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Influence of charge distribution on the discrepant MS/MS fragmentation of the native and oxidized FMRF: evidence for the mobile proton model

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Using the mobile proton model as a framework, the influence of charge distribution on the discrepant fragmentation of peptides FMRF, FM(O)RF and FM(O_2)RF (with united peptide sequence) was explored by mass spectrometry experiments and quantum chemical calculations. With the added O atoms, more negative charges were prompted to deposit in the main protonation sites of the oxidation products. Consequently, the solvated proton to the oxidized peptides could flow to the amide bonds in an easier manner and made these bonds fragment easily. Oxidation also induced the discrepant fragmentation of these bonds in a predictable manner: the more negative charges deposited in an amide bond, the more daughter ions (a_n, b_n, y_n ions and their derivatives) were produced. The combined methods proposed here refined the mobile proton model for peptide fragmentation and opened the way to probe the discrepant fragmentation of peptides in peptide/protein identification. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide identification; MS/MS; peptide fragmentation; mobile proton model; charge distribution

Introduction

Peptide tandem mass spectra (MS/MS) generated from low-energy fragmentation processes have been widely used in peptide identification [1–8]. To confirm the sequences of target peptides, theoretical fragmentation patterns based on the assumed sequences are usually generated and compared to the experimental spectra [2,6–8]. Although the theoretical MS/MS data can be searched very rapidly, this methodology is limited to obtaining sufficient sequence information for most protonated peptides due to the incomplete fragments and noise peaks [9–11]. Thus, mechanistic understanding of peptide fragmentation is significant for peptide sequencing and identification [3–4,12–15].

Extensive progress has been made through the efforts of several research groups in predicting the fragmentation mechanism of protonated peptides [9–11,13–18]. By systematically changing peptide sequences, Wysocki and coworkers proved that the cleavage processes of protonated peptides depended on peptide composition, gas-phase basicity, protonation sites and the number of added protons [13–15,17]. Using quantum chemistry calculation, Paizs *et al*. verified that the protonated amide bonds activated the cleavage processes of charge-induced fragment ions [4,16]. On the basis of these pioneering achievements, a prevalent 'mobile proton model' was proposed to interpret the fragmentation mechanism of peptides. This model claims that the primary fragmentation pathways of most protonated peptides are charge-directed and an input of energy is required to move the proton from the more basic sites to the amide bonds (the main cleavage sites), producing the b-type and y-type ions [14,17–18]. Having the ability to predict the presence of such fragment ions, this model markedly optimizes the algorithms for database search and was widely applied in peptide/protein identification [8,19–20].

However, to analyse the MS/MS spectra in an effective way, quantitative information aspects (ion intensity) of peptide fragmentation is still an issue that needs to be addressed [8,20–21]. This is because the existing theoretical fragmentation patterns based on the mobile proton model cannot provide relative intensities (as important as qualitative information) for different fragment ions. Attempts aimed at predicting the discrepant fragmentation of peptides will refine this model and provide more effective algorithms for database search. Being charge-directed, the fragmentation of peptides induced by a mobile proton can be highly influenced by their charge distributions in certain fixed ways: sites with more negative charges might be prone to solvate the proton and cleavage. Moreover, the precise charge distributions of peptides can be obtained by quantum chemical calculations [22–24]. Therefore, exploring the influence of charge distribution on peptide fragmentation is an apposite strategy for quantitative evaluation.

In the work presented here, model peptide Phe-Met-Arg-Phe (FMRF) with a Met residue (quite susceptible to oxidation) was subjected to H_2O_2 oxidation. Side-chain oxidation on peptide FMRF would change its charge distribution [25] and simultaneously resolve the inconsistent amide bond sequence/protonation sites of previous studies [13–14]. The MS/MS data (fragmentation energy curves and fractional abundances of fragment ions) of the native and oxidized peptides and their charge distributions obtained

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by quantum chemical calculations were incorporated to study the influence of oxidation on the charge distribution and the discrepant fragmentation of the native and oxidized FMRF.

Materials and Methods

Materials

Peptide Phe-Met-Arg-Phe (FMRF, >95% pure) was purchased from GL Biochem Inc. (Shanghai, China) and had a molecular weight of 599.3 Da. Thiourea, methionine (Met), trifluoroacetic acid, NH_4HCO_3, and 30% H_2O_2 were ordered from Sinopharm Chemical Reagent Inc. (Shanghai, China), and HPLC acetonitrile from Merck (Darmstadt, Germany). A solution of FMRF at a concentration of 0.06 mmol/L and other reagents were prepared with ultrapure water using a Millipore Ultrapure water system.

Oxidation of Model Peptide FMRF

To prepare the oxidized sample, a mixture of 0.05 mmol/L FMRF and 5 mmol/L H_2O_2 was incubated at 25 °C for 30 min. The oxidation reaction was terminated by adding the stop solution (Thiourea 100 mmol/L, Met 2 mmol/L) and the final concentration of FMRF was 0.04 mmol/L. The intact and oxidized samples were stored at 0–4 °C before MS and MS/MS analysis.

MS and MS/MS Analysis

An LCQ Fleet mass spectrometer (Thermo–Fisher) equipped with an electrospray ionization source was optimized for the mass fingerprinting of native and oxidized peptides in positive ion mode (source voltage 3.5 kV, cone voltage 50 V, source block 110 °C, desolvation heater 350 °C, and Ar collision gas). Samples were adjusted to 0.02 mmol/L with acetonitrile (containing 0.1% trifluoroacetic acid) and directly injected into the mass spectrometer at a flow rate of 3 µl/min using a syringe pump. The instrument was tuned using a known mass of the unmodified peptide FMRF (599.8 Da). Data acquisition was controlled with the X-calibur 2.0 software (Thermo–Fischer) and the scan ranges were automatically adjusted according to the molecular weights of the parent ions.

Quantum Chemical Calculations of Charge Distribution

The charge distributions of the native and oxidized FMRF were calculated at the B3LYP level of theory using the Gaussian 03 W program. A 6-31G(d) basis set has been implemented in order to perform efficient and accurate calculations. The fully optimized structures for the peptides were obtained through minimum-energy geometry optimizations. Calculations of electronic and structural properties allowed direct comparisons with experimental measurements, including the analysis of charge distributions.

Results and Discussion

Side-chain Oxidation Products Analysis

Samples of oxidized FMRF were subjected to MS and MS/MS analysis to identify the oxidized products. This is because oxidation can shift the peptide mass, and the mass difference between the intact and oxidized peptides can be observed [26–27]. Figure 1A

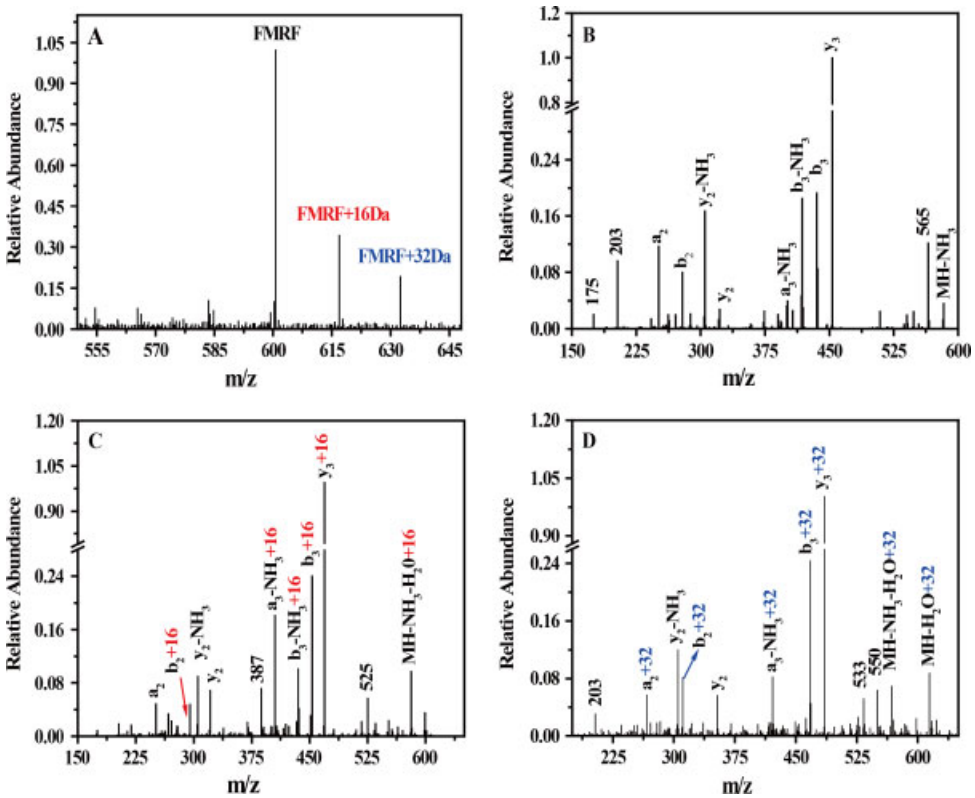

Figure 1. (A) Mass spectrum of the oxidized FMRF solution that was exposed to H_2O_2 oxidation for 30 min. (B–D) MS/MS spectra of ions at *m/z* 601.3, 617.3 and 633.3 with the collision energy at 30 eV. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

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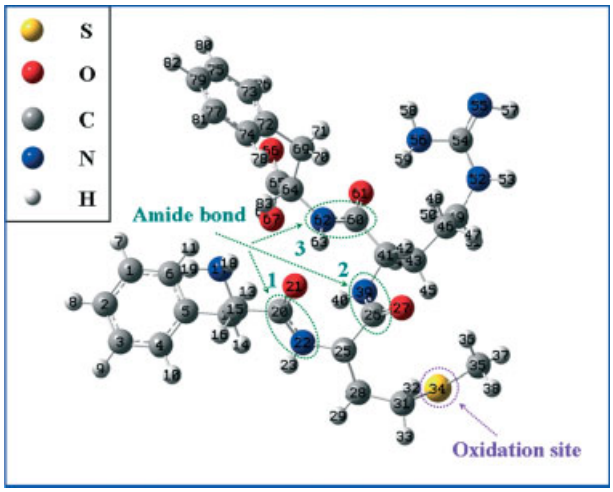

Scheme 1. A representative optimized conformation for FMRF. Amide bonds 1, 2 and 3 are referred to below.

illustrates the ESI/MS detection of a sample of oxidized FMRF and the main quasi-molecular ions are at *m/z* 600.3, 616.3 and 632.3. Obviously, the ion at *m/z* 600.3 should be attributed to $[FMRF+H]^+$. The ions at *m/z* 616.3 and 632.3 should be ascribed to its oxidation products $[FMRF+O+H]^+$ and $[FMRF+2O+H]^+$, considering the atomic weight of O (16 Da).

To identify the oxidation sites within FMRF, we also obtained the tandem mass spectra of these single-charged peptides with the collision induced dissociation (CID) technique. From Figure 1B, it could be seen that the native FMRF fragments in a predictable manner due to the predominant a-, b- and y-type ions. These ions, mainly generated by the cleavage of the amide bonds within FMRF (a-type ions were the secondary fragment of b ions), could provide sufficient structure information for the identification of FMRF and its oxidation products. Daughter ions upstream from $[FMRF+O+H]^+$ showed that a shift of +16 Da was detected on the b_2 + 16 Da, b_3 (a_3) +16 Da and y_3 + 16 Da fragment ions (Figure 1C), but y_2 + 16 was not detected, suggesting the added O atom was located at the Met residue. The MS/MS spectrum of $[FMRF+2O+H]^+$ (Figure 1D) also revealed that the Met residue was the unique oxidation site. This is because, the 32 Da mass adding was detected on the b_2, b_3 (a_3) and y_3 ions, but not detected for y_2 ions. The oxidation mechanisms of Met for the formation of the +16/32 Da products had been explored in depth: the O atom was prone to incorporating into the S atom in the side-chain and formed Met sulfoxide (+16 Da) and Met sulfone (+32 Da) [27–28]. Therefore, we concluded that reactions with H_2O_2 resulted in the side-chain oxidation of FMRF, producing FM(O)RF (sulfoxide) and FM(O_2)RF (sulfone).

Influence of Side-Chain Oxidation on the Charge Distribution of FMRF

According to the mobile proton model, the fragmentation of protonated peptides was charge-directed and largely influenced by the nature of the protonation sites [3,17]. Being positively charged, the migration of protons to the potential protonation sites could be influenced by the charge distribution of peptides [22]. For this reason, we investigated the specific influence of side-chain oxidation on the charge distributions of the native and oxidized peptides with the benefit of quantum chemical calculations.

Table 1. Charge distribution of the potential protonation sites in native and oxidized FMRF

Potential protonation sites		Mulliken atomic charges (Q)		
		FMRF	FM(O)RF	FM(O_2)RF
Amide bond 1[a]	O_{21}[b]	−0.462092	−0.533316	−0.532316
	N_{22}	−0.552279	−0.647613	−0.648646
Amide bond 2	O_{27}	−0.488338	−0.538459	−0.548473
	N_{39}	−0.592551	−0.618205	−0.619073
Amide bond 3	O_{61}	−0.438050	−0.541169	−0.541168
	N_{62}	−0.712507	−0.641563	−0.642461
N-terminus	N_{17}	−0.712799	−0.723950	−0.723424
Side-chain of Arg	N_{56}	−0.717158	−0.778938	−0.779143

[a] 1, 2 and 3 stand for the three amide bonds marked in Scheme 1.
[b] Atom numbers stand for the protonation sites marked in Scheme 1.

A representative conformation of FMRF (Scheme 1) was energy-optimized at the B3LYP/6-31G(d) level and the conformation-dependent charge distribution was obtained as well. As previously concluded, the S_{34} atom in the Met residue was the definite oxidation site and the oxidation products were FM(O)RF and FM(O_2)RF. On the basis of the above strategy, the optimized conformations and the charge distributions of FM(O)RF and FM(O_2)RF were acquired as well. In order to probe the specific influence of side-chain oxidation on peptide fragmentation, a simple comparison of the relative charges obtained for the basic sites in the native and oxidized peptides is listed in Table 1. Compared to the native peptide, oxidation on the S_{34} atom prompted more negative charges to deposit in most potential protonation sites of FM(O)RF and resulted in significant decrease in their relative values (increase only occurs to N_{62} in the third amide bonds). When the oxidation extent was increased (with two O atoms simultaneously added at S_{34}), the charge distribution of these sites changed with the above trend (with even more negative charges deposited in these sites).

Relationship between Charge Distribution and the Fragmentation Efficiency Curves

Fragmentation efficiency curves were usually used to evaluate fragmentation processes of protonated peptides, as they could provide a measure of the relative fragmentation energetics for the precursor ions and the relative intensities of the fragmented ions [13,15,29]. The fragmentation efficiency curves of the single-charged FMRF, FM(O)RF and FM(O_2)RF were investigated by ESI/CID and is illustrated in Figure 2. A comparison of these curves showed that FMRF required higher collision energies for efficient fragmentation, whereas FM(O)RF and FM(O_2)RF required lower energies for the same extent of fragmentation. Moreover, the fragmentation efficiency curve for FM(O_2)RF with a greater extent of oxidation shifted to even lower energies compared to FM(O)RF. The discrepant fragmentation of these peptides could also be characterized by the shape of these curves. Compared with the shallow curve of FMRF, the curves of the oxidized peptides have steeper trends with a greater extent of oxidation, suggesting that dissociations of FM(O)RF and FM(O_2)RF were kinetically favoured [13].

In view of the charge distributions of these peptides, we found that oxidation on the Met side-chain prompted more negative

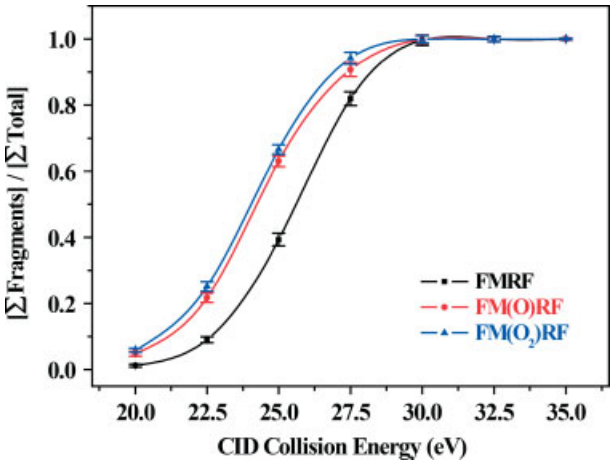

Figure 2. Fragmentation efficiency curves for the singly charged peptides. The values were expressed as the means of three experiments.

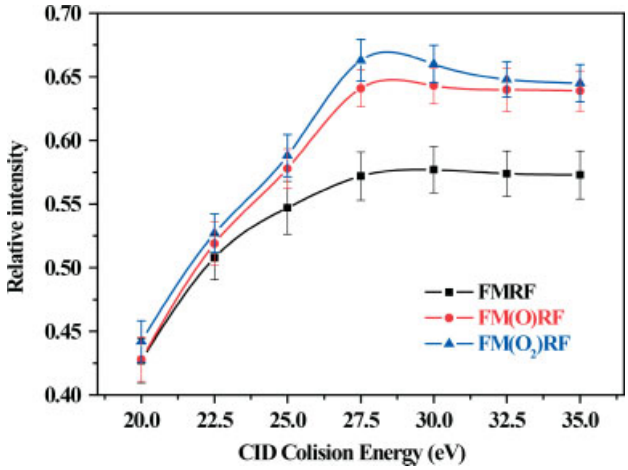

Figure 3. Fractional abundance curves for the basic fragment ions of the singly charged peptides. The values were expressed as the means of three experiments.

charges to deposit in most protonation sites in FM(O)RF, FM(O_2)RF (Table 1), implying that these sites would have a higher chance of being protonated. According to the 'mobile proton model', the primary fragmentation pathways of these peptides were charge-directed and their enhanced affinity to protons could induce easier fragmentation of peptides. Though more negative charges were deposited in the N_{56} atom and went against the fragmentation of peptides, this adverse influence was feeble because of the slight differences, compared with the total trend of the other protonation sites. The discrepancy between the curves of FM(O)RF and FM(O_2)RF also could be interpreted by charge distribution: with a higher oxidation extent, most protonation sites in FM(O_2)RF had more negative charges (compared with FM(O)RF), making the curve shift to the lower CID collision energies.

Relationship between Charge Distribution and the Fractional Abundances of the Basic Fragment Ions

Owing to the concomitantly undesignated fragments (hard to be used for database search), the fragmentation efficiency curves could not quantitatively interpret the changed fractional abundances of the basic ions (a-, b- and y-type ions) [9–10]. To quantitatively evaluate the discrepant fragmentation processes of these peptides, the fractional abundances of their basic fragment ions were obtained at different collision energies by setting the intensity of the total fragment ions as 1 (Figure 3). Results showed that the increased input energies promoted the higher fragmentation degrees of the amide bonds in all peptides, forming more basic fragment ions. However, when input energies exceeded 30 eV, the fractional abundances of the basic fragment ions fell. The inconsequent trends should result from the fact that other bonds in these peptides, more than the amide bonds, were prone to cleavage at higher energies. Compared with FMRF, the oxidized peptides had higher curve positions with increased extents of oxidation, also suggesting that dissociations of the amide bonds in FM(O)RF and FM(O_2)RF were kinetically favoured (consistent with the results of fragmentation efficiency curves).

Changes between the charge distributions of these peptides could also explain the above phenomenon under the framework of the mobile proton model: the negatively charged amide bonds in FM(O)RF and FM(O_2)RF had stronger affinity to protons and promoted the cleavage processes of their amide bonds. Compared to FM(O)RF, the corresponding amide bonds in FM(O_2)RF were slightly negatively charged and thus had a little higher fractional abundances of its basic fragment ions.

Relationship between Charge Distribution and the Fractional Abundances of the Basic Fragment Ions Corresponding to Each Amide Bond

To quantitatively evaluate the discrepant fragmentation of amide bonds in these peptides, the fractional abundances of the basic fragment ions corresponding to each bond were obtained by setting the intensity of the total basic fragment ions as 1. Figure 4A shows the fractional abundances of the basic fragment ions corresponding to the three amide bonds of FMRF. With the increase in CID collision energy, the fragmentation degrees of amide bonds one and two gradually increased, indicating that the input energy was in favour of the fragmentation of these amide bonds. But for amide bond three, it had lower fractional abundances. According to 'mobile proton model', the fragmentation processes of peptides depended on the mobile proton and the conformation in the vicinity of their amide bonds as well [12,30–31]. In view of the settled charge distribution of FMRF, the selective fragmentation of these amide bonds should be attributed to the special conformation of these bonds.

Though charge distribution could not explain the different fragmentation trends among the amide bonds in the same peptide, it could be used to evaluate the discrepant fragmentation processes of the FMRF with and without oxidation. Considering the fractional abundances of the amide bonds in FM(O)RF and FM(O_2)RF, we found that oxidation promoted the cleavage processes of amide bonds one and two, but went against for the third amide bond (Figure 4B–D). This phenomenon can be explained in view of the changed charge distribution of the oxidized peptides: with the added O atoms, the protonation sites in the amide bonds one and two of FM(O)RF and FM(O_2)RF were more negatively charged and fragment easily, whereas the amide bonds three were less negatively charged and fragment in a difficult mode.

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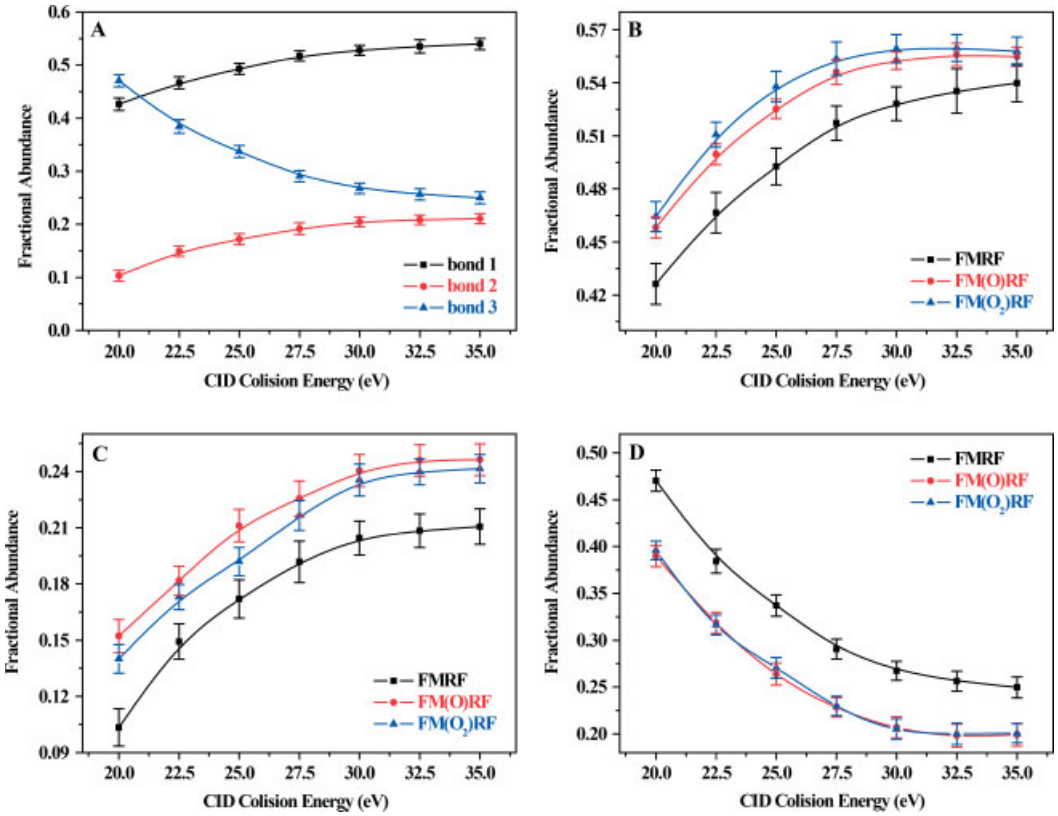

Figure 4. (A) Fractional abundance curves for the basic fragment ions corresponding to each amide bond in the singly charged FMRF. (B–D) Fractional abundance curves for the basic fragment ions corresponding to amide bond 1, 2 and 3 in the singly charged peptides. The values were expressed as the means of three experiments.

Conclusions

The mobile proton model, supported by MS/MS results and quantum chemical calculations, possessed a central role in evaluating the influence of charge distribution on peptide fragmentation. According to the experimental results, the Met residue in FMRF was directly oxidized into Met sulfoxide and Met sulfone, forming the side-chain oxidation products FM(O)RF and FM(O_2)RF. With the added O atoms, more negative charges were deposited in the main protonation sites in FM(O)RF and FM(O_2)RF. As a result, the added protons could flow to the amide bonds in a free manner and made the cleavage of the oxidized peptides much easier. The corresponding results were the lower fragmentation efficiency curves and higher fractional abundances of the total basic fragment ions. Oxidation also changed the charge distributions of amide bonds in different trends and induced the discrepant fragmentation of these bonds in a predictable manner: an amide bond with less deposited negative charges will fragment with difficulty. The methods described in this article provides an experimental basis to further substantiate the mobile proton model as well opens the way for the quantitative analysis of the MS/MS spectra of dissociated peptides.

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