The Relative Charge Ratio Between C and N Atoms in Amide Bond Acts as a Key Factor to Determine Peptide Fragment Efficiency in Different Charge States

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The influence of charge state on the peptide dissociation behavior in tandem mass spectrometry (MS/MS) is worthy of discussion. Comparative studies of singly- and doubly-protonated peptide molecules are performed to explore the effect and mechanism of charge state on peptide fragmentation. In view of the charge-directed cleavage of protonated peptides described in the mobile proton model, radiolytic oxidation was applied to change the charge distribution of peptides but retain the sequence. Experimental studies of collision energy-dependent fragmentation efficiencies coupled with quantum chemical calculations indicated that the cleavage of ARRA and its side-chain oxidation products with oxygen atoms added followed a trend that doubly-protonated peptides fragment more easily than singly-protonated forms, while the oxidation product with the guanidine group deleted showed the opposite trend. By analyzing the charge distribution around the amide bonds, we found that the relative charge ratios between C and N atoms (Q_C/Q_N) in the amide bonds provided a reasonable explanation for peptide fragmentation efficiencies. An increase of the Q_C/Q_N value of the amide bond means that a peptide fragments more easily, and vice versa. The results described in this paper provide an experimental and calculation strategy for predicting peptide fragmentation efficiency. (J Am Soc Mass Spectrom 2010, 21, 1857–1862) © 2010 American Society for Mass Spectrometry

Tandem mass spectrometry (MS/MS) has become a technology for protein or peptide identification and characterization due to its high accuracy and sensitivity [1–5]. The experimental MS/MS data are compared against a database of theoretical peptide MS/MS spectra to find the best sequence match and the success of this approach depends heavily on the accurate prediction of the theoretical spectra [4, 6, 7]. However, peptide fragmentation is frequently far from ideal (where amide bonds are cleaved, yielding perfect product ion spectra) due to incomplete fragmentation [4, 8–10]. Thus, clarifying the cleavage mechanism of peptides can provide a theoretical basis for peptide identification.

Previous studies have shown that peptide dissociation behavior in MS/MS is a complex function of many factors including the amino acid sequence, residue content, and charge state [11–15]. Determining the effects of sequence and specific residues on peptide

dissociation behavior requires various peptide types, while the charge state has an effect applying to all sequences and residues. In addition, for the same peptide, both its sequence and residue content are difficult to change, while the different charge states can be easily observed in the same experiment. Therefore, evaluating the effect of charge state on peptide fragmentation is feasible. Dongre et al. [16] found that doubly-protonated peptides fragment more easily than the singly-protonated forms of the same peptides, while Jockusch et al. [17] indicated that a multiply protonated molecule at a higher charge state did not necessarily fragment more easily than that at a lower charge state. Therefore, it is significant to study the effect of charge state on peptide fragmentation.

The mobile proton model [11, 16, 18–20] has been employed to rationalize selective and nonselective fragmentation in protonated peptides; the charge state will affect the charge distribution of each atom in a peptide, and thus affect the protonation sites and cleavage mechanism [13]. To simulate the charge distribution of peptides, a strategy of quantum chemical calculation is required, which results in more precise and in-depth

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understanding of the effect of the charge state on peptide fragmentation.

Here, we concentrated our work on small peptides that have few reaction channels, rendering the peaks in the spectra easy to assign [21–23]. Arginine-containing peptides provide a strong signal in positive ion ESI-MS and are often used as model peptides [24, 25]. Oxidation of Arg, which is expected to be quite susceptible to attack by hydroxyl radicals, will significantly change the charge distribution of Arg-containing peptides with no change in peptide amide bonds. In this paper, a simple model peptide containing two arginine residues, Ala-Arg-Arg-Ala, was selected for side-chain oxidation and MS analysis to explore the fragmentation differences between singly and doubly-protonated molecules. In this way, the model peptides retain the same amide bonds and the results can be reliably compared.

Experimental

Materials

Peptide Ala-Arg-Arg-Ala (ARRA) was purchased from GL Biochem Inc. (Shanghai, China), and had a purity of at least 95%. Thiourea and trifluoroacetic acid (TFA) were ordered from Sinopharm Chemical Reagent Inc. (Shanghai, China). HPLC acetonitrile was purchased from Merck (Darmstadt, Germany). Solutions of peptide at a concentration of 40 μM were prepared with ultrapure water from a Millipore Ultrapure Water System (Shanghai, China).

Oxidation of Model Peptide

The peptide solution was exposed to a cobalt-60 γ -ray source (5 Gy/min) at the Agricultural Sciences Academy of Shandong Province. For 40 μM ARRA, 600 μL of the solution in a 1.5 ml Eppendorf tube was exposed for 2 min. After exposure to radiation, 30 μL thiourea (0.2 M) was added into the Eppendorf tube to eliminate the surviving oxidants and finally the sample was stored at 0–4 °C before mass spectroscopic analysis.

Mass Spectrometric Analysis

The native and radiolytically oxidized peptide were analyzed directly by electrospray MS without chromatographic separation. The solution concentration was adjusted to 20 μM with acetonitrile (containing 0.1% TFA) and was infused directly into the ESI-MS at a flow rate of 3 $\mu\text{L}/\text{min}$. Mass spectra were acquired on a LCQ Fleet mass spectrometer equipped with an ion trap of ThermoFisher (New York, USA). The protonated peptides were formed by electrospray ionization (ESI). The ESI source conditions were set as follows: needle voltage 3500 V, sample cone voltage 55 V, extraction cone voltage 0.5 V, source temperature 110 °C, cone gas (N_2) 30 L/h. The instrument was tuned using a known mass of the unmodified peptide ARRA.

To obtain the various fragment ions of model peptides, the collision-induced dissociation (CID) MS/MS spectra were acquired. Data acquisition was controlled with the Xcalibur 2.0 software (ThermoFisher) and the scan ranges were automatically adjusted according to the molecular weights of the parent ions. Each value was expressed as the means of at least 100 scans.

Quantum Chemical Calculations

An extensive conformational search was performed on the natural peptide ARRA, by means of a Boltzmann jump search method using the Materials Studio software (ver. 4.4), designed to select more and more stable conformers through sequential geometry optimization of trial structures at increasing levels of calculation theory. By this procedure, more than 1000 conformational structures were generated. Ten relative low-energy structures were encoded in Gaussian 03W [26] input files and further refined by performing density functional theory (DFT) optimizations, using the B3LYP functional and the 6-31G(d) basis set. All of the charge distribution results reported in this paper was calculated at the B3LYP level of theory using the Gaussian 03W program. A 6-31G(d) basis set was implemented to perform efficient, yet accurate, calculations. Partial charges on the amide nitrogen and carbonyl oxygen atoms were obtained by averaging over the optimized low-energy conformers. Calculations of electronic and structural properties allow direct comparisons with experimental measurements, including the analysis of charge distribution.

Results and Discussion

Side-Chain Oxidation Products of ARRA

Our model compound was the Ala-Arg-Arg-Ala peptide (ARRA), which has a molecular mass of 472. In its mass spectrum (Figure 1a) the peaks at m/z 237.27 and 473.29 belong to the singly- and doubly-protonated molecules. Because ARRA contains two Arg residues, it can be oxidized to produce multiple types of products. Arginine is expected to be more susceptible than alanine to attack by hydroxyl radicals because the reactivity order of the amino acid side chains is Arg > Ala [27]. We explored the positive ESI-MS spectrum of ARRA exposed to γ -rays (Figure 1b) to study the side-chain oxidation products. Compared with the native peptide, the two peaks at m/z 489.45 and 505.18 are the singly-protonated oxidation products with one or two oxygen atoms added, respectively, while the peaks at m/z 245.22 and 253.13 are the doubly-protonated molecules of the above oxidation products [28]. Radiolysis also results in the loss of a guanidine group from the side chain of an Arg residue, giving rise to a characteristic product with a –43 Da mass change (peak at m/z 430.26 and 216.21) [25]. In addition, there is another relatively high peak at m/z 443.27 and 222.29, which is the oxidative decarbox-

Figure 1. Positive ESI mass spectrum of (a) native ARRA and (b) ARRA exposed to γ -rays for 2 min.

ylation product with the formation of an aldehyde group at the carbon next to the original carboxyl group [28]. In this paper, our focus was only on the side-chain oxidation products. The MS/MS spectra of singly and doubly-protonated molecules for the native peptide and its side-chain oxidation products are shown in Supplementary Data, Figure S-1, which can be found in the electronic version of this article. It can be seen that the main fragment ions of all peptides are a_n , b_n , and y_n ions, suggesting that fragmentation of all these peptides follows similar pathways and mostly occurs at the amide bonds.

The mechanism of radiolytic oxidation for the formation of various products is presented in Scheme 1. It should be noted that for the side-chain oxidation products with an O atom added or guanidine group loss, the +16 Da mass or -43 Da mass change could occur in each of the Arg residues. The product with a +32 Da

mass change is obtained following the addition of one O atom at each of the two Arg residues.

Comparative Fragmentation Efficiency Curves for Singly- and Doubly-Protonated Peptides

Peptides containing Arg residue generally yield singly- or doubly-protonated molecules upon electrospray ionization and the different charge states may also have a significant effect on the fragmentation behavior of peptide ions [13]. Fragmentation efficiency curves (percent fragmentation of a given peptide as a function of laboratory collision energy) are a measure of how easily a protonated peptide fragments in the gas phase. In view of the above, we compared the fragmentation efficiency curves of singly- and doubly-protonated pep

Scheme 1. Proposed mechanism for formation of radiolytic oxidation products from ARRA.

tides for native ARRA and its multiple side-chain oxidation products (Figure 2). Doubly-protonated ARRA and its oxidation products with O atoms added can fragment to the same extent at lower collision energy, while the singly-protonated ones at higher collision energy (Figure 2a–c). For the oxidation product with the guanidine group loss (Figure 2d), the two curves of singly- and doubly-protonated molecules intersect. The lower threshold energy of doubly-protonated peptide means that some fragment ions are easily produced in this doubly-protonated peptide, while the relative stability towards fragmentation of the peptide is determined by the energy when peptide fragments completely. It can be seen from Figure 2d that the doubly-protonated peptide has a higher terminal energy, indicating that the doubly-protonated peptide needs more energy to fragment completely than the singly-protonated one. It has previously been shown that the shapes of fragmentation efficiency curves are characteristic of the reaction entropy [29]. Kinetically unfavored reactions are associated with shallow slopes, while fast (kinetically favored) fragmentation results in steep slopes. Following this mode of interpretation, the curve of singly-protonated peptide in Figure 2d was steeper than that of the doubly-protonated form, indicating that the dissociation of its singly-protonated molecule is kinetically favored compared with the doubly-protonated molecule (in contrast to what is occurring in Figure 2a–c).

Comparative Charge Distribution in the Amide Bonds of Singly- and Doubly-Protonated Peptides

Under low-energy collision conditions, protonated peptide molecules fragment primarily at amide bonds along the peptide backbone [30]. The mobile proton model, one of the central tenets in peptide fragmentation mechanisms, indicates that cleavage of amide bonds is initiated by migration of the charge along the peptide backbone [16, 18, 31]. In view of the above, we calculated the charge distribution on the amide bonds of the studied peptides (Table S-1) using quantum chemical calculations at the B3LYP/6-31G(d) level. Previous studies [32–34] have led us to believe that this level of theory can give consistently reliable, conformationally dependent charges and energies for peptides. We list a comparison of the relative charge on the C and N atoms (Q_C/Q_N) in the amide bonds of the native and side-chain oxidized peptides in Table 1. By analyzing the $\Delta(Q_C/Q_N)$ of singly-protonated peptides compared with the doubly-protonated peptides, we found that for the model peptide and its oxidation products with +16 and +32 Da mass changes, each Q_C/Q_N value in the three amide bonds of the singly-protonated peptides tended to decrease. However, for the oxidation product with –43 Da mass reduction, the three Q_C/Q_N values all increased instead. This phenomenon shows that the effect of charge state (singly- or doubly-protonated) on peptide fragmentation efficiency does not always follow a univer-

Figure 2. The comparative fragmentation efficiency curves of singly and doubly-protonated peptides (ESI) for (a) ARRA, (b) ARRA+16 Da, (c) ARRA+32 Da, (d) ARRA-43 Da. Each curve was averaged over three experiments.

Table 1. Relative charge comparison between C and N atoms in the amide bonds

	$(Q_C/Q_N)_1^b$	$(Q_C/Q_N)_2^b$	$(Q_C/Q_N)_3^b$	$\Delta(Q_C/Q_N)_1$	$\Delta(Q_C/Q_N)_2$	$\Delta(Q_C/Q_N)_3$
M^a						
AR ⁺ R ⁺ A ^d	−0.99674	−0.99347	−1.04226	α_1^c	α_2^c	α_3^c
AR ⁺ RA ^d	−0.99747	−1.00276	−1.05508	$\alpha_1 - 0.00073$	$\alpha_2 - 0.00929$	$\alpha_3 - 0.01282$
ARR ⁺ A ^d	−1.00908	−0.99271	−1.04372	$\alpha_1 - 0.01234$	$\alpha_2 + 0.00077$	$\alpha_3 - 0.00146$
M^a+16Da						
AR ⁺ O ⁺ R ⁺ A ^d	−0.99762	−0.99286	−1.04286	β_1^c	β_2^c	β_3^c
AR ⁺ O ⁺ RA ^d	−0.99802	−1.00276	−1.05571	$\beta_1 - 0.00040$	$\beta_2 - 0.00990$	$\beta_3 - 0.01284$
AR ⁺ O ⁺ R ⁺ A ^d	−1.00794	−0.99312	−1.04452	$\beta_1 - 0.01032$	$\beta_2 - 0.00026$	$\beta_3 - 0.00166$
AR ⁺ R ⁺ O ⁺ A ^d	−0.99644	−0.99455	−1.04151	γ_1^c	γ_2^c	γ_3^c
AR ⁺ R ⁺ O ⁺ A ^d	−0.99545	−1.00490	−1.05483	$\gamma_1 + 0.00099$	$\gamma_2 - 0.01036$	$\gamma_3 - 0.01332$
ARR ⁺ O ⁺ A ^d	−1.00719	−0.99403	−1.04322	$\gamma_1 - 0.01075$	$\gamma_2 + 0.00052$	$\gamma_3 - 0.00171$
M^a+32Da						
AR ⁺ O ⁺ R ⁺ O ⁺ A ^d	−0.99484	−0.99314	−1.04261	δ_1^c	δ_2^c	δ_3^c
AR ⁺ O ⁺ RO ⁺ A ^d	−1.00076	−1.00561	−1.05617	$\delta_1 - 0.00591$	$\delta_2 - 0.01248$	$\delta_3 - 0.01356$
AR ⁺ O ⁺ RO ⁺ A ^d	−1.00524	−0.99450	−1.04381	$\delta_1 - 0.01040$	$\delta_2 - 0.00136$	$\delta_3 - 0.00120$
M^a−43Da						
⁺ AR ⁺ R ^{−43} A ^d	−1.04210	−1.01360	−1.07174	λ_1^c	λ_2^c	λ_3^c
AR ⁺ R ^{−43} A ^d	−0.99436	−1.00263	−1.06044	$\lambda_1 + 0.04775$	$\lambda_2 + 0.01097$	$\lambda_3 + 0.01129$
⁺ AR ^{−43} R ⁺ A ^d	−1.05534	−1.00468	−1.05571	σ_1^c	σ_2^c	σ_3^c
AR ^{−43} R ⁺ A ^d	−1.00587	−0.99325	−1.04469	$\sigma_1 + 0.04946$	$\sigma_2 + 0.01143$	$\sigma_3 + 0.01102$

^aM stands for the model peptide.^b1, 2, 3 stand for the three amide bonds numbered from the N-terminal side to C-terminal side.^c α , β , γ , δ , λ , σ stand for the fiducial $\Delta(Q_C/Q_N)$ values of the six doubly-protonated peptides.^dIncludes symbols ⁺, O, ^{−43} which stand for the binding sites of protons, O atoms and the loss of the guanidine group, respectively.

sal trend, which is consistent with the results of the fragmentation efficiency curves in Figure 2.

The Relationship Between (Q_C/Q_N) Values in the Amide Bonds and Peptide Fragmentation Efficiency

Based on an overall analysis of the experimental results and theoretical calculations, we conclude that there is a close relationship between the Q_C/Q_N values in the amide bonds and peptide fragmentation efficiency. An increase of the Q_C/Q_N value in the amide bond means that the peptide fragments easily, and vice versa. Therefore, this charge ratio can be used to predict the relative cleavage extent of peptides.

Conclusions

By the powerful combination of experimental results and theoretical calculations, we have been able to shed further light on the key factor of peptide fragmentation in different charge states. The fragmentation efficiency curves presented here show that the effect of charge state on peptide fragment efficiency does not always follows a universal trend: for the native ARRA and its oxidation products with oxygen atoms added to the side chain, the doubly-protonated peptides fragment more easily than the singly-protonated forms; while for the oxidation product with the guanidine group removed from the side chain of Arg residue, the singly-protonated molecule fragments more easily. This phenomenon is also supported by quantum chemical calculations, and may be interpreted by the compara-

tive Q_C/Q_N values in the amide bonds. The increase of Q_C/Q_N results in a high fragmentation efficiency of a peptide, and vice versa. The results described in this paper clarify the relationship between charge distribution and peptide fragmentation. This work has increased our understanding of the mobile proton model to the extent where it can now serve as a predictive model to probe peptide fragmentation.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2010.06.022](https://doi.org/10.1016/j.jasms.2010.06.022).

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