The pH Dependence of Product Ion Spectra Obtained from Precursor Ions with the Same Charge Number in ESI of Carbonic Anhydrase 2

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The effect of solvent conditions, including pH, on product ion spectra obtained from precursor ions produced by electrospray ionization (ESI) was examined. Bovine carbonic anhydrase 2 was used as a model protein and the product ions generated by collision induced dissociation of the whole protein were measured under several different solvent conditions (pH 5.0, 3.7, and 0.1% HCOOH (pH 2.6)/MeCN (1/1)). The product ion spectra from precursor ions with the same charge number, the observed m/z values and the relative intensities of the product ions were similar. It therefore appears that the solvent conditions used have no effect on the product ion that is generated. On the other hand, different profiles of the product ion were obtained from precursor ions having different charge numbers. This indicates that the charge number of the precursor ion appears to be a major determinant of the product ion species and its relative intensity in product ion spectra of proteins.

Keywords: CID, ESI, carbonic anhydrase 2

INTRODUCTION

Electrospray ionization (ESI), a well recognized method for soft ionization in mass spectrometry (MS), is used in studies of many kinds of bio-polymers such as proteins, nucleic acids and oligosaccharides. ESI produces ions with consecutive charge numbers and the ions show a distribution in intensity for each multiply charged ion in a mass spectrum. This charge state distribution is known to be dependent on the nature of the sample solution, and is affected by conditions, such as pH, temperature, and the type of solvent used. 1–5

The charge state distribution in the ESI MS of a protein is considered to reflect the folding states in the gas-phase, the solvent system used is known to be an influencing factor. The charge state distribution resulting from a denaturing acidic solvent results in the production of ion peaks corresponding to a higher charge state (lower m/z) and vice versa. 1–3,6,7 However, characterizing the folding state of a proteins in solution and the gas-phase remains a controversial issue, and several studies related to this have appeared in the literature. 1–3,6,7 Specifically, Robinson reported a correlation between the degree of folding and charge state. 10 In the case of ESI mass spectra of proteins measured under different solvent conditions, ions with same charge number z are frequently found in all of the spectra. From the folding analysis point of view, the issue of whether these ions have same folding structure or not is an interesting topic. If these ions share the same conformation, the charge number z can be used as an indicator of the folding state of the protein.

Collision-induced dissociation (CID) is useful for examining the structural characteristics of ions produced by ESI. Utilizing the CID method, target protein ions can be specifically selected and the product ion spectrum can provide not only information concerning the primary structure, but also the folding states of a protein. We recently reported on product ion spectra of a whole protein and the influence of the charge number z of the precursor ions generated at a fixed solvent condition of pH 3.7 on the CID products using bovine carbonic anhydrase 2 (CA2) as the protein. 7 The ESI mass spectrum of the protein showed a bimodal charge state distribution, and the resulting CID patterns from the precursor ions with different charge numbers reflected the gas-phase folding states of CA2 ions.

On the other hand, if the patterns of the product ion spectra obtained from precursor ions with the same charge number z, but with different solvent conditions, are different from each other in the observed m/z values and the intensity profiles of the product ions, a comparison of the product ion spectra might be a new approach for evaluate the folding state of proteins. To the contrary, if the pattern of the product ion spectra is independent from solvent conditions such as pH, it presumably provides intrinsic information concerning the folding state of an ion at that charge number. This suggests that the charge number is a major determinant of the folding state of the each multiply charged ion of a protein.

In the present study, we measured product ion spectra of the complete CA2 protein, and selected precursor ions that were produced under different pH and solvent conditions and had the same charge number z.
EXPERIMENTAL

Materials
CA2 from bovine erythrocytes was purchased from Sigma (St. Louis, MO). All other reagents, including acetic acid and acetonitrile, were of the highest grade available.

Sample preparation
An approximately 100 pmol/µL solution of CA2 was prepared by dissolving the protein in water. To dilute the CA2 solution for MS measurements, the following solvents were prepared: (1) 0.3% acetic acid the pH of which was 3.7. (2) 0.3% acetic acid, the pH of which was 5.0. The pH of these solutions was adjusted with aqueous ammonia. (3) A 1:1 (v/v) mixture of 0.1% formic acid (pH 2.6) and acetonitrile. A CA2 concentration of 5.0 pmol/µL was used in acquiring ESI and product ion spectra. The CA2 solutions were maintained at ambient temperature for at least 20 min before infusion into the mass spectrometer.

ESI MS
Mass spectra were obtained with a SYNAPT G2 HDMS quadrupole time-of-flight mass spectrometer equipped with an electrospray ion source and a MassLynx data processor (Waters Corp., Milford, MA). The data acquisition parameters were as follows; electrospray capillary voltage of 3.0 kV, sample cone voltage of 30 V, source temperature of 100°C, and desolvation temperature of 200°C. The collision cell was maintained at 0.024 mbar with collision gas (Ar) during the CID measurements. And the following collision energies were employed for the measurements; ramping from 90 to 95 eV, 65 to 70 eV, and 35 to 40 eV for the precursor ions of m/z 2419 (12+), m/z 1705 (17+), and m/z 1210 (24+), respectively. The flow rate for the direct infusion of the sample solutions was 5.0 µL/min.

RESULTS AND DISCUSSION

ESI mass spectra of CA2 at different pH conditions
ESI mass spectra of CA2 measured at different pH values and solvent conditions are shown in Fig. 1. Consistent with our previous studies, multiply charged ions [M+nH+Zn]n+ corresponding to holo-CA2 were predominant at pH 5.0 (Fig. 1a). A bimodal charge state distribution showing local maxima at 12+ (m/z 2149.8) and 19+ (m/z 1528.6), and local minimum at 14+ (m/z 2074.2) was observed at pH 3.7 (Fig. 1b). Multiply charged ions corresponding to apo-CA2 ([M+nH]+) were predominantly observed in both lower and higher charge state ensembles, while the ions corresponding to holo-CA2 were still observed but were small. The observed bimodal charge state distribution at pH 3.7 consisting of ions with a high charged, such as 19+ and ions with a low charge, such as 11+, suggests that CA2 can exist at least two molecular ensembles with different folding states. The highly charged CA2 ions observed at pH 3.7 are considered to correspond to disordered states where the protein may not be completely denatured. On the other hand, the folding state of the lower charged protein appears to exist in relatively compact folding states, which is consistent with previous studies. In addition, multiply charged apo-CA2 ions with a higher charge state distribution with a maximum at 29+ (m/z 1001.8) were observed when a mixture of 0.1% formic acid (pH 2.6) and acetonitrile was employed as a solvent (Fig. 1c). This suggests that, when the formic acid and acetonitrile mixture is used as a solvent, the protein exists in a nearly unfolded state. Moreover, ions corresponding to holo-CA2 were no longer observed under this condition.

Product ion spectra of a fixed charge number at different pH conditions
The CID experiments were performed on the multiply charged CA2 ions produced under different solvent conditions described above. The product ion spectra obtained from the precursor ions of 12+, 17+, and 24+ are shown in Figs. 2, 3, and 4, respectively. Among these, the 12+ ions are assigned to the lower charged ion ensemble and the 17+ and 24+ ions are members of a higher charged ion ensemble. In the product ion spectrum obtained from the precursor ion of m/z 2425 (12+, holo-CA2), generated at pH 5.0, y67 (4+, 5+, 6+) and b192 (7+, 8+) ions were observed (Fig. 2a). To date, tandem mass spectrometry (MS/MS) of proteins, has been reported using several dissociation modes. In the case of CA2, MS/MS analyses have been performed by Fourier transform ion cyclotron resonance mass spectrometry. The product ions obtained in this study are consistent with these reports, and they were assigned accordingly. The product ions of y67 and b192 were C-terminal and N-terminal side ions that originated from the cleavage at the Tyr192–Pro193 peptide bond, respectively. This is in agreement with the previous observation that the peptide bond of Xxx–Pro is readily cleaved in CID measurements. Further, y67 and b192 represents a complementary ion pair covering the entire amino acid sequence of CA2 and the production of this type of ion pair has also been reported for other proteins. The sum of the charge number of the pairs (y67 (4+) and b192 (8+)) and (y67 (5+) and b192 (7+)) was 12, which is equal to the charge number of the precursor ion at m/z 2425 (12+). The resulting b192 ions can be assigned as holo-ions based on the m/z values. All three His residues that are bound to Zn, i.e. His93, His95, and His118, are included in the N-terminal side product ion b192. In a previous report, MS/MS
measurements of CA2 were performed and the holo-b192 ion was detected at nearly neutral conditions (pH 6.8). In this study, the holo-b192 ion was detected at an even more acidic condition (pH 5.0). Similar product ions were detected at pH 2.6, when a 1:1 (v/v) mixture of 0.1% formic acid and acetonitrile and at pH 3.7 were used as well (Figs. 2b and 2c). The profile of the relative intensities of the product ions of y67 and b192 was also similar among these spectra and the b192 ion was assigned as an apo-ion. Despite the fact that the precursor ions of Figs. 2b and 2c are derived from apo-CA2, it is interesting to note that the intensity profiles of the product ion spectra are almost the same as that from holo-CA2 (Fig. 2a). This indicates that the binding of Zn has little effect on the generation of b192, which originates from the dissociation between Tyr192 and Pro193. This is also the case for the product ion spectra. From another point of view, the relative intensities of the multiply charged y67 and b192 (12+), y67 (4+), 6+, 7+), y68 (5+), and b192 (12+, 13+) were generated from the precursor ion at m/z 1708 (17+, apo-CA2) using a solvent at pH 3.7 (Fig. 3a). A similar product ion spectrum, with respect to the m/z values and the intensity profile of the product ions, was obtained when the mixed solution of formic acid and acetonitrile (1/1 (v/v)) (c). The aqueous CA2 solution was diluted with 0.3% acetic acid and adjusted to pH 3.7 with aqueous ammonia. The CA2 concentration was 5.0 pmol/µL. Ions marked with an asterisk are the precursor ion selected for the measurements.

![Fig. 2. Product ion spectra obtained from the 12+ precursor ion of CA2.](image)

![Fig. 3. Product Ion spectra obtained from the 17+ precursor ion of CA2 at m/z 1708.](image)

![Fig. 4. Product ion spectra obtained from the 24+ precursor ion of CA2 at m/z 1210.](image)

The findings reported herein indicate that the charge state distributions of ESI mass spectra are shifted, depending on the nature and pH of the solvent. However, the m/z values and the relative intensity of the product ions obtained from precursor ions with the same charge number z are similar and the type of solvent used has negligible influence on the product ion spectra. From another point of view, the folding states and protonation site of the precursor ions are presumed to be similar among ions with the same charge number. The relative intensities of the multiply charged y67 and b192 ions are shown in Fig. 5. In the case of y67 ions from the 12+ precursor ion at m/z 2425, y67 (4+, 6+, 7+), and y67 (6+) were observed, and their intensities were y67 (5+) > y67 (4+) > y67 (6+). For b192, b192 (7+) and b192 (8+) were observed and the intensity of b192 (7+) was higher than that for b192 (8+). The same intensity relationships were obtained in the case of all other solvent conditions employed in this study (Fig. 5a). This was true for the 17+ (m/z 1708) and 24+ (m/z 1210) precursor ions, indicating that the charge state distribution of the product ions is not influenced by the solvent used (Figs. 5b and 5c). This finding suggests that precursor ions having the same charge number are in the same folding state, even when they are generated in different solvent conditions. This indicates that the determining factor for folding state of each multiply charged CA2 ion is the charge number z. A similar observation was...
reported for ubiquitin.\(^2\)\(^3\) In contrast, our finding is not applicable to proteins whose conformation is stabilized by strong intramolecular interactions.\(^2\)\(^3\) However, the findings in these prior reports as well as our present study indicated that the product ion spectrum provides an adequate description of the folding state of a protein.

**CONCLUSION**

The effect of the solvent conditions, including pH and solvent composition on the product ion spectra of a whole protein was examined using CA2 as a model protein. A comparison of the product ion spectra indicated that the pH and solvent composition of the sample solution has no or a negligible effect on the product ion species and the relative intensities obtained from the precursor ions having the same charge number. On the other hand, the product ion spectra obtained from the precursor ions having a different charge number were different. Thus, the charge number of the precursor ion appears to be an intrinsic factor in determining the product ion species and its relative intensity in a product ion spectrum and can be a determining factor for the folding state of multiply charged protein ions in the gas phase.

**REFERENCES**