA). The *p*-methoxybenzylpyridinium chloride was synthesized by condensation of the *p*-methoxybenzyl chloride with pyridine.

Preparation for MALDI Experiments. For preparation of 2,5-DHB and 1,5-DAN matrix solution, 2,5-DHB and 1,5-DAN

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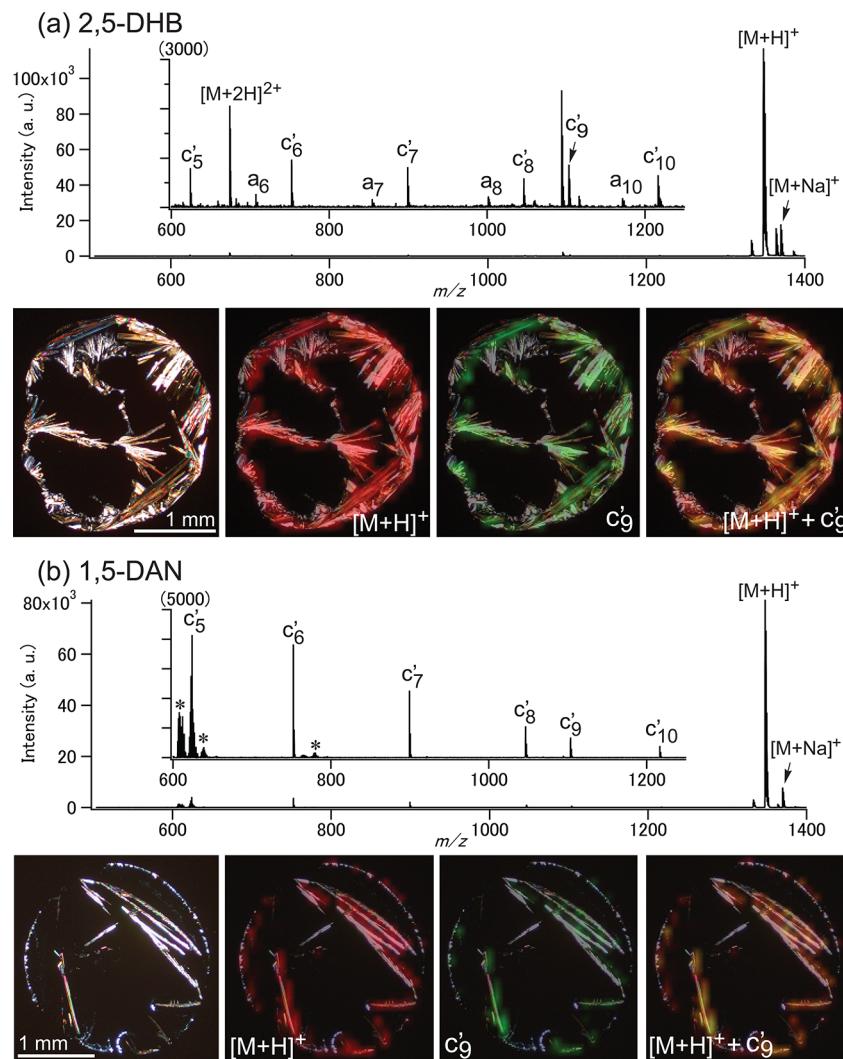


Figure 1. MALDI-MSD spectra of substance P obtained using (a) 2,5-DHB and (b) 1,5-DAN as a matrix. Lower panel: From left to right, picture of the spot, ion image of the protonated analyte $[M+H]^+$ (red), ion image of the c_9' ISD fragment (green), and ion images of both $[M+H]^+$ and the c_9' ISD fragment.

were dissolved in water/ACN (1/1, v/v) with 0.1% formic acid at a concentration of 20 and 10 mg/mL, respectively. For the preparation of a mixture of 2,5-DHB and 1,5-DAN, 2,5-DHB (40 mg/mL) and 1,5-DAN (20 mg/mL) were dissolved in water/ACN (1/1, v/v) with 0.1% formic acid and mixed at 1/1 and 1/2 (v/v) ratios. The mixture of 2,5-DHB and 1,5-DAN (2/1, molar ratio) formed an ionic liquid and was prepared shortly before the experiments because of the instability of ionic liquid of 2,5-DHB and 1,5-DAN.

For the imaging experiments (Figures 1 and 2), a volume of 1 μ L of substance P solution (20 pmol/ μ L in water) was deposited onto an ITO-coated microscopic slide, and 1 μ L of matrix solution was then added. For the single mass spectrum acquisitions (Figures 3 and 4, Tables 1 and 2), a volume of 0.5 μ L of analyte peptide solution (20 pmol/ μ L in water) or *p*-methoxybenzylpyridinium chloride (100 pmol/ μ L in water/ACN, 1/1, v/v) was deposited onto a MALDI target plate, and 0.5 μ L of matrix solution was then added.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. All MALDI mass spectrometry analyses were carried on an UltraFlex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a frequency-

tripled Nd:YAG laser (355 nm). All the experiments were operated in positive polarity in reflectron mode with the ion accelerating voltage set at 25 kV and a delayed extraction of 30 ns. The laser fluence was set to about 10–20% higher than the detection threshold of $[M+H]^+$, and was optimized in order to obtain MALDI-MSD mass spectra that had high signal-to-noise ratios (S/N) of the ISD ion peaks. For each condition, the same laser fluence was used for MALDI imaging experiments and single mass spectrum acquisitions. Calibration was performed externally using a peptide mixture (Bruker Daltonics, Bremen, Germany) deposited on the MALDI target plate.

For MALDI analyses except for imaging experiments, a total of 1000 laser shots was accumulated for each mass spectrum acquisition. The survival yields and reduced product yields were evaluated by averaging 7 and 10 measurements, respectively.

For MALDI-MSD imaging experiments, optical images of analyte/matrix spots were obtained using a polarized light microscope Olympus BH-2 using crossed Nicols (Olympus, Japan). The imaging spatial resolution was set to 100 μ m in FlexImaging 2.1 software, and ISD mass spectra were acquired. For all ISD mass spectra, 700 individual laser shots were

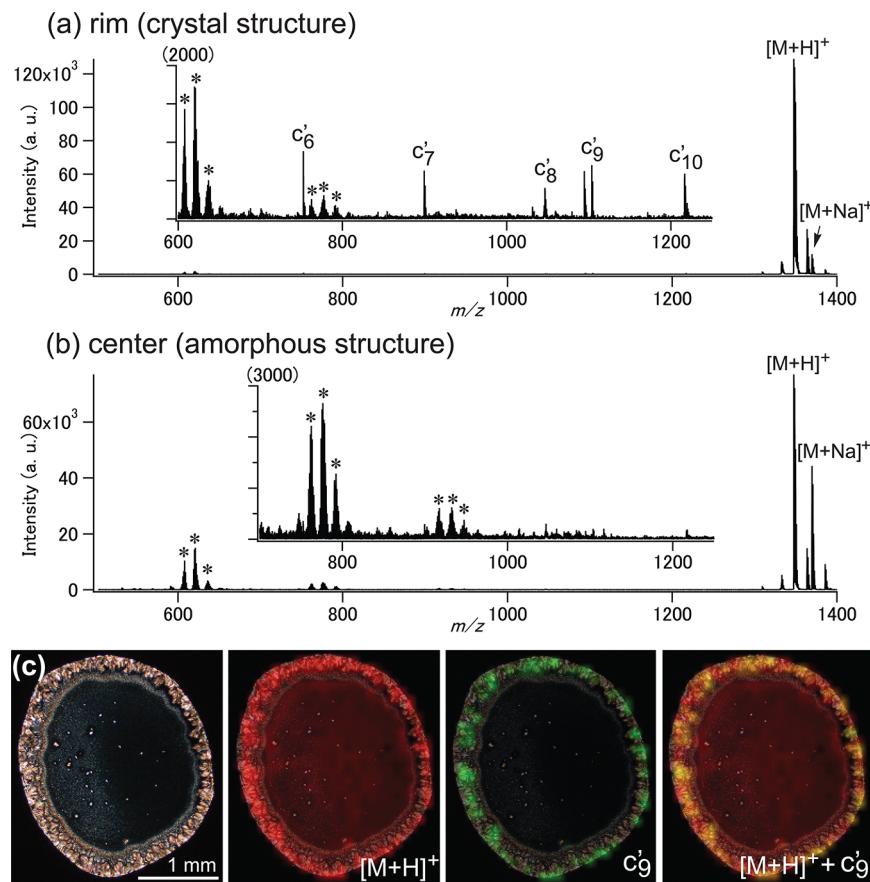


Figure 2. MALDI mass spectra of substance P obtained with (a) the crystal structure and (b) the amorphous structure of a 2,5-DHB/1,5-DAN (1/1 molar ratio) mixture matrix. (c) From left to right, picture of the spot, ion image of the protonated analyte $[M+H]^+$ (red), ion image of the c'_9 ISD fragment (green), and ion images of both $[M+H]^+$ and the c'_9 ISD fragment.

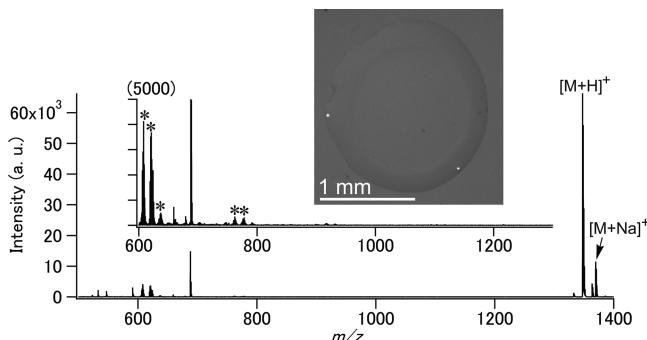


Figure 3. MALDI mass spectra of substance P obtained with a 2,5-DHB/1,5-DAN (2/1 molar ratio) ionic liquid matrix. Asterisks indicate matrix clusters. Inset panel: picture of the spot.

collected at each pixel position at a laser frequency of 200 Hz. Results were interpreted using FlexImaging 2.1 software.

Notation. We employed herein the unambiguous notation of Zubarev when naming the fragment ions.¹⁴ According to this notation, homolytic N–C_α bond cleavage yields the radical fragments $c\bullet$ and $z\bullet$, and loss of a hydrogen atom from a $c\bullet$ or $z\bullet$ fragment produces a c or z fragment, respectively. The product of a hydrogen atom transfer to a $c\bullet$ or $z\bullet$ fragment is denoted c' and z' , respectively. Thus, c and z fragments are 1.0078 Da lower in mass compared to $c\bullet$ and $z\bullet$ fragments, respectively. The c' and z' fragments are 1.0078 Da larger than

the $c\bullet$ and $z\bullet$ fragments. Unless noted otherwise, all assigned peaks represent singly protonated molecules.

RESULTS AND DISCUSSION

It has been previously described that the use of 2,5-DHB and 1,5-DAN was efficient to induce MALDI-ISM of peptides and proteins.^{15–17} Figure 1 shows the MALDI-ISM spectra of substance P obtained with 2,5-DHB and 1,5-DAN. MALDI-ISM spectra with 2,5-DHB and 1,5-DAN show c' ions with strong intensities. In order to better understand the interaction of the matrix with analytes, we performed imaging experiments to evaluate the influence of laser shot location on the ISD ion yield of substance P. The lower panel of Figure 1 shows ion images of the protonated molecule $[M+H]^+$ and the ISD fragment c'_9 . A similar ion image distribution was observed for other ISD fragments. From the ion 2D maps, it is clear that ISD fragmentation as well as protonation essentially occurs when laser irradiations are performed on matrix crystals. These results therefore confirm the importance of the matrix crystal in the occurrence of protonation and ISD processes. According to previously published X-ray crystallography data, 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.¹⁸ The hydrophilic (100) surface of the matrix crystal is covered by peptides via intermolecular hydrogen bonds.⁶ This suggests that the intermolecular hydrogen bonds between peptide and matrix in the 2,5-DHB

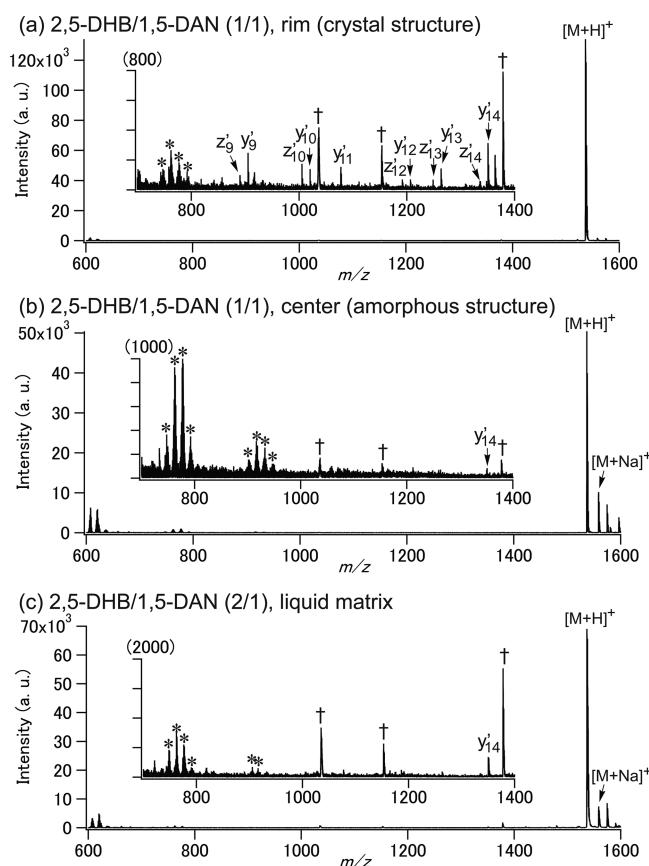


Figure 4. MALDI mass spectra of fibrinopeptide A obtained with (a) the crystal structure and (b) the amorphous structure of a 2,5-DHB/1,5-DAN (1/1 molar ratio) mixture matrix and (c) a 2,5-DHB/1,5-DAN (2/1 molar ratio) ionic liquid matrix. Asterisks and daggers indicate matrix clusters and postsource decay signals, respectively.

crystal are important for proton and hydrogen radical transfer reactions.

Next, we used a mixture of 2,5-DHB and 1,5-DAN at a 1/1 molar ratio as a matrix. Figure 2 shows the MALDI-ISD spectra of substance P, the optical image of the spot, and the ion images. From the optical images obtained by polarized light microscopy, we can conclude that crystals are formed at the rim of the spot, whereas an amorphous structure is present in its center. Figure 2 indicates that protonated molecules were produced from both crystal and amorphous regions. In contrast, ISD fragment ions are only observed when laser irradiation is performed on matrix crystals as confirmed by imaging experiments. This result suggests that hydrogen transfer reaction occurs in the matrix crystal region. In order to distinguish the reactions occurring within the matrix crystal and in the MALDI plume, we used a mixture of 2,5-DHB and 1,5-DAN (2/1, molar ratio) which forms an ionic liquid instead of crystals. The optical image of this spot is shown in the inset of Figure 3. No crystals were observed by polarized light microscopy, except for very few small crystals. Figure 3 shows the MALDI spectrum of substance P using the 2,5-DHB/1,5-

DAN ionic liquid matrix. Although the use of ionic liquid matrixes generates protonated substance P and matrix cluster ions signals, c' ions are not observed, even if the laser power was increased.

As the existence of a hydrogen-abundant peptide radical containing enough internal energy for decay is the condition for ISD fragmentation, we decided to investigate these two parameters in order to determine why ISD is suppressed in 2,5-DHB/1,5-DAN amorphous structure and ionic liquid matrix. First, we focused our attention on the internal energy of analyte ions generated by MALDI with 2,5-DHB, 1,5-DAN, and 2,5-DHB/1,5-DAN mixtures. A method to measure the internal energy of ions in mass spectrometry is the survival yield method, which is based on the fragmentation of "thermometer molecules" (benzyl substituted benzylpyridinium salts), and that was utilized by Colette et al.¹⁹ Briefly, we measured the internal energy of ions in MALDI with 2,5-DHB, 1,5-DAN, and 2,5-DHB/1,5-DAN mixtures. Figure S1 (Supporting Information) shows MALDI mass spectra of *p*-methoxybenzylpyridinium chloride, containing both the analyte (M^+) and its fragment (F^+), which is produced through a simple fragmentation pathway (Scheme 1). Survival yields (Y) of *p*-methoxybenzylpyridinium chloride are calculated by eq 1 and summarized in Table 1.

$$Y = I(M^+)/[I(M^+) + I(F^+)] \quad (1)$$

In agreement with previous reports,^{20,21} the internal energy of ions generated by MALDI is about 3.0 eV for 2,5-DHB and 1,5-DAN and 2.8 eV for 2,5-DHB/1,5-DAN mixtures. The difference of internal energies obtained with those matrixes is only about 0.2 eV, which does not contribute to ISD fragmentation.

Next, we focused on the formation efficiency of hydrogen-abundant peptide radicals. The use of a reducing matrix shows the reduction of a disulfide bond, leading to reduced product.²² The MALDI-ISD efficiency of a matrix can be correlated to its tendency to reduce the disulfide bond of peptide.¹⁷ To estimate the reduction of a disulfide bond, we used a peptide, calcitonin (salmon I), containing one disulfide bond. Figure S2 (Supporting Information) shows the comparison of positive-ion MALDI mass spectra of calcitonin obtained with four different matrixes: 2,5-DHB, 1,5-DAN, 2,5-DHB/1,5-DAN (1/1 molar ratio), and 2,5-DHB/1,5-DAN ionic liquid (2/1 molar ratio). The abundance of reduced products was calculated using theoretical isotopic contributions of all signals, as summarized in Table 2. The disulfide bond was strongly reduced by using 2,5-DHB, 1,5-DAN, and their mixture crystal compared to the observed reduction using ionic liquid and an amorphous structure of the 2,5-DHB/1,5-DAN mixture. This indicates that suppression of ISD fragmentation using ionic liquid and an amorphous structure of the 2,5-DHB/1,5-DAN mixture originates from a low efficiency of hydrogen-abundant peptide radical formation. Although the use of ionic liquid and an amorphous structure of the 2,5-DHB/1,5-DAN mixture does not generate ISD fragments, a reduced product of calcitonin is observed in Figure S2d and e (Supporting Information). This is

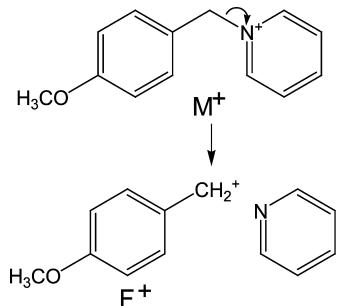
Table 1. Survival Yields (Y) of *p*-Methoxybenzylpyridinium Chloride (%)

2,5-DHB	1,5-DAN	2,5-DHB/1,5-DAN (1/1), crystal	2,5-DHB/1,5-DAN (1/1), amorphous	2,5-DHB/1,5-DAN (2/1), liquid matrix
88.2 \pm 1.8	88.8 \pm 3.5	93.2 \pm 0.6	92.8 \pm 1.0	93.3 \pm 0.9

Table 2. The Ratio of Signal Intensity of Reduced Calcitonin $[M + 2H + H]^+$ to That of Non-Reduced Ion $[M+H]^+$ of Peptides (%)

2,5-DHB	1,5-DAN	2,5-DHB/1,5-DAN (1/1), crystal	2,5-DHB/1,5-DAN (1/1), amorphous	2,5-DHB/1,5-DAN (2/1), liquid matrix
16.4 ± 7.8	91.6 ± 2.4	29.1 ± 2.9	7.1 ± 2.9	8.7 ± 2.3

Scheme 1. Fragmentation of *p*-Methoxybenzylpyridinium Cation



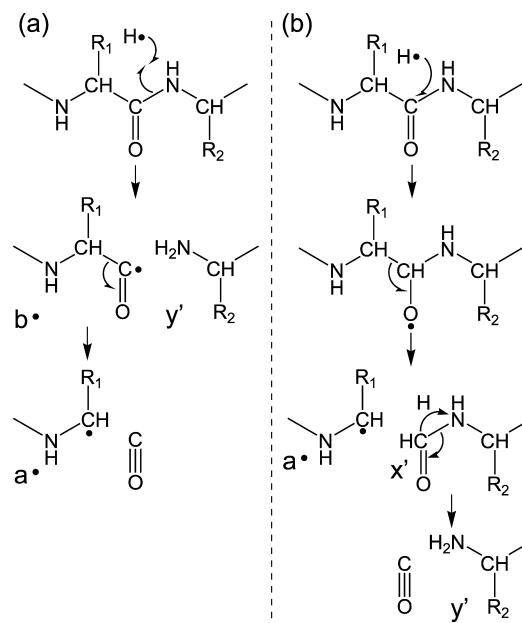
probably due to the higher hydrogen affinity of the disulfide bond compared to amide carbonyl.²³ Hydrogen attachment to the disulfide bond probably occurs in the ionic liquid matrix and/or MALDI plume.

The low efficiency of peptide radical formation can be the consequence of a low hydrogen radical production or of a low reactivity of the hydrogen radicals produced. In order to obtain information about hydrogen production from matrix molecules, we focused our attention on the matrix signals in MALDI mass spectra. Positive- and negative-ion MALDI mass spectra of 1,5-DAN, 2,5-DHB, and their mixtures are shown in Figure S3 (Supporting Information). The mixtures of 2,5-DHB/1,5-DAN only produced positively charged 1,5-DAN and negatively charged 2,5-DHB, probably due to the basic and acidic nature of 1,5-DAN and 2,5-DHB, respectively. It should be noted that positively charged 1,5-DAN and negatively charged 2,5-DHB were detected from all the spots of this mixture (1/1 molar ratio). It suggests the 2,5-DHB and 1,5-DAN presence in both crystal and amorphous structures. In order to confirm the composition of 2,5-DHB and 1,5-DAN in the spots of this mixture (1/1 molar ratio), we used Raman spectroscopy to establish the profile of the two compounds across the spots. The experimental conditions are described in the Supporting Information. As expected, 2,5-DHB and 1,5-DAN show similar distributions in the spot (Supporting Information, Figure S4). It has been reported that 1,5-DAN and 2,5-DHB gave $[M - 2H]^{2\bullet}$, which are formed by releasing a hydrogen atom from 1,5-DAN and 2,5-DHB.^{10,22} The $[M - 2H]^{2\bullet}$ of 2,5-DHB were observed in MALDI spectra of 2,5-DHB/1,5-DAN mixtures. Additionally, crystals, amorphous structure, and ionic liquid of 2,5-DHB/1,5-DAN mixtures gave similar ion patterns. This suggests that, following laser irradiation, the same reactions were induced by crystals, amorphous structure, and ionic liquid of 2,5-DHB/1,5-DAN mixtures, leading to release of hydrogen radicals. However, only crystals of the 2,5-DHB/1,5-DAN mixture gave ISD fragment ions. Intermolecular hydrogen bonds between matrix and peptide in the crystal are thus essential in order to induce efficient hydrogen transfer reaction, leading to the formation of hydrogen-abundant peptide radical. Therefore, we concluded that the first step of MALDI-ISD mainly occurs in matrix crystals.

We next turned to examination of C-terminal side fragments by MALDI-ISD with the 2,5-DHB/1,5-DAN mixture. Figure 4 shows MALDI mass spectra of fibrinopeptide A containing Arg residue at the C-terminus obtained with 2,5-DHB/1,5-DAN mixtures. As expected, z' ions were observed in the MALDI mass spectrum obtained from the rim of the 2,5-DHB/1,5-DAN mixture (1/1 molar ratio), whereas the ionic liquid and amorphous structure of 2,5-DHB/1,5-DAN mixtures did not produce z' ions. The suppression effect of the hydrogen transfer reaction between peptide and matrix was confirmed by the results shown in Figure 4.

Finally, we focused our attention on the formation mechanism of y' fragments. To date, it is believed that the y' fragments are generated by the thermal activation process in the MALDI plume.^{24,25} However, if the y' ions are often observed in MALDI-ISD spectra, their counterpart b ions are absent. In other words, peptide bond cleavage by thermal activation, leading to the b/y' fragment pair, is less likely to occur in the MALDI-ISD process. Moreover, $a\bullet$ ions are usually present in MALDI-ISD spectra instead of b ions. Although all tested matrixes produced analyte ions with similar internal energy (Table 1), the formation of y' fragments was suppressed by using 2,5-DHB/1,5-DAN in the ionic liquid and amorphous structure. It tends to prove the involvement of hydrogen-abundant peptide radicals in the formation of $a\bullet/y'$ fragments during the MALDI-ISD process. In order to explain this phenomenon, a mechanism similar to the one proposed by Zubarev et al.²³ to explain the occurrence of $a\bullet/y'$ ion pairs in electron capture dissociation experiments can be envisaged (Scheme 2a). This process involves the formation of a $b\bullet$ fragment pair and then a fragmentation of the $b\bullet$ fragments,

Scheme 2. Proposed Formation Pathways of the $a\bullet/y'$ Fragment Pair via Hydrogen Attachment



which give only $a\bullet$ fragments by loss of a CO molecule. Concerning MALDI-ISM experiments, it was previously shown that intermediate $b\bullet$ fragments are formed by the cleavage at Xxx-Pro bonds (Xxx = Ala,¹¹ Arg,¹¹ Ile,¹¹ Ser,¹¹ Tyr,¹⁰ and Val²⁶) by using an oxidizing matrix, and that these $b\bullet$ fragments can give both a and b fragments. This therefore indicates that $b\bullet$ fragments have enough stability to form a b fragment by radical recombination. In consequence, the $b\bullet$ fragments would be expected to give b' and b fragments, whereas those fragments are absent in MALDI-ISM spectra with reducing matrix. This suggests that the formation of a $b\bullet/y'$ fragment pair is not likely to occur.

On the other hand, formation of an $a\bullet/x'$ fragment pair in MALDI-ISM was previously reported by Smargiasso et al.²⁷ Because the transfer of a hydrogen atom from a matrix molecule to the carbonyl group of a peptide backbone initiates the MALDI-ISM,⁶ the existence of an oxygen-centered radical is possible. In that case, the $a\bullet/x'$ fragment pair would be generated by the α -cleavage of this oxygen-centered radical. A proposed pathway for the formation of an $a\bullet/x'$ fragment pair is shown in Scheme 2b. The $a\bullet/y'$ fragment pair might be formed via this mechanism, in which the x' fragments undergo the loss of CO after the backbone cleavage. However, x' ions, which are intermediate species in the proposed mechanism, were not observed in Figure 4, probably due to their low stability. This point will require further investigation for better understanding of the MALDI-ISM process.

CONCLUSION

In this study, we focused on the hydrogen transfer reaction between matrix molecules and analytes that produces hydrogen-abundant peptide radicals and their internal energy. For a similar internal energy content of "thermometer" molecule, the behavior of peptide ions is quite different according to the physical state of the matrix.

The use of either 2,5-DHB or 1,5-DAN matrixes leads to the formation of peptide radicals via hydrogen attachment to the peptide backbone or disulfide bond, whereas that is suppressed by the use of ionic liquid matrixes and an amorphous structure of 2,5-DHB and 1,5-DAN mixture used as a matrix. It indicates that the hydrogen-abundant peptide radicals are exclusively formed when favorable intermolecular hydrogen bonds can be formed between peptides and matrix, which is the case in the matrix crystals. Subsequently, $c'/z\bullet$ fragment pairs are formed by the radical-induced cleavage, and then, radical $z\bullet$ fragments undergo either gain of a hydrogen atom or loss of a side chain in the MALDI plume.^{7–9} Our data also suggests that the $a\bullet/y'$ fragment pair is formed via a hydrogen-abundant peptide radical. An oxygen-centered radical is probably formed by hydrogen attachment to the peptide. Subsequently, radical-induced cleavage at the C_{α} -C bond leads to the formation of an $a\bullet/x'$ fragment pair, and then, x' fragments undergo the loss of CO after the backbone cleavage. Considering the presence of oxygen-centered peptide radicals allows a better understanding of the MALDI-ISM process.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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