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Mass Spectrometry Analysis of *In Vitro* Nitration of Carbonic Anhydrase II†

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Protein tyrosine nitration is considered as an important indicator of nitrosative stresses and as one of the main factors for pathogenesis of inflammation and neuronal degeneration. In this study, we investigated various nitrosative modifications of bovine carbonic anhydrase II (CAII) through qualitative and semi-quantitative analysis using the combined strategy of Fourier transformation ion cyclotron resonance mass spectrometry (FT-ICR MS) and ion-trap tandem mass spectrometry (IT-MS/MS). FT-ICR MS and its spectra were used for the search of the pattern of nitrosative modifications. Identification of nitrosatively modified tyrosine sites were executed through IT-MS/MS. In addition, we also tried to infer the reason for the site-specific nitrosative modifications in CAII. In view of the above purpose, we have explored- i) the side chain accessibility, ii) the electrostatic environment originated from the acidic/basic amino acid residues neighboring to the nitrosatively modified site and iii) the existence of competing amino acid residues for nitration.

Key Words : Mass spectrometry, FT-ICR MS, High resolution MS, Nitration, Nitrosylation

Introduction

Increased tyrosine nitration on proteins serves as an important indicator to responses for nitrosative stresses and plays a causative role in pathogenesis of inflammation and neuronal degeneration.[1-3] Recently, the novel nitrosative modification on tyrosine residues, which is the introduction of a nitroso group (-NO) on tyrosine residues by peroxynitrite and tetranitromethane (TNM), has been reported.[4]

Protein tyrosine nitration exhibits a certain degree of selectivity, as not all tyrosine residues of proteins are the substrates for nitration.[5] It has been known that the targeted nitration of certain tyrosine residues may be biochemically correlated with the structure-function relationship of nitrated proteins.[6,7] The nitration of protein tyrosine residues may result in dramatic changes in protein structure and consequently alter its function, which link nitrosative stress to altered molecular functions in disease[8-11] The nitrated sites (356Y and 366Y) of sphingosine-1-phosphate lyase 1(SIP lyase 1) are located in the catalytic domain of the protein.[12] Nitration of leukocyte immunoglobulin-like receptor subfamily A member 4(LIRA4) also occurs in the antigen-interacting domain, subsequently regulating the interaction between antigen and LIRA4.[12]

Recent studies have shown that the amino acid sequence or specific nitrating environments are the regulating factors for the site- and protein-specific tyrosine nitration.[13,14] Protein tyrosine nitration usually occurs at Tyr residues which are in close proximity to the acidic residues and also to the loop regions on protein.[15,16] Another regulating factor for nitration is the presence of tryptophan (Trp), cysteine (Cys) and methionine (Met). Trp, Cys and Met residues, which compete for nitration with Tyr, affect the nitration of Tyr residues.[17,18] The location of tyrosine residues, in favorable environment for nitration within the secondary and tertiary protein structure, may have a critical influence on the site-specific nitration.

In the current study, we have analyzed *in vitro* nitrated-bovine carbonic anhydrase II (CAII) with the high resolution FT-ICR mass spectrometry (MS) and the LC-ion trap (IT) tandem mass spectrometry (MS/MS) to investigate the sites of nitrosative modifications on CAII. Our FT-ICR study showed the peaks corresponding to mono- and bis-nitrated CAII as the major species along with the minor peaks corresponding to nitrosylated, tri-nitrated or combinations of nitrosylated and nitrated CAII. Our observations indicate that TNM mediated nitrosative modifications generated variety of combinations of nitration and nitrosylation. LC-IT-MS/MS identified that Tyr114 and Tyr70 are the predominant sites of nitrosative modifications while Tyr51 and Tyr88 are slightly modified among the entire eight tyrosine residues of CAII. The modified sites commonly had neighboring acidic and basic residues and the absence of tryptophan in proximity. Besides, the surface localization was not a critical factor. These results suggest that the nitrosative modifications prefer

†This paper is to commemorate Professor Myung Soo Kim's honourable retirement.

tyrosine residues within certain environment.

Experimental

Sample Preparation. For *in vitro* nitration, bovine carbonic anhydrase II (CAII, Sigma-Aldrich, St. Louis, USA) was incubated with 10 mM tetranitromethane (TNM, Sigma-Aldrich) for 1 h at 37 °C in 5 mM Tris-HCl (pH 8.7). After SDS-PAGE was run with the CAII modified nitrosatively, the band of CAII was in-gel digested with 10 ng/μL sequencing grade trypsin (Promega, Madison, USA) in 50 mM NH_4HCO_3 (pH 8.0). The digested peptides were analyzed by mass spectrometer. For FT-ICR MS analysis, the TNM-treated CAII was purified by liquid chromatography with C8 reverse phase column.

MS Analysis. The digested peptides were separated by nano-flow liquid chromatography (nanoLC) with a micro-capillary C18 column (75 μm id, 100 mm length). The peptides were subsequently ionized by electrospray ionization (ESI) and analyzed by linear ion trap mass spectrometer (LTQ, Thermo, Waltham, MA, USA). All spectra were acquired with data-dependent mode. Each full MS scan was followed by nine MS/MS scans, corresponding from the most intense peak to ninth intense peak of full MS scan. For the observation of the whole nitrosative modificatioin on CAII, we used 12T Fourier transform MS (FT-ICR MS, Varian, Santa clara, CA, USA) with syringe perfusion. CAII was dissolved in 50% MeOH and 0.1% trifluoroacetic acid (TFA) and the concentration was 1 μg/μL.

Data Analysis. For the identification of nitrosative modification, all MS/MS spectra of ESI-linear ion-trap MS were searched on CAII database consisting of the amino acid sequence of CAII, using the SEQUEST algorithm. Dynamic modifications were admitted for oxidized methionine (+16 Da), carbamidomethylated cysteine (+57 Da), nitrosylated tyrosine (+29 Da), nitrated tyrosine (+45 Da), and di-nitrated tyrosine (+90 Da). SEQUEST criterion for searching nitrosatively modified peptides was XCorr value which must be greater than 1.2, 2.3, and 3.5 for +1, +2, and +3 charge state peptides, respectively.

Results

Determination of the Extent of Nitrosative Modifications. To characterize the pH effects on the tyrosine nitration, *in vitro* nitration of CAII with TNM was performed under pH 5.7, 7.2 or 8.7 and analyzed the extent and sites of nitration. Although the slightly increased nitration was found at pH 5.7, the extent of tyrosine nitration was not affected notably by the change of pH, (data not shown). The optimal concentration of TNM on the nitration was determined as 10 mM. At the lower or higher concentrations than 10 mM of the reagent, the extent of nitration was reduced significantly. Based on these observations, the nitration reactions were optimally carried out with 10 mM of TNM at pH 8.7 throughout the experiment.

To characterize the heterogeneous nitrosative modifications,

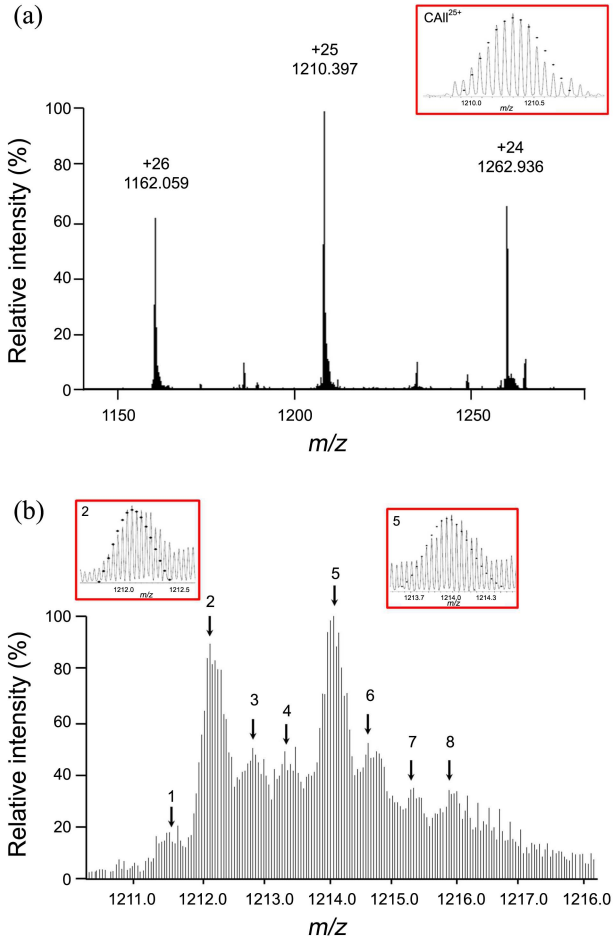

Figure 1. FT-ICR MS spectrum of CAII. A. Charge distribution of CAII. Three major peaks - having +24, +25 and +26 charges, respectively - were observed. The spectrum in the box display isotopically resolved CAII ion in the +25 charge state ($CAII^{25+}$) and the dots represent the theoretical isotopic distribution for CAII. B. Nitrosative distribution of CAII. Each peak represents one state of nitrosative modification. This spectra is the expansion of m/z range corresponding to the peak having +25 charges. Each box indicate nitrosative modification spectrum.

the changes of molecular weights of CAII treated with TNM were determined with FT-ICR mass spectrometer. Figure 1 shows the FT-ICR mass spectrum obtained from TNM-treated CAII. The molecular ions corresponding to the +24, +25 and +26 charge states were observed (Fig. 1(a)). Among the charge distribution spectrum in Figure 1(a), the spectra of the +25 charge state were analyzed to characterize the TNM mediated CAII nitration. Figure 1(b) shows that several nitrosative modifications occurred on TNM-treated CAII. These molecular ion peaks corresponding to different nitrosative modifications on CAII suggested that the modifications on CAII were heterogeneous. Previously, we observed that the TNM-treatment resulted in both nitrosylation as well as nitration.[4] The addition of NO, NO_2 and $2NO_2$ resulted in the mass increase of 28.99, 44.99, and 89.97 Da, respectively. Assignment of the peaks to the corresponding nitrosative modifications on CAII was summarized in Table 1.

In case the ionization efficiency was barely affected by the

Table 1. Interpretations of FT-ICR MS spectrum for nitrosative modifications

Paek number[a]	Center of mass	Charge (positive)	Protein mw. (cal)[b]	Mass diff.[c]	Modification	Mass shift[d]	Delta M[e]	Resolution (ppm)[f]
1	1211.55	25	30263.45	28.73	NO	28.99	0.27	9
2	1212.17	25	30279.13	44.4	NO_2	44.99	0.59	19
3	1212.84	25	30295.78	61.05	2NO	57.98	3.07	101
4	1213.34	25	30308.25	73.53	NO+NO_2	73.98	0.45	15
5	1214.09	25	30327.03	92.3	2 NO_2	89.97	2.33	77
6	1214.63	25	30340.55	105.83	2NO+NO_2	102.97	2.86	94
7	1215.3	25	30357.2	122.48	NO+2 NO_2	118.96	3.51	116
8	1215.88	25	30371.8	137.08	3 NO_2	134.96	2.12	70

[a]'Peak number' indicates that of peaks of Figure 1(b). [b]'Protein mw. (cal)' is the calculated mass of CAII based on the peaks of FT-ICR MS. [c]'Mass diff.' is the difference between the protein mw. (cal) and reference mass of CAII. The reference mass was derived from the *m/z* of 1210.397 of Figure 1(a) with the charge of +25. The value of the reference mass is 30234.73. [d]'Mass shift' is the additive monoisotopic mass of specific nitrosative modification group. When this modification group is added, loss of single proton occurs. [e]'Delta M' is the difference is the absolute value of the difference between 'mass diff.' and 'mass shift'. [f]'Resolution' is the ratio of 'delta M' to 'protein mw'. The values are represented as ppm.

nitrosative modifications, the relative intensities corresponding to each of the nitrosative modification were correlated to the relative amounts of the proteins modified by the each modification. The nitrated (peak #2) and the bis-nitrated (peak #5) CAII were the major nitrosatively modified species measured from the TNM-treated CAII. In addition, nitrosylation (peak #1), tris-nitration (peak #8) and combined modifications of nitrosylation and nitration (peaks #4, 6 and 7) of tyrosine residues were also observed as minor species.

Characterization and Identification of Nitration on Carbonic Anhydrase II. To identify the nitration sites on CAII after *in vitro* nitration reaction, the nitrated protein was digested with trypsin and the resulted tryptic peptides were analyzed by LC-IT-MS/MS. Database search of the acquired MS/MS spectra identified various nitrosatively modified peptides of CAII. Among the 8 tyrosine residues, a total of 4 tyrosine residues were modified by TNM. Table 2 shows that the Tyr51, Tyr70, Tyr88 and Tyr114 were the sites of modification, which were validated by their XCorr values and also through the manual verification. The representative MS/MS spectra of nitrated, bis-nitrated and nitrosylated peptides of CAII were shown in Figure 2. Other MS/MS spectra corresponding to the nitrosatively modifications were listed in Supplementary Figure 1. The extent of tyrosine nitration was presumed to be proportional to the ratios of the number of MS/MS spectra which correspond to the modified peptides and those of MS/MS spectra assigned to the non-modified peptides with the same amino acid sequence of nitrated peptides. Table 2 shows the pattern of nitrosative modification of tyrosine residues in CAII. Among 8 Tyr residues of CAII, 4 tyrosine residues (51, 70, 88, and 114) were involved in the various nitrosative modifications as identified. Since the nitrosative modifications were heterogeneous as identified by MS/MS analysis and high resolution mass analysis, it includes nitrosylation, nitration and bis-nitration. Among the modified Tyr residues, Tyr114 and Tyr70 were the major subjects of heterogeneous modifications listed in Table 2. All identified residues except Tyr88 were the targets for the heterogeneous nitrosative modifications. Bis-nitration of Tyr51, Tyr70 and Tyr114 were identified as well along with the nitrosylation and nitration.

The major nitrosative modification was nitration which represented *ca.* 74% of the nitrosative modifications. Bis-nitraton and nitrosylation (*ca.* 13% of each modification) were also identified. The bis-nitrated Tyr residues contained two nitro groups on the tyrosine residue resulting 3,5-dinitrotyrosine[19]

Regulating Factors of Nitrosative Modifications *in vitro*. Generally, the residues localized in the protein surface are expected to be involved in the nitrosative modifications due to their higher chance to interact with the nitrating agents at a diffusion-controlled rate. It may explain the reason why the most deeply buried Tyr residues, Tyr190 and Tyr193, were not nitrated in this experimental condition (Data not shown). The side chain accessibility is an indicator of the surface localization.[20,21] Interestingly, although the side chain

Table 2. The pattern of nitrosative modifications of tyrosine (Tyr) residues in CAII. CAII has totally 8 of Tyr reisdues. Among these Tyr residues, nitrosative modifications at Tyr7, Tyr190 and Tyr193 were not observed

Position of tyrosine	Amino acid sequence	Number of peptides			
		unmodified	NO-	NO_2-	$2NO_2$-
51Y	PLALVYGEATSR	40	1	1	1
70Y	MVNNGHSFNVEYDDSQDK	25	2	7	1
88Y	DGPLTGTYR	8	-	1	-
114Y	YAAELHLVHWNTK	2	5	37	6
127Y	YGDFGTAAQQPDGLAVVGVFLK	9	-	-	-

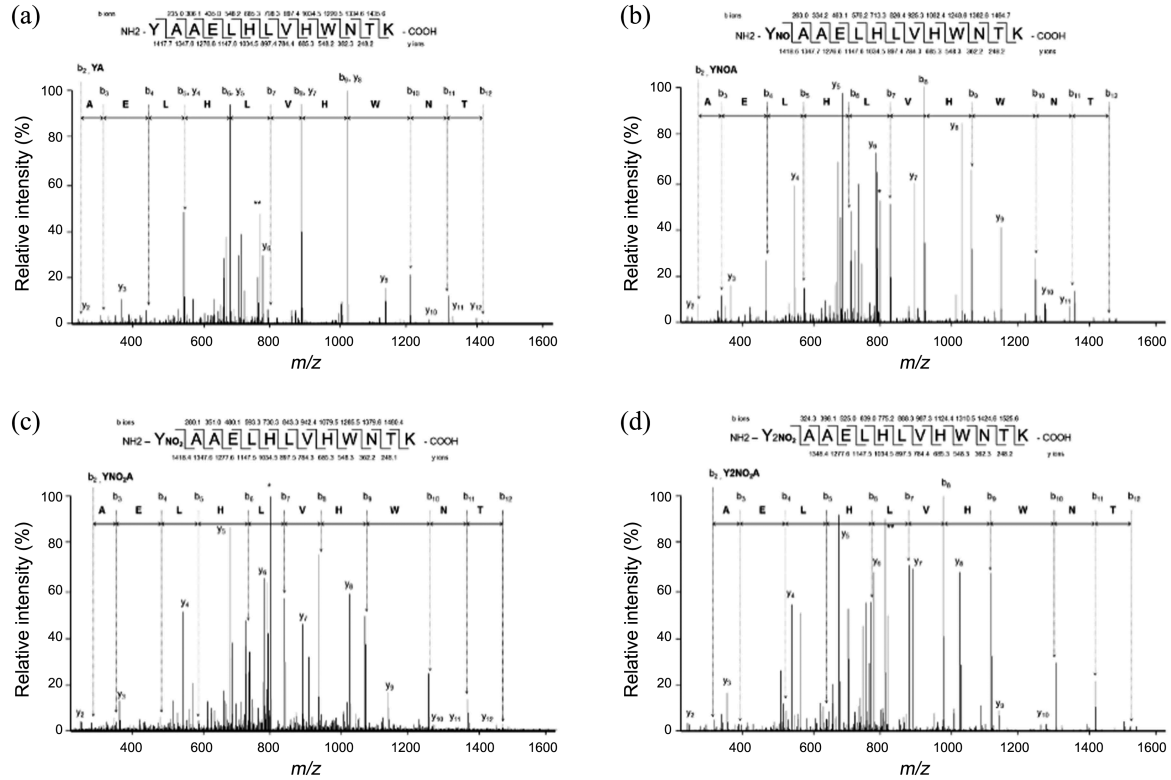

Figure 2. MS/MS spectrum of the tryptic peptide having Tyr114. These spectra show the various nitrosative modifications of Tyr114. The amino acid sequence of this peptide is YAAELHLVHWNTK. MS/MS spectra were obtained from linear ion-trap (LTQ, Thermo). A. Unmodification B. Nitrosylation (-NO) C. Nitration (-NO_2) D. Bis-nitration (-$2NO_2$). In the figures,* means the peak of $[M + 2H^+ - H_2O]^{2+}$, and ** is that of $[M + 2H^+ - 2H_2O]^{2+}$.

accessibility of Tyr127 is the highest value of 32.1, the nitrosative modifications on Tyr127 were not observed in our experiment (Table 2). Besides, Trp is another site of nitrosative modification. Therefore, the presence of Trp (122W), localized within the 10 Å distance of the Tyr127, is a critical regulating factor for the nitration on Tyr127 (Table 3).

Tyr114 residue was subjected to a variety of nitrosative modifications including nitrosylation, nitration and bis-nitration. This residue is expected to be located to the surface of the folded protein, which has the second highest side chain accessibility. Interestingly, the extent and the diversity of nitrosative modifications on Tyr114 were most substantial although this residue was not the most exposed residue. More than 90% of identified MS/MS spectra corresponding to the peptide were nitrosatively modified. However, only the higher side chain accessibility and the absence of competing residues such as Trp are not enough to be modified by TNM. Among the modified residues, Tyr51, Tyr70 and Tyr88 were partially buried as determined by the side chain accessibility ranging from 1.0 to 6.5, whereas that of Tyr114 was 22.8 (Table 3). These results suggest that there would be several other factors regulating the nitrosative modifications on Tyr residues in addition to the localization of the Tyr residues.

Table 3. Physico-chemical environments of tyrosine residues in CAII. 'Side chain accessibility' is a parameter for the possibility of the existence on the surface of protein. This value was obtained from Accelrys DS Visualizer (Accelrys Software Inc., San Diego, USA)

Position	Side chain accessibility	Within 10 distance from Y		
		Acidic residue	Basic residue	Tryptophan
7Y	3.49		244R	4W, 96W, 243W
51Y	6.51	52E, 178D		
70Y	1.01	68E, 70D, 71D, 178D	56R, 75K	122W
88Y	6.13	71D, 74D, 137D	79K, 88R	122W
114Y	22.81	100D, 101D, 116E, 212E	111K, 112K, 147K	
127Y	32.1	128D, 137D	126K	122W
190Y	0	40D, 188D	55R	190W
193Y	0	105E	252R, 255R	207W

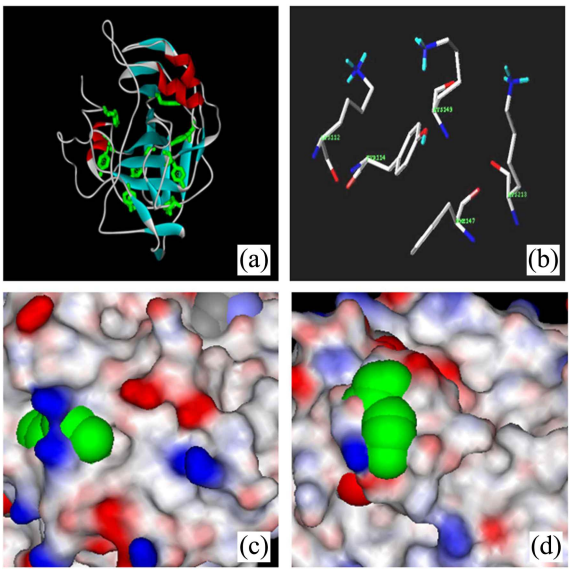

Figure 3. Structural analysis of CAII. A. Tertiary structure of CAII. Green sticks mean 8 tyrosine of CAII. B. The distribution of basic residues around Tyr114. Ring structure in the center is the phenyl ring of tyrosine. Three basic (e-amine of lysine) groups are observed around the phenyl ring. C. Surface exposure of Tyr114 and the distribution of electrostatic potential around Tyr114. (blue : positive, red : negative) D. Surface exposure of Tyr127 and the distribution of electrostatic potential around Tyr127. These images were shown by Accelrys DS Visualizer (Accelrys Software Inc., San Diego, USA).

The efficient TNM-treated modifications on several tyrosine residues with the low side chain accessibility may be explained by the electrostatic microenvironment of the target residues. In particular, electrostatic potential influences the local concentration of the nitrating agent, $ONOO^-$ to facilitate or to prevent the nitrosative modifications of specific tyrosine residues.

Among the nitrosatively modified residues supposed to be buried inside the CAII, Tyr70 with an accessibility value of 1.00 has 4 acidic residues and 2 basic residues within 10 Å distance determined by structural analysis program, Accelrys DS Visualizer (Table 3 and Figure 3). Tyr70 was subjected to nitrosylation and nitration as shown in Table 2. These results suggest that the acidic environment is favorable for the nitrosative modifications of the target Tyr.

Molecular modeling demonstrates that the Tyr114 is surrounded by three Lys residues within 10 Å distance (Figure 3(c)). The sensitivity of Ty114 to TNM-mediated nitration is characterized by the solvent accessible location on the protein surface and the proximity to the basic residues as well as the absence of Trp. In contrast, Tyr127 is located on the surface of the protein possesses an accessibility value of 32.1 and has 2 acidic residues and 1 basic residue within 10 Å distance but is not modified by TNM. Since Tyr127 is entirely exposed to the solvent, therefore, its orientation is not favorable to interact electrostatically with neighboring acidic and basic residues in an effective way.

Taken together, these results indicate that the favorable environment of the targeted tyrosine residues within the folded proteins may play a critical role in determining the final outcome of the reaction.

Discussion

Although tyrosine nitration is known to be regulated by diffusion-controlled process, this study suggests that TNM-mediated nitration depends on the presence of acidic or basic residues surrounding the target Tyr residues and the presence of Trp which competes with Tyr for the nitration in addition to surface exposure. As CAII contains total of 8 Tyr residues of which 2 Tyr residues are exposed to the surface of CAII, 4 Tyr residues are partially buried and 2 Tyr residues are fully buried, this protein is a suitable model to confirm whether microenvironments of Tyr affect the extent of protein tyrosine nitration. Among them, only 4 Tyr residues are nitrosatively modified by TNM.

One of two fully exposed Tyr residues, Tyr114 in the microenvironment with 7 acidic or basic residues within 10 Å distance and no Trp vicinity was heavily modified. In contrast, another fully exposed Tyr127 was not modified by TNM treatment. Other nitrosatively modified residues (Tyr51 and Tyr70) also have acidic and basic residue but they do not have Trp close to their vicinity. Although they are not surface exposed residues yet they are subjected to heterogeneous nitrosative modifications like Tyr114. Interestingly, Tyr88, which has a Trp residue (Trp122) in proximity, are slightly modified. However, its extent of nitration is very low, only one MS/MS spectrum was assigned to the nitrated tryptic peptide, in contrast, eight MS/MS spectra were assigned as unmodified tryptic peptides. Among the four unmodified residues, two Tyr residues are fully buried (Tyr190 and Tyr193), one is partially buried (Tyr7) and the other is fully exposed (Tyr127). All unmodified Tyr residues encompass one to three Trp residue(s) close to a 10 Å proximity. The monoisotopic mass of the tryptic peptide (STDFPNFDPGSLLPNVLDYWTYPGSLTTPPLLESVT WIVLK), which comprises Tyr190 and Tyr193 residues with a total of 41 amino acids, is 4592.34. MS/MS analysis of such a mass is not suitable to be analyzed by an ion trap mass spectrometer. Therefore, It was not possible to determine whether the sites in this peptide are nitrated or not. Therefore, these data indicate that surface exposure of Tyr residues is not the only factor for Tyr nitration.

Tyrosine residues have a relatively high tendency for locating within a carboxylic acid-rich environment,[16] and neighboring negative charge on carboxyl group of acidic residues participates to form hydrogen bonds with the adjacent hydroxyl group of tyrosine with 2-4 Å distance. These electrostatic interactions may have effects on the local concentration of the nitrating agents proximal to tyrosine residues for directing site-specific nitration.

Here we have observed the FT-ICR MS spectrum of the nitrosatively modified CAII protein and the MS/MS spectrum of its corresponding tryptic peptides. Consequently, we investigated the relationship between the nitrosative modification and the environment occurring from the location of

the modified site and its neighboring specific amino acids such as acidic/basic residue and tryptophan. Our study shows the possibility of using the combined FT-ICR MS and tandem MS for the qualitative and/or quantitative research on protein modifications.

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