Folding analysis of the zinc protein, carbonic anhydrase 2 (CA2), was performed using electrospray ionization ion mobility spectrometry coupled with collision-induced dissociation (ESI IMS/CID). Multiply protonated ions with a bimodal charge state distribution were observed indicating the presence of at least two folding states for gas-phase CA2 ions as was described in a previous study (Nabuchi, Y.; Murao, N.; Asoh, Y.; Takayama, M. Anal. Chem. 2007, 79, 8342–8349). In the IMS driftgram, several ions with different mobility were observed for each multiply charged ion, and this suggests that CA2 ions consist of several components with different folding states. IMS/CID spectra were acquired against precursor ions separated by mobility. The CID spectra gave several characteristic product ions including those from the N- and C-terminal region of CA2. A shift to larger charge number for the most abundant of the several product ions was observed for ions having a larger drift time. This charge number shift indicates that the folding state of the ion is more unfolded. Furthermore, differences in the production of an ion corresponding to the N-terminal side fragment gave information about the unfolding process of CA2.

The study of folding and conformation analysis of proteins and peptides has been performed using soft ionization mass spectrometry. For this purpose, electrospray ionization (ESI) is the most common ionization method employed in many studies.\(^1\) ESI can directly transfer ions from solution to the gas phase. Though the sample solution passes through the electrospray needle which has a high electric potential, analytes are kept in solution until the final ionization step. In the IMS driftgram, several ions with the same charge number. In the absence of IMS, different folding states are found in ion ensembles containing both high and low charge states. However, with the use of IMS, it is possible to detect small differences in folding of protein ions with the same m/z value by measuring the drift time \(t_d\) and then using this parameter as an index of shape of the protein. IMS can potentially allow observation of differences in folding of a multiply charged ion produced via the ESI process. Furthermore, this method is considered to have the potential to allow elucidation of the folding states of proteins which have not been revealed by conventional mass spectrometry.\(^8\) In addition, the shift in charge state distribution of multiply protonated proteins, \([M+nH]^+\), allows monitoring of different conformations of the analytes. For example, a conformation corresponding to an unfolded protein will lead to the formation of higher charge state distributions, whereas a folded protein leads to lower charge states. Furthermore, mild denaturing conditions often result in multimodal charge state distributions indicating the coexistence of different conformations.\(^2\)–\(^7\)

Recently, ion mobility spectrometry (IMS) has been put to practical use and applied to the analysis of protein folding.\(^14\)–\(^16\) The IMS method can distinguish the molecular size and/or shapes of ions such as those of folded and unfolded states even in the cases of ions with the same \(m/z\) values. With the use of IMS, \(m/z\)-selected analyte ions can be separated on the basis of differences in their mobility. In practical terms, this is measured as a drift time \(t_d\) and can be considered as a parameter related to the size and/or shape of an ion.\(^16\)–\(^17\)

With the use of IMS, it is possible to ascertain whether several ions with different shape in terms of mobility have a mass with the same charge number. In the absence of IMS, different folding states are found in ion ensembles containing both high and low charge states. However, with the use of IMS, it is feasible to detect small differences in folding of protein ions with the same \(m/z\) value by measuring the drift time \(t_d\) and then using this parameter as an index of shape of the protein. IMS can potentially allow observation of differences in folding of a multiply charged ion produced via the ESI process. Furthermore, this method is considered to have the potential to allow elucidation of the folding states of proteins which have not been revealed by conventional mass spectrometry.

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**Ion Mobility and Collision-Induced Dissociation Analysis of Carbonic Anhydrase 2**

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8890 Analytical Chemistry, Vol. 82, No. 21, November 1, 2010

**Analytical Chemistry, Vol. 82, No. 21, November 1, 2010**

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Published on Web 10/14/2010
In the present study, we applied IMS and collision-induced dissociation (CID) coupled with IMS to evaluate the folding states of a protein. By comparison of charge state distribution and/or peak abundance of the product ions produced from precursor ions with different mobility, further evaluation of the folding change is potentially possible. For this purpose, bovine carbonic anhydrase 2 (CA2) was used as the analyte protein. CA2 is a zinc (Zn) metalloenzyme consisting of 259 amino acids which catalyzes the reversible hydration of CO\(_2\)\(^{18,19}\). The Zn can be released from the holo-protein with loss of activity\(^{19,20}\). It is known that the ESI mass spectrum of the protein shows a bimodal charge state distribution in certain pH conditions, and ions corresponding to both apo- and holo-protein are observed\(^{21}\). Here we measured the IMS driftgram of CA2 in several solvent conditions, and ions with the same \(m/z\) but having different drift times were observed. The folding states of the CA2 ions are discussed on the basis of the ESI IMS/CID spectra of the protein.

**EXPERIMENTAL SECTION**

**Materials.** CA2 from bovine erythrocytes was purchased from Sigma (St. Louis, MO). All other reagents used in the study such as acetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), formic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and acetonitrile (Kanto Chemical Co., Inc., Tokyo, Japan) were of the highest guaranteed grade.

**Sample Preparation.** CA2 solution of approximately 100 pmol/\(\mu\)L was prepared by dissolving the protein in \(H_2O\). To dilute the CA2 solution for MS measurements, the following solvents were prepared: 0.2% acetic acid which was \(pH\) adjusted with aqueous ammonia to \(pH\) 3.6, \(pH\) 5.0, and \(pH\) 5.1. The CA2 concentration prepared for acquiring the IMS spectra was 1.8 pmol/\(\mu\)L while 5.0 pmol/\(\mu\)L was employed for acquisition of IMS/CID spectra. CA2 solutions used for the analysis were kept for 20 min at ambient temperature before infusion into the mass spectrometer.

**ESI MS.** Mass spectra were obtained with a SYNAPT HDMS quadrupole time-of-flight mass spectrometer equipped with an electrospray ion source and MassLynx data processor (Waters Corp., Milford, MA). Instrument acquisition parameters used were as follows: an electrocapillary voltage of 2.8 kV, a sample cone voltage of 30 or 50 V, a source temperature of 100 °C, and a desolvation temperature of 200 °C. The collision cell was maintained at 0.047 mbar with collision gas (Ar) during the IMS/CID measurements. For the ion mobility measurements, nitrogen was used as the gas in the ion mobility cell and the cell pressure was maintained at 0.5 mbar for IMS measurements and 1.0 mbar for IMS/CID. The IMS wave velocity was 300 m/s, and wave pulse heights of 7.9 and 3.0 V were used for IMS and IMS/CID, respectively. The rate of direct infusion of the sample solution was 20 \(\mu\)L/min during all MS measurements. To obtain the product ion spectra from a peak separated by mobility difference in the IMS/CID measurements, the following peak processing was conducted using MassLynx: each peak A or B in the driftgrams was separated at the valley of the doublet peaks, and each product ion spectrum was corrected with adequate subtraction of the spectrum of the overlapped peak.

**RESULTS AND DISCUSSION**

**ESI IMS/Time of Flight (TOF) MS of CA2.** Ion mobility measurements were performed for each charge state of CA2 ions produced at \(pH\) 5.1 and \(pH\) 3.6. Similar to the results from a previous study\(^{21}\) multiply charged ions [\(M + nH + Zn\)]\(^{+}\) corresponding to holo-CA2 were found to be predominant at \(pH\) 5.1 (Figure 1a). In contrast, a bimodal charge state distribution was observed at \(pH\) 3.6 (Figure 1b). Multiply charged ions corresponding to apo-CA2 (\([M + nH]\))\(^{+}\) were predominantly observed in both charge state ensembles. In comparison, the folding state of the lower charged ions ensemble is to be relatively more compact than the higher charged ensemble in accordance with previous studies\(^{2,3,5}\).

In the driftgrams, definite doublet peaks were found in several charge states such as 12\(^{+}\) (Figure 1a), 13\(^{+}\), and 19\(^{+}\) (Figure 1b), while broad shaped peaks having more than two peak tops were also observed. This indicates that the multiply charged CA2 ions are composed of several ions with different mobility even at the same \(m/z\) value. The mobility reflects the size and/or shape of the sample. Thus, the mobility is considered to reflect the folding state of proteins, because folded proteins are suggested to be more compact than unfolded proteins\(^{22,23}\). This means that a protein ion with larger mobility is in a more folded state. From this, the resulting IMS driftgrams in the insets of Figure 1 indicate that the gas-phase CA2 ions with certain charge numbers consist of at least two different folding states. In contrast, some peaks did not give any separation in the ion mobility spectra. There are two possible reasons for this observation. One is due to insufficient resolution from the IMS conditions employed in the study. The other possibility is that the mobility of the ions does not converge on a certain size, and the \(m/z\)-selected ions have a broad distribution with respect to mobility.

**ESI IMS/CID/TOF MS of CA2.** In order to obtain information about the folding state of CA2 ions with different mobility, CID experiments coupled with ESI IMS were performed on the series of ions giving clear doublet peaks as described above. In this study, the peaks with short and long drift time in each doublet peak are distinguished as A and B, respectively. Typical product ion spectra obtained from the ESI IMS/CID experiments include apo-CA2 ions with charge states of \(m/z\) 1529 (19\(^{+}\)) and 2234 (13\(^{+}\)) and a holo-CA2 ion of \(m/z\) 2425 (12\(^{+}\)), and these are shown in Figures 2–4, respectively. The CID spectra of peaks A and B in the IMS driftgrams showed multiply charged product ions with characteristic charge state distributions corresponding to \(y47 (3^{+}, 4^{+}), y61 (3^{+}, 4^{+}, 5^{+}, 6^{+}), y67 (3^{+}, 4^{+}, 5^{+}, 6^{+}, 7^{+}), y68 (3^{+}, 4^{+}, 5^{+}, 6^{+}),\) and \(b192 (7^{+}, 8^{+}, 9^{+}, 10^{+}).\) These \(y\)- and \(b\)-series ions with several charge state distributions originate from cleavage at the peptide bonds of Glu212-Pro213, Thr198-Pro199, Tyr192-Pro193, Thr191-Tyr192, and Tyr192-Pro193, respectively. The

principal cleavage sites are shown in Figure 5. The product ion b192 corresponds to the N-terminal side fragment of 1–192 and the product ion y67 corresponds to the C-terminal side fragment of 193–259. Thus, the product ions of b192 and y67 are a complementary pair covering the whole amino acid sequence of CA2. In the CID spectrum obtained from the precursor ion at \( m/z \) 2425 (12+), the complementary pair product ions of b1927+ and y675+, b1928+ and y674+, y675+ and y674+ were observed (Figure 4). Thus, the sum of the charge numbers of the pairs (b1927+ and y675+) and (b1928+ and y674+) is 12. These sum totals are equal to the charge number of the precursor ion at \( m/z \) 2425 (12+). A similar complementary relationship was observed in the CID of the precursor ion at \( m/z \) 2234 (13+) that resulted in the product ions (b1927+ and y676+), (b1928+ and y675+), (b1929+ and y674+), and (b19210+ and y673+) (Figure 3). These results indicate that a precursor ion with a certain charge number is cleaved through CID into N- and C-terminal side product ions with charge numbers \( n \) and \( c \) and that the sum of the charge

Figure 1. ESI mass spectra and IMS driftgrams of CA2 ions produced at (a) pH 5.1 and (b) pH 3.6. The driftgrams shown are (a) two different charge states (11+, 12+) of holo-CA2 ions and (b) three different charge states (13+, 15+, 19+) of apo-CA2 ions.

Figure 2. ESI IMS/CID mass spectra of the precursor ion at \( m/z \) 1529 (19+) at pH 3.6. The measurements were performed against two precursor ions of apo-CA2 (A and B), which have the same mass but different mobility on a driftgram. A CA2 concentration of 5.0 pmol/µL was used for the measurements.
number of the product ions of the N-terminal side b-series ion and C-terminal side y-series ion may be conserved, i.e., $z = n + c$. However, other complementary pairs such as y61 and b198 were not observed in the measurements.

With regard to tandem mass spectrometry (MS/MS) analysis of proteins with high molecular mass, it becomes feasible via several dissociation modes.\(^{(24-29)}\) In the case of CA2, an MS/MS analysis has been performed using ESI/Fourier transform ion cyclotron resonance (FTICR) MS.\(^{(25)}\) The results obtained in this study are consistent with the product ions from the previous report. The cleaved peptide bonds for the product ions y61 and y67 were Tyr192-Pro193 and Thr198-Pro199, respectively. These are in agreement with the previous observation that the peptide bond of Xxx-Pro is readily cleaved in CID measurement.\(^{(26)}\)

Comparison of the product ion spectra obtained from precursor ions with the same m/z values but different mobility (peak A and peak B) provides insights into the structural features of CA2. The observed product ions y61 and y67 are characteristic of the cleavage at the b-series ions, indicating the presence of Pro in the sequence. These findings are consistent with the previous reports, which demonstrated the cleavage of Pro bonds in CID measurements.


B) shows no obvious difference in the cleavage sites. In the bimodal charge state distribution shown in Figure 1b, the IMS/CID spectra obtained from higher charge state precursor ions of apo-CA2 (19+\textsuperscript{+}) mainly showed y-series ions of y\textsubscript{61} and y\textsubscript{67} (Figure 2). Comparing the CID patterns of peaks A and B of the 19+ ion (Figure 2) shows that peak A gave product ions of y\textsubscript{61} (3\textsuperscript{+}, 4\textsuperscript{+}, 5\textsuperscript{+}) and y\textsubscript{67} (3\textsuperscript{+}, 4\textsuperscript{+}, 5\textsuperscript{+}), whereas peak B consisted of y\textsubscript{61} (3\textsuperscript{+},4\textsuperscript{+}, 5\textsuperscript{+}, 6\textsuperscript{+}) and y\textsubscript{67} (4\textsuperscript{+}, 5\textsuperscript{+}, 6\textsuperscript{+}, 7\textsuperscript{+}). The peak abundance of the higher charge state product ions such as y\textsubscript{61} (5\textsuperscript{+}) and y\textsubscript{67} (5\textsuperscript{+}) in peak B was higher than that of the same product ions in peak A. Furthermore, the lowest charge state product ion y\textsubscript{67} (3\textsuperscript{+}) observed in the CID spectra of peak A in Figure 2 was absent from that of peak B. Similarly, the ion intensity of y\textsubscript{61} (3\textsuperscript{+}) in peak B was weaker than that in peak A. In the comparison of the IMS/CID spectra of peak A and B, it was observed that product ions of peak B with long drift time shifted to a higher charge state distribution than those ions in peak A with a short drift time. Such a shift in the charge state distribution with respect to the peak abundance of the product ions can also be observed in Figures 3 and 4. The results obtained here indicate that the product ions of peak B with longer drift time gave higher charge states with higher peak abundance than those in peak A with a shorter drift time. This suggests that gas-phase CA2 ions from peak B are in unfolded states compared to those from peak A.

The charge state distributions for the product ions of y\textsubscript{61} and y\textsubscript{67} are shown by plotting the relative abundance against charge number (Figure 6). In the all product ion spectra examined here, the most abundant product ion of peak A with short drift time was y\textsubscript{67}\textsuperscript{4+}, while that of peak B with long drift time was y\textsubscript{67}\textsuperscript{5+}. It is interesting that a charge number shift of the most abundant product ion occurs in the CID of peaks A and B. For the product ion of y\textsubscript{61}, a similar charge number shift was observed in the precursor ion of 19+ (Figure 6a), although the most abundant ion in both peaks A and B was 4+ for the y\textsubscript{61} ion derived from 13+ and 12+ (Figure 6b,c). This charge number shift suggests that the IMS/CID spectra can be related to the gas-phase folding states of the CA2 ion and that peaks A and B correspond to relatively folded and unfolded states, respectively.

It is of interest to compare the product ions and the charge state distributions of apo-CA2 with those of holo-CA2. The IMS/CID spectra of the lower charge state precursor ion of apo-CA2 (13+) at m/z 2234 showed product ions with several charge states such as b\textsubscript{192} (7\textsuperscript{+}, 8\textsuperscript{+}, 9\textsuperscript{+}, 10\textsuperscript{+}), y\textsubscript{61} (3\textsuperscript{+}, 4\textsuperscript{+}, 5\textsuperscript{+}, 6\textsuperscript{+}), and y\textsubscript{67} (3\textsuperscript{+}, 4\textsuperscript{+}, 5\textsuperscript{+}, 6\textsuperscript{+}).

**Figure 5.** Assignments of product ions obtained from ESI IMS/CID/TOF MS measurements of CA2. Primary and secondary structures of CA2 were obtained from the RCSB protein data bank (1V9E). Symbols used to indicate the secondary structure are a wave for a helix, an arrow for a β-sheet, and a bell-shaped curve for a turn structure. Amino acid residues marked by an asterisk are high proton affinity residues.

**Figure 6.** Charge number of the most abundant ion of product ions obtained from precursor ions having different mobilities. Relative intensities of y\textsubscript{61} and y\textsubscript{67} obtained from precursor ion peaks A and B, which have the same mass but different mobility, were plotted against the charge number. The plots a–c represent the results of the precursor ions, m/z 1529 (19+), m/z 2234 (13+), and m/z 2425 (12+), respectively.
(3+, 4+, 5+, 6+) ions, as shown in Figure 3. In contrast, the IMS/CID spectra of holo-CA2 (12+) showed a relatively narrow range of charge state distributions such as b192 (7+, 8+, y61 (3+, 4+, 5+, 6+), and y67 (3+, 4+, 5+, 6+), as shown in Figure 4. A conserved sum total of the charge number of b- and y-series ions was observed in the IMS/CID. The IMS/CID spectra of apo-CA2 (13+) showed wider charge state distribution than those of holo-CA2 (12+). The difference in the charge state distribution of y- and b-series ions between holo- and apo-CA2 may originate from the folding state of the CA2 ions. Thus, the apo-CA2 ions are in a relatively unfolded state compared with the holo-CA2 ions. However, there is no difference in the cleavage sites between apo- and holo-CA2. Since the known Zn binding sites of CA2 are His93, His95, and His118,18 these His residues are not included in the y-series ions but in the b-series ions. Thus, Zn binding is assumed to have little effect on the dissociation site in CID. In other words, the effect of Zn binding on the folding state of the C-terminal region is considered to be small.

The IMS/CID spectra of the lower charged precursor ions shows the presence of complementary pair product ions of y67 and b192, as shown in Figures 3 and 4 and as summarized in Table 1. In the product ions from the higher charged precursor ions, however, the intensity of ions assumed to b192 was too weak to assign (Figure 2). This may suggest that the folding state of the precursor ion has certain effects on the yield and/or stability of b192 ions. Since the precursor ions of 19+ are the ions from the higher charged ion ensemble from the bimodal charge state distribution of CA2 (Figure 1b), the folding state is considered to be less folded.21 In the other complementary pair of y61 and b198, however, b198 ions were not observed in any of the CID spectra, whereas the product ion of y61 was clearly observed (Table 1). It is difficult to explain the lack of the b198 ion from the viewpoint of a difference in the cleavage sites between Thr198-Pro199 and Tyr192-Pro193 (Figure 5) because both the C-terminal side product ions y67 and y61 have almost the same primary structure with the same number of basic residues, four Arg and four Lys, and having similar charge state distributions (Table 1). Basic amino acid residues such as Arg, Lys, and His are known as preferential protonation sites in ESI processes. In particular, the Arg residue tends to bind a proton more easily than Lys and His residues because of its higher proton affinity.30 This indicates that in the lower charge state precursors (13+ and 12+) both y67 and y61 ions have similar charge state distributions including 3–6 protons on the 4 Arg and 4 Lys residues of the C-terminal side regions 193–259 or 199–259 and 7–10 protons on the Arg, Lys, and His residues of the product ions of the N-terminal side regions 1–192 or 1–198. The N-terminal side regions of 1–192 and 1–198 of CA2 have 11 His, 5 Arg, and 14 Lys residues.25 However, it should be noted that a significant distinction between Thr198-Pro199 and Tyr192-Pro193 is that Tyr192-Pro193 is in a b-sheet region containing the b-strand Tyr189-Gly194, while Thr198-Pro199 is in a turn region (Figure 5). From this, it may be expected that the cleavage of the peptide bond at Tyr192-Pro193 might lead to the distortion of the b-sheet and would spatially separate the precursor ions into the two product ions b192 and y67. In contrast, the cleavage of the peptide bond at Thr198-Pro199 would not lead to the spatial separation of the precursor ions due to the presence of the b-sheet. Although this proposition rationalizes the low abundance or low CID yields of y61 and y47 ions, the lack of observation of b ions in the CID spectra with the exception of the b192 ion cannot be explained.

The process of the change of folding of CA2 when going from pH 4.4 to 3.6 is described in our previous report.21 The proposed process derived from the observations in the IMS/CID measurements in the present study can be described as follows. At pH 5.0, CA2 ions can exist as holo-CA2 [M + Zn + nH]n+ with a charge state distribution consisting of 10+, 11+, and 12+ in the ESI mass spectrum (Figure 1a). In the subsequent IMS/CID measurement of those lower charge state precursor ions, the product ions of b192, y67, and y61 could be observed. At pH 3.6, in contrast, CA2 ions were observed as apo-CA2 [M + nH]n+ through loss of Zn from the holo-form and the resulting ions showed a bimodal distribution consisting of the lower and the higher charge state ion ensembles (Figure 1b). The lower charge state ensemble had similar charge numbers to those observed at pH 5.0. The IMS/CID measurements of the lower charge state ensemble gave similar product ions to those from holo-CA2, i.e., b192, y67, and y61. However, the charge state distribution of an N-terminal side product ion, b192, shifted to higher charge number (Table 1). This suggests that the b192 ion at pH 3.6 is in a more unfolded state than at pH 5.0. In other words, the N-terminal side region changes to an unfolded state(s) with the pH change from pH 5.0 to 3.6. The ion ensemble with higher charge state distribution is suggested to be a more unfolded state than that with a lower charge state distribution. In the IMS/CID spectra of the higher charge state ensemble, the C-terminal side product ions y67 and y61 were observed, but the N-terminal side ion b192 was not observed in the ensemble. The lack of b192 ion may be due to unfolding of b192 with the distortion of the b-sheet. Thus, the ESI IMS/CID measurement was demonstrated to have two indicators that detect change in the folding of the protein.

**CONCLUSIONS**

In the case of CA2 ions produced by ESI processes, the ions having higher charge number are considered to have more unfolded states.21 From the present study using IMS, it was revealed that the CA2 ions produced by ESI consist of several ions with different mobility even at the same m/z value. Furthermore, CA2 ions with larger mobility (shorter drift time) are suggested to be in a more folded state because the size or shape of these ions is more compact than those with smaller mobility (longer drift time). From comparison of CID spectra between shorter and longer drift time ion components, the charge number

### Table 1. Charge State Distribution of Major Product Ions of b192, y67, and y61, Which Were Present in the ESI IMS/CID Spectra As Definite Doubles (Peaks A and B) in the IMS Driftgrams of apo- and holo-CA2 Ions

<table>
<thead>
<tr>
<th>precursor ion peak</th>
<th>charge state distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>b192</td>
<td>3+, 4+, 5+, 6+, 7+, 8+, 9+, 10+</td>
</tr>
<tr>
<td>y67</td>
<td>3+, 4+, 5+, 6+, 7+, 8+, 9+, 10+</td>
</tr>
</tbody>
</table>

(3+), (4+), (5+), (6+), (7+), (8+), (9+), (10+), (11+), (12+), (13+), (14+), (15+), (16+) ions.
of the most abundant ion of several product ion ensembles shifted from lower to higher charge number. These charge number shifts tend to occur in higher charge state ions produced in ESI processes. With the comparison of the product ion formation and its charge state distribution, the unfolding process of CA2 is proposed as follows: upon change from pH 5.0 to 3.6, loss of Zn from holo-CA2 and extensive unfolding of the N-terminal side of the protein occurs. With the use of the charge state distribution of product ions as an indicator, the ESI IMS/CID measurements demonstrate the difference in the folding state among ion ensembles and illustrate the unfolding process of the protein. Thus, the ESI IMS/CID method and comparison of the resulting product ion spectra are valuable tools for probing the folding state not only of CA2 but also of other proteins.

SUPPORTING INFORMATION AVAILABLE
Tertiary and secondary structures of CA2 obtained from the RCSB protein data bank (1V9E). This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 25, 2010. Accepted September 20, 2010.
AC101482B