Probing the Unfolding and Refolding Processes of Carbonic Anhydrase 2 Using Electrospray Ionization Mass Spectrometry Combined with pH Jump

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A novel method for proving the time course of the unfolding and refolding processes of metalloprotein bovine carbonic anhydrase 2 (CA2) is demonstrated using electrospray ionization mass spectrometry (ESI MS) combined with pH jumps between 3.6 and 4.4. The shift in mass accompanied by the release or coordination of a zinc ion and the change in the charge state distribution were measured to evaluate the folding process. The time course of the ESI mass spectra revealed the existence of four types of ions in the experimental system, i.e., lower charged apo-CA2 and holo-CA2 ions and higher charged apo-CA2 and holo-CA2 ions. The deconvolution spectrum of the ion peak ensemble for each type of ion was processed and time course plots of the relative intensities of the four ions were prepared in order to analyze the folding processes. These analyses revealed the coexistence of two folding states of the lower and higher charged apo-CA2 under the condition of pH 3.6. The lower and higher charged apoproteins spontaneously refolded to the lower charged holoprotein by a pH jump from 3.6 to 4.4 without the addition of an extra zinc ion. The higher charged holoprotein observed during both the unfolding and refolding processes was considered to be an intermediate of the change in folding. The present study indicates that ESI MS combined with pH jump would be a powerful method to probe the unfolding and refolding of proteins. This method simultaneously measures mass spectra and analyzes the folding processes as a function of time using deconvolution spectra constructed by selecting a suitable *m/z* range for the analysis from the peaks of charge state distributions.

For functional proteins, the formation of a well-defined specific conformation is considered to be necessary for biological functions. However, there are proteins that are unstructured or partially structured under physiological conditions and disordered proteins that fold upon binding to their biological targets such as receptors and deoxyribonucleic acids. Proteins in partially folded states are structurally heterogeneous and undergo conformational changes in a time-dependent manner. The conformational disorder allows the proteins to bind nonspecifically, perhaps in different conformations and to many different partners.[1–4] Thus, studies of partially folded protein structures and the folding process will lead to an understanding of the folding state and clarification of the biological function.

There are several methods for investigating the conformation and folding behavior of proteins such as circular dichroism (CD), nuclear magnetic resonance (NMR), X-ray crystallography, and mass spectrometry (MS). Many achievements involving proteins have been reported from utilizing electrospray ionization mass spectrometry (ESI MS). ESI MS monitors different conformations of a protein given by different charge states detected as multiply protonated proteins, $[M + nH]^{n+}$. For example, a conformation corresponding to an unfolded protein will lead to the formation of higher charge state distributions; mild denaturing conditions often exhibit multimodal charge state distributions indicating the coexistence of different conformations in the solution.[5–10] Thus, ESI MS has been considered a suitable method for the detection of the conformation and folding processes of proteins. One of the established methods is to measure the amide hydrogen/deuterium (H/D) exchange rate of the peptide backbone.[11–15]

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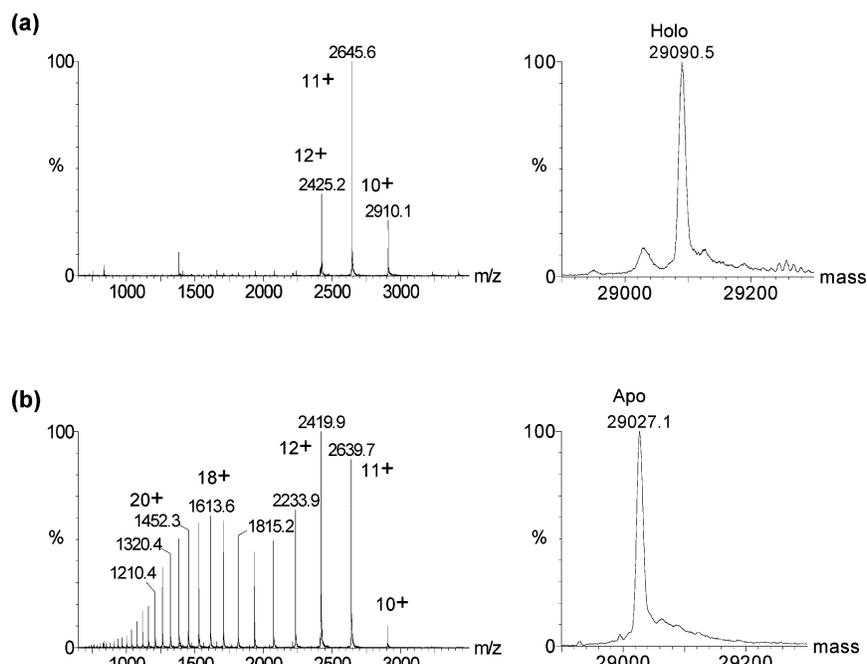† Chugai Pharmaceutical Co., Ltd.

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Figure 1. ESI mass spectra of CA2 measured at (a) pH 4.4 and (b) pH 3.6. The measurements were performed under 0.2% acetic acid containing aqueous ammonia for pH adjustment. The deconvolution spectra are shown in the right side.

However, because the H/D exchange rate is sensitive to pH, the H/D method is not suitable for probing the folding processes caused by pH change.[16] The other method for a protein folding study is time-resolved ESI MS, which requires a multiple on-line mixing system connected to a mass spectrometer. In such studies, ESI mass spectra of the reaction mixture are recorded at different times after initiation of the reaction. Konermann et al. have elucidated the folding and unfolding processes of proteins using this method.[17−20]

In the present study, we continuously monitored the change in folding processes resulting from pH jump using ESI MS. A metalloprotein is considered suitable for this method because a conformation change in the protein causes the release of metal ions from the protein or coordination of the metal ion with the protein. Folding reactions are detecting by the shift in mass from the behavior of the metal ions as well as the charge state distribution observed with ESI MS. Here we selected a carbonic anhydrase (CA), bovine carbonic anhydrase 2 (CA2), as the model metalloprotein. CA2 is a zinc metalloenzyme, and the zinc can be released from the protein as apo-CA2.[21,22] Moreover, the protein has been reported to have partially folded structures under certain conditions[23,24] and catalyzes the reversible hydration of CO_2. The
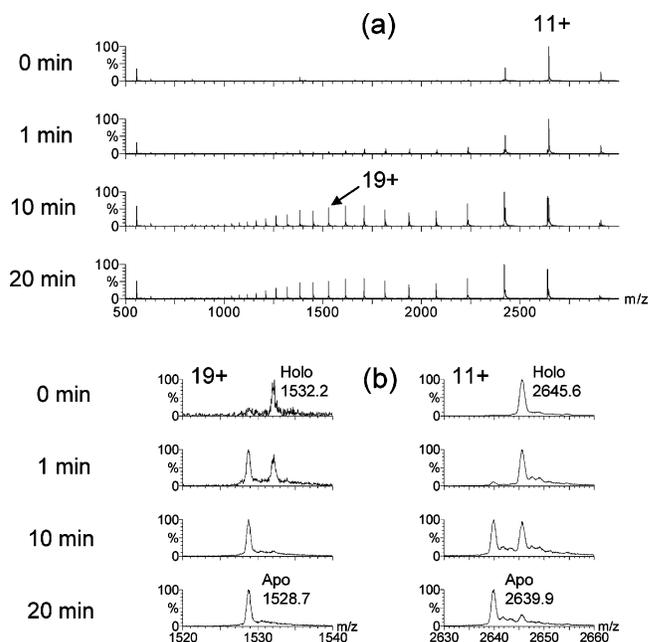

Figure 2. Time-dependent changes of mass spectra resulting from a pH jump from 4.4 to 3.6. Unfolding was initiated by the addition of acetic acid to the CA2 solution at pH 4.4. (a) The mass spectra showing whole charge state distribution. (b) The ions of the charge states 19+ and 11+ were extracted from the corresponding mass spectrum in part a.

CA isozymes have been characterized according to subcellular distribution: cytoplasmic (CA1, CA2, CA3, and CA7), plasma membrane glycosylphosphatidylinositol-anchored to plasma mem-

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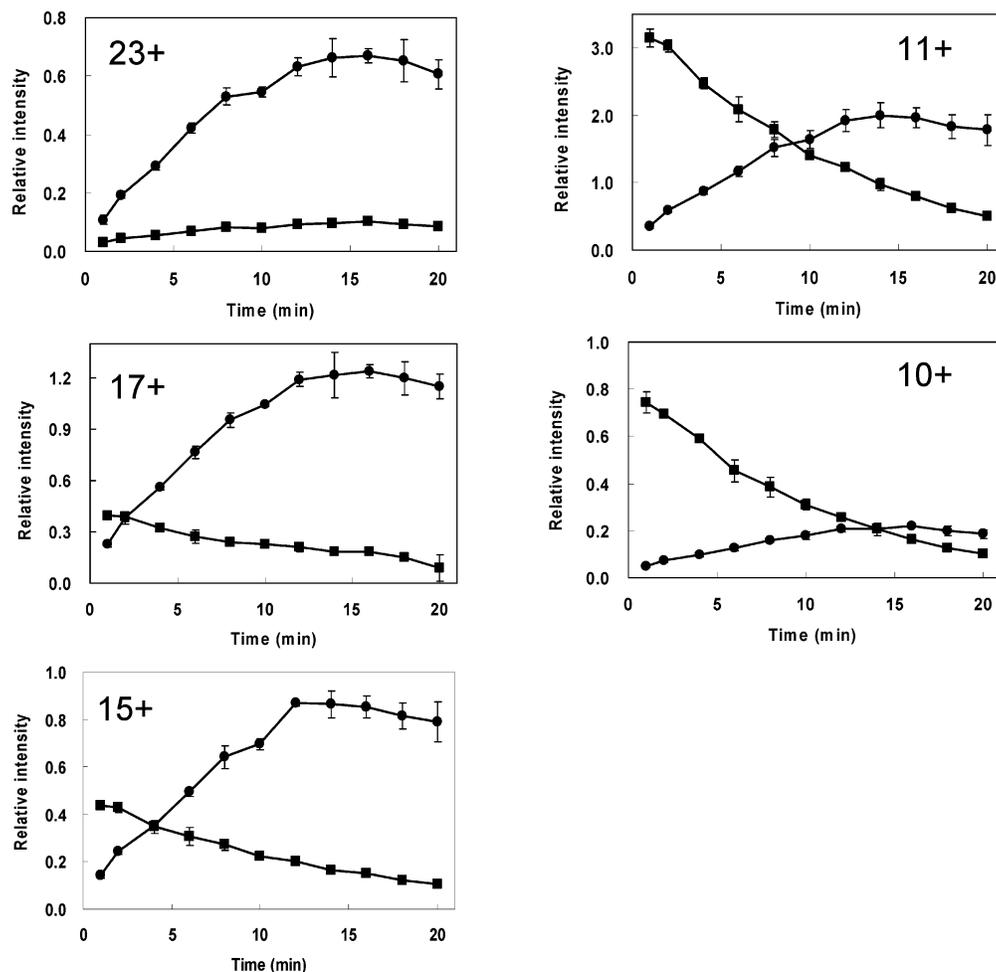(24) Andersson, D.; Hammarstrom, P.; Carlsson, U. *Biochemistry* **2001**, *40*, 2653−2661.

Figure 3. Change in relative intensity of each charge state of apo- and holo-CA2 in the unfolding process. Unfolding was initiated by the addition of acetic acid to the CA2 solution at pH 4.4. The relative intensity of apo-CA2 (-●-) and holo-CA2 (-■-) ions were plotted for each charge state as a function of time. Each value represents the mean ± SD of triplicate measurements.

branes of specialized epithelial and endotherial cells (CA4), in mitochondria (CA5), or in salivary secretions (CA6). CA isozymes are found widespread in nature in animals, plants, and certain bacteria. In animals, CA plays an important role in respiration by facilitating transport of CO_2 and is involved in the transfer and accumulation of H^+ and HCO_3^-.[25,26] The bovine CA2 used in this study exhibits high activity from erythrocyte, consists of 259 amino acid residues and a zinc ion that is essential for the activity, and is tetrahedrally coordinated to the imidazoles of the three histidine (His) residues at His93, His95, and His118 and to a water molecule.[27]

Here we observed charge state distributions and a shift in mass due to transitions of the CA2 protein from apo to holo and vice versa. In addition, the range of pH jump selected allowed the folding changes to continue for several minutes and thus we were able to continuously probe both the unfolding and refolding processes of CA2 as a function of time. The information obtained would be helpful in understanding not only the essential structure of CA2 but also the disordered state of the protein.

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EXPERIMENTAL SECTION

Materials. CA2 was purchased from Sigma (St. Louis, MO). Leu-enkephalin (Leu-Enk) was obtained from Peptide Institute Inc. (Osaka, Japan). All other reagents used in the study were of the highest guaranteed grade.

Probing the Unfolding Process. One hundred pmol/μL of CA2 solution was prepared by dissolving the protein in H_2O. To measure the unfolding process, 3.5 μL of the CA2 solution was added to 200 μL of 0.2% (v/v) acetic acid at pH 4.4. The pH was adjusted with aqueous ammonia. Then 3.5 μL of 10 μg/mL of Leu-Enk was added to the solution. Leu-Enk was used in this study as the internal standard of the ion abundance measurement for the time course of the unfolding process. The mass of CA2 in the solution was measured to confirm that holo-CA2 was the main species in the solution. To 100 μL of the solution was added approximately 240 μL of 0.2% (v/v) acetic acid to start the unfolding of CA2 (pH jump from 4.4 to 3.6). The mass of CA2 was measured for 20 min at ambient temperature. The pH values were measured using another sample set which had the same ratio of 0.2% (v/v) acetic acid and aqueous ammonia as the samples for the measurement of mass. The measurements were performed in triplicate. From the spectrum of each sampling time, the ratio of CA2 to the Leu-Enk ion peak height was calculated to
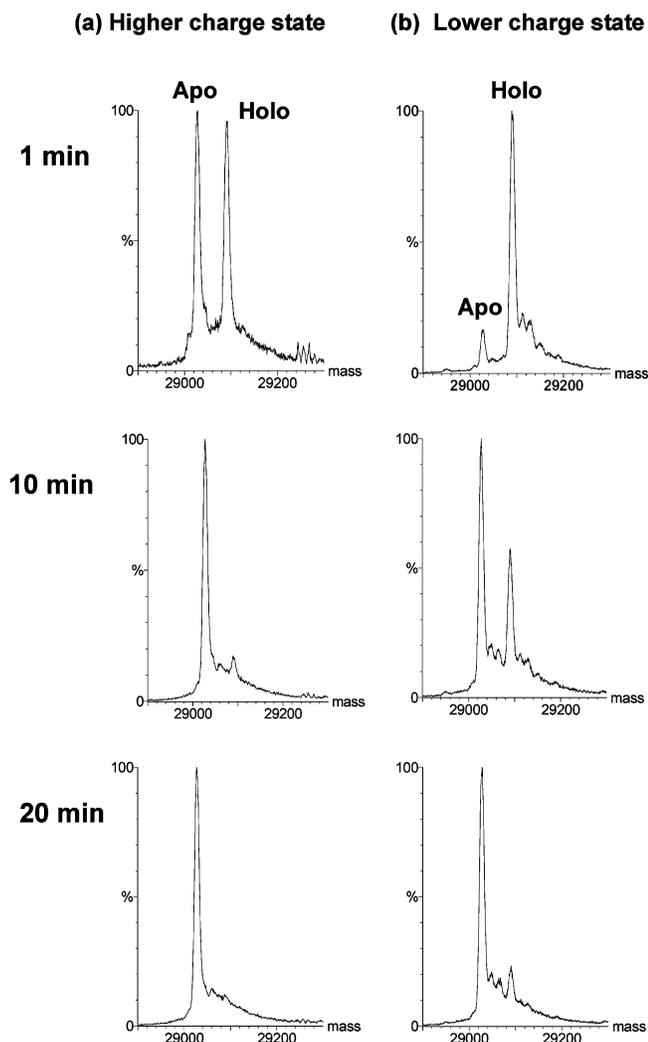
(a) Higher charge state   **(b) Lower charge state**

Figure 4. Deconvolution spectra of CA2 in the unfolding process, (a) representative spectra of the higher charge state (deconvoluted from m/z 800 to 2000) and (b) representative spectra of the lower charge state (deconvoluted from m/z 2000 to 3000).
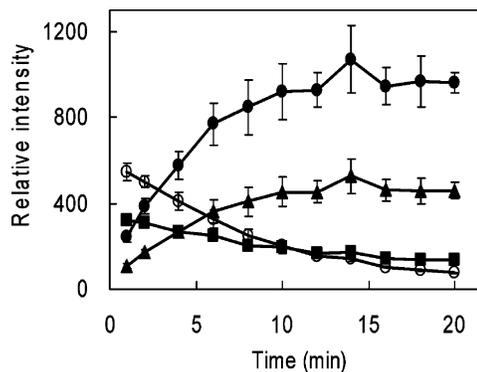

Figure 5. Change in relative ion intensity of the four types of ions obtained from deconvolution spectra in the unfolding process. The relative intensities of lower charged apo-CA2 (-▲-), lower charged holo-CA2 (-○-), higher charged apo-CA2 (-●-), and higher charged holo-CA2 (-■-) ions were plotted as a function of time. Each value represents the mean ± SD of triplicate measurements.

a desolvation temperature of 100 °C were employed for the measurements. Nitrogen was used as the gas for nebulizing and desolvation. The rate of direct infusion of the sample solution was 10 μL/min during all mass measurements. Mass spectra were cumulatively acquired for 0.5 min at each sampling time. Deconvolution spectra were obtained to evaluate the time course of each ion ensemble observed in the mass spectra. Processing was performed from m/z 800 to 2000 and m/z 2000 to 3000 for apoproteins. In order to evaluate the holoproteins, m/z 800 to 2300 and m/z 2300 to 3000 were set for the deconvolution ranges.

RESULTS AND DISCUSSION

ESI MS of Bovine Carbonic Anhydrase 2. ESI mass spectra of CA2 measured at pH 4.4 and pH 3.6 are shown in Figure 1a and Figure 1b, respectively. At pH 4.4, multiply charged ions [M + nH + Zn]$^{n+}$ corresponding to holo-CA2 were found to be predominant, consisting mainly of charge states from 10+ to 12+ with 11+ the most abundant (Figure 1a). In contrast, at pH 3.6 multiply charged ions [M + nH]$^{n+}$ corresponding to apo-CA2 were predominant and higher charge state peaks were observed in the mass spectrum. The charge state distribution at pH 3.6 formed a bimodal distribution which consists of a higher charge state region with 18+ the most abundant, a lower charge state region with 12+ the most abundant, and a boundary valley with a charge state of 15+ (Figure 1b). This suggests that CA2 has two folding states for the apoprotein under the condition of pH 3.6. According to previous studies, the folding state of the lower charged protein is suggested to be relatively rigid.[5,6,8] The values of molecular mass obtained with deconvolution for the apo- and holo-CA2 ion peaks were 29 027.1 and 29 090.5 Da, respectively. These values are consistent with the values of the calculated relative molecular mass of 29 024.3 Da for apo-CA2 and 29 087.3 Da for holo-CA2.[21]

Probing the Unfolding Process of CA2 with a pH Jump from 4.4 to 3.6. The time course of the ESI mass spectra obtained by probing the unfolding process of CA2 is shown in Figure 2. Measurement was conducted for 1 min after the initiation of the pH jump from 4.4 to 3.6 for the CA2 solution. Each ion peak observed in the mass spectra suggest that the resulting multiply charged CA2 ions, [M + nH]$^{n+}$ for apo-type and [M + nH + Zn]$^{n+}$ for holo-type, were divided into four groups: lower

reduce interspectrum variation of the ion abundance. The peak height ratio (relative intensity) was plotted against the sampling time and evaluation of the folding change was performed.

Probing the Refolding Process. To measure the refolding process, the procedure was similar to the unfolding experiment. To 2 μL of 100 pmol/mL CA2 solution, 400 μL of 0.2% (v/v) acetic acid pH 3.6 (pH adjusted with aqueous ammonia) was added. Then 2 μL of 10 μg/mL of Leu-Enk (as the internal standard) was added to the solution and incubated at 30 °C for 30 min. The mass of CA2 in the solution was measured to confirm that apo-CA2 was the main species in the solution. Then, to 300 μL of the solution, 45 μL of 0.2% (v/v) aqueous ammonia was added to start the refolding of CA2 (pH jump from 3.6 to 4.4). The mass of CA2 was measured for 20 min at ambient temperature. The measurements were performed in triplicate. The relative intensity of each peak was plotted against the sampling time and evaluated.

ESI MS. Mass spectra were obtained with a Q-TOF Ultima quadrupole time-of-flight mass spectrometer equipped with an electrospray ion source and MassLynks data processor (Micromass, Manchester, UK). An electrocapillary voltage of 3.0 kV, a sample cone voltage of 100 V, a source temperature of 50 °C, and
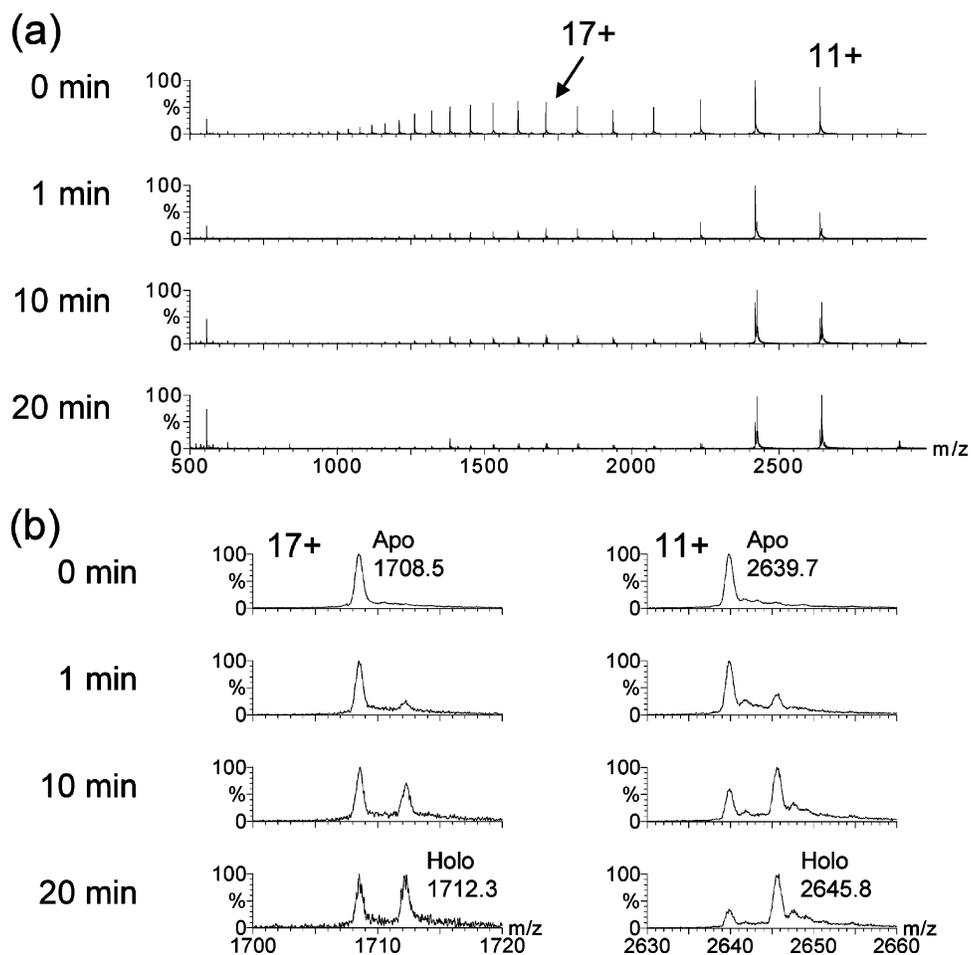
Figure 6. Time-dependent changes of mass spectra resulting from a pH jump from 3.6 to 4.4. Refolding was initiated by the addition of aqueous ammonia to the CA2 solution at pH 3.6. (a) The mass spectra showing whole charge state distribution. (b) The ions of charge states 17+ and 11+ were extracted from the corresponding mass spectrum in part a.

charge state apo-CA2 such as $[M + 11H]^{11+}$ at m/z 2639.9, lower charge state holo-CA2 such as $[M + 11H + Zn]^{11+}$ at m/z 2645.6, higher charge state apo-CA2 such as $[M + 19H]^{19+}$ at m/z 1528.7, and higher charge state holo-CA2 such as $[M + 19H + Zn]^{19+}$ at m/z 1532.2, as shown in Figure 2b. These four types of ions were easily distinguished from each other. The time course of the mass spectra shows that the ions corresponding to holo-CA2 change to apo-CA2 with time. The peaks corresponding to higher charged apo-CA2 increased in abundance with time and a bimodal spectral pattern was formed, as shown in the spectrum at 20 min in Figure 2a. The relative intensity of holo-CA2 and apo-CA2 in several charge states such as 10+, 11+, 15+, 17+, and 23+ was plotted against time (Figure 3). The time course plots show that the decrease in abundance of the holo-ions and the increase in abundance of the apo-ions occurred simultaneously. It is likely that the transition from holo-CA2 to apo-CA2 would proceed in the same charge state in solution, e.g., $[M + 11H + Zn]^{11+} \rightarrow [M + 11H]^{11+}$. However, in lower charged states the sum of the relative intensities of holo-ions and apo-ions at the initial (1 min after initiation) was larger than that at the final (20 min after initiation) in the same charge state, whereas in higher charged states the sum at the final was larger than that at the initial. This suggests that another transition pathway, intercharge transition from lower charged holo-CA2 to higher charged holo-CA2, exists in the unfolding process. Thus, analysis that will further probe

the time courses of the four ion types is necessary to clarify the unfolding pathway in detail. Additionally, the ionization efficiency of holo- and apo-ions is considered to be different. It is assumed to be of the order apo > holo, so the protein amounts of apo- and holo-CA2 cannot be compared to each other from the ion abundances.

To analyze the time course of the four ion types in detail, we considered mass spectra deconvoluted over an appropriate range of charge states to be effective. The boundary selected as the appropriate range of charge states was 15+ for apo-CA2 and 13+ for holo-CA2 (Figures 1a and 3). Thus, the deconvolution was performed on peaks from 15+ to 36+ and from 10+ to 14+ for apo-CA2 and from 10+ to 13+ and from 14+ to 36+ for holo-CA2. The time courses in the mass spectra deconvoluted for higher and lower charge states are shown in Figure 4a and Figure 4b, respectively. An abundant peak corresponding to the apo-CA2 ion was observed even 1 min after the initiation in the higher charge state (Figure 4a). This indicates that the CA2 molecule releases the zinc ion easily in solution and tends to produce higher charged ions using the ESI process. The peak abundance of holo-CA2 ion at 29 089.4 Da in the lower charge state decreased gradually over time (Figure 4b), whereas that in the higher charge state decreased rapidly (Figure 4a). The time courses of the abundance of the four types of ions, lower and higher charge state apo-CA2 and lower and higher charge state holo-CA2, are shown
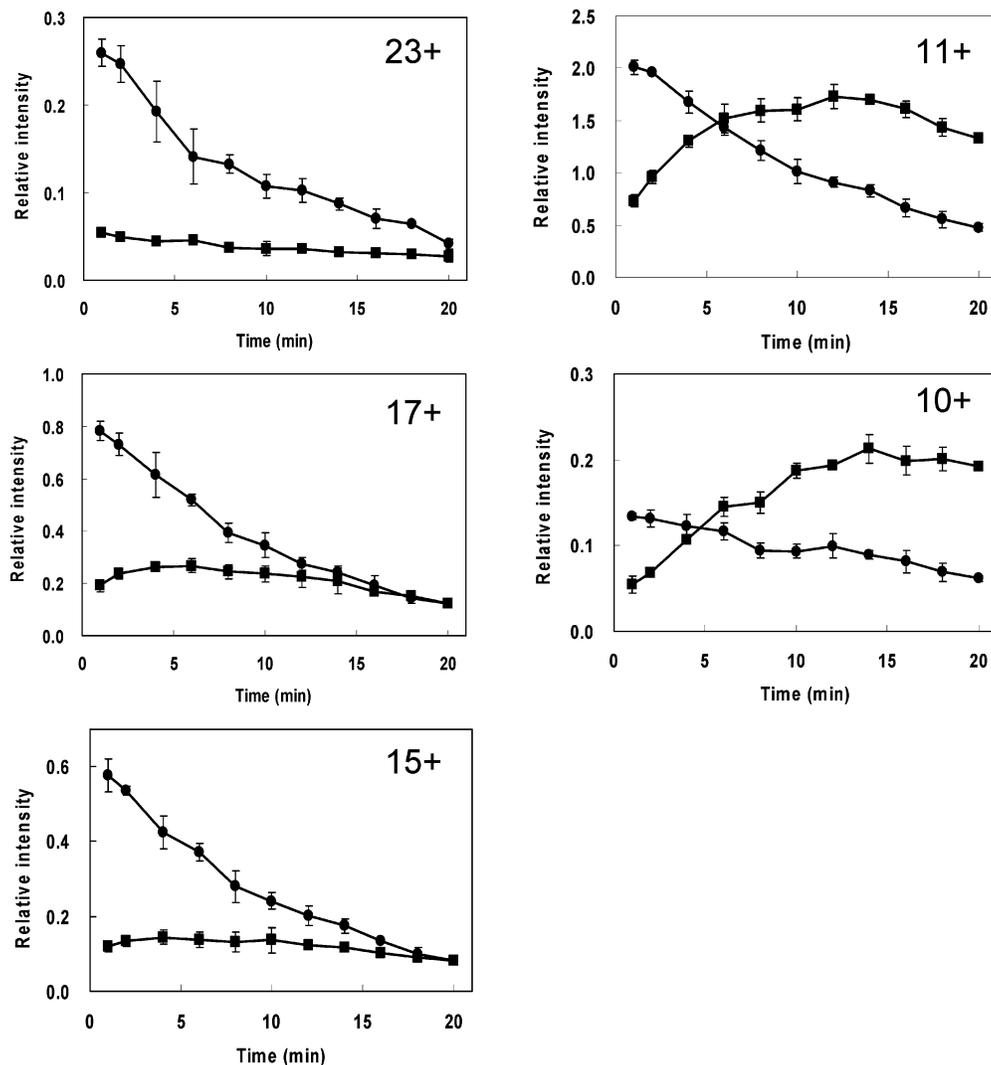
Figure 7. Change in relative ion intensity of each charge state of apo- and holo-CA2 in the refolding process. Refolding was initiated by the addition of aqueous ammonia to the CA2 solution at pH 3.6. The relative intensity of apo-CA2 (-●-) and holo-CA2 (-■-) ions were plotted for each charge state as a function of time. Each value represents the mean ±SD of triplicate measurements.

in Figure 5. The time courses indicate that with a pH jump from 4.4 to 3.6, the predominant process is the transition from lower charged holo-CA2 to higher charged apo-CA2. This process may occur from the release of the zinc ion from holo-CA2 and from unfolding. In summary, the unfolding processes detected with ESI MS were confirmed by the mass shift caused by the release of the zinc ion and the formation of the bimodal apoprotein ion distribution. In this study, mass spectra were cumulatively acquired for 0.5 min at each sampling point. This acquiring time might be long to exhibit the feature of the mass spectrum as a snapshot at each sampling point, especially for the early sampling time point. However, the folding change of CA2 was discussed using all mass spectra measured for 20 min. Thus, the acquiring time setting gave no affect on the obtained pathway of the CA2 folding change.

Probing the Refolding Process of CA2 with a pH Jump from 3.6 to 4.4. With the use of the same procedure described above, the refolding process of CA2 with a pH jump from 3.6 to 4.4 was revealed. The time course of the ESI mass spectra obtained is shown in Figure 6. The time courses of the typical higher charged ion 17+ and the lower charged ion 11+ are shown

in Figure 6b. The peaks corresponding to the higher charged ion decreased with time, and the predominant change was the disappearance of the bimodal spectral pattern resulting from the higher charged apo-CA2 changing to the lower charged holo-CA2. Thus, refolding of CA2 from an apo- to holo-ion was suggested to have occurred. The relative intensities of the holo- and apo-ions at the different charge states of 10+, 11+, 15+, 17+, and 23+ were plotted against time (Figure 7). The time course plots show that the lower charged holo-ions, especially from 10+ to 11+, increased with time, while the higher and lower charged apo-ions decreased drastically. The deconvoluted spectra of the four ion types (higher charge state apo- and holo-CA2 and lower charge state apo- and holo-CA2) were evaluated for the refolding process (Figure 8), and the time course for each type of ion was plotted against time (Figure 9). Figure 8 shows that apo-CA2 ions in both higher and lower charge states remain even after 20 min. Figure 9 also shows that the increase in holo-CA2 was slight compared to the decrease in apo-CA2. Although this suggests that the refolding with a pH jump from 3.6 to 4.4 was not complete at the 20-min sampling point, the final product of the refolding will possibly be the lower charged holo-ion. Note that, as shown in
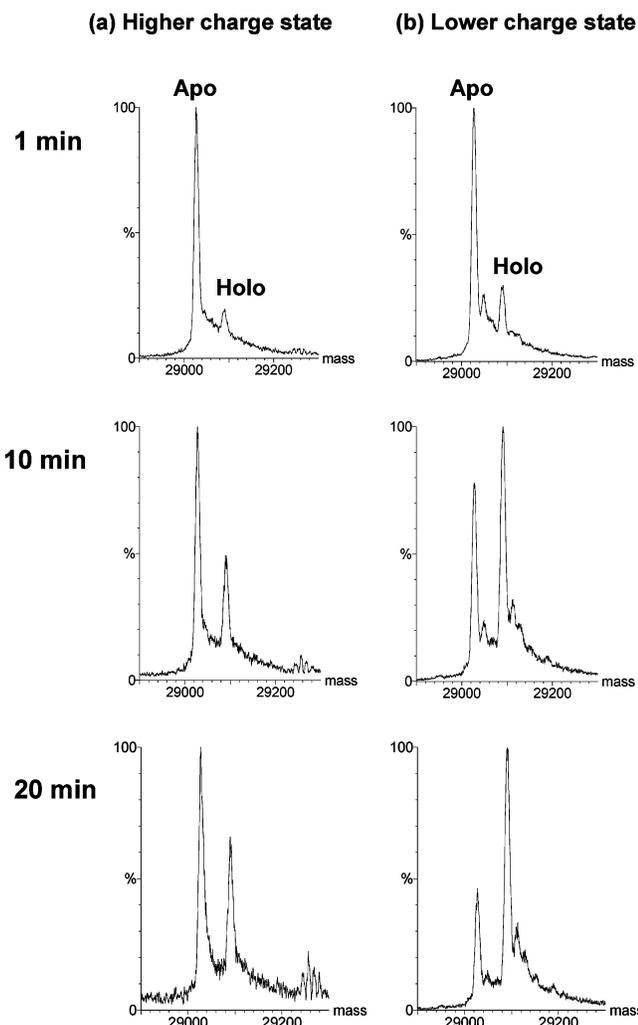
(a) Higher charge state

(b) Lower charge state

Figure 8. Deconvolution spectra of CA2 in the refolding process, (a) representative spectra of the higher charge state (deconvoluted from m/z 800 to 2000) and (b) representative spectra of the lower charge state (deconvoluted from m/z 2000 to 3000).
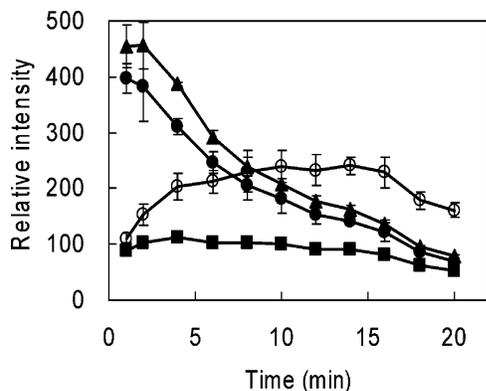

Figure 9. Change in relative ion intensity of the four molecular species obtained from deconvolution spectra of the refolding process. The relative intensity of lower charged apo-CA2 (-▲-), lower charged holo-CA2 (-○-), higher charged apo-CA2 (-●-), and higher charged holo-CA2 (-■-) ions were plotted as a function of time. Each value represents the mean ±SD of triplicate measurements.

Figure 9, the intensity of the lower charged holo-ion started to decrease at the 16-min time point. It was speculated that this was due to the formation of a state with lower ionization efficiency.

Scheme 1. Proposed Pathways of the Folding Change of CA2 under the Conditions Employed in the Study

H_{Apo} : Higher charged apo ion
H_{Holo} : Higher charged holo ion
L_{Apo} : Lower charged apo ion
L_{Holo} : Lower charged holo ion
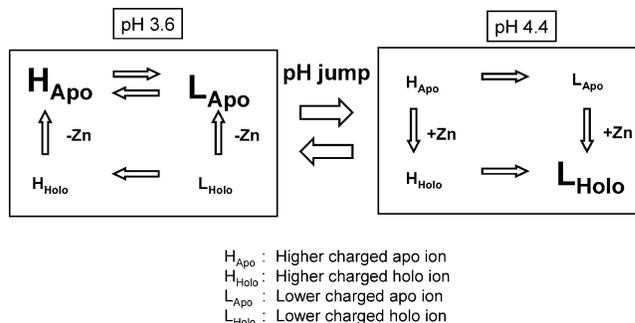
Proposed Schematic Pathway for the Folding Processes of CA2 with a pH Jump. Using a pH jump from 3.6 to 4.4, we successfully probed the refolding process of CA2 by ESI MS. In general, the rate of secondary structure formation has been reported to be rapid on a millisecond time scale.[28,29] Thus, if the process continues for more than 20 min, it is indicative of the formation of a tertiary structure, similar to the time course refolding process observed in a solution containing guanidine hydrochloride.[23,24] The lower charged holo-CA2 ions observed resulting from the decrease of both the higher and lower charged apo-CA2 ions suggests that there were two folding pathways, the folding of a polypeptide without Zn and the Zn binding to the polypeptide, leading to the formation of the lower charged holo-CA2 ions. It is interesting that in the time course measurements, the formation of holoprotein proceeds without the addition of an extra zinc ion, speculating that the zinc ion stays close to the polypeptide with a weak interaction at pH 3.6, and during the measurement of mass, the interaction would be pegged out.

From this study with a pH jump from 3.6 to 4.4, we propose a refolding pathway as shown in Scheme 1. In the initial state at pH 3.6, two different apoproteins in the folding state, H_{apo} and L_{apo}, might exist in equilibrium and form the bimodal spectral pattern. The pH jump to 4.4 initiates the zinc binding with those apoproteins. The resulting higher charged holoprotein H_{holo} refolds to the lower charged holoprotein L_{holo}. Thus, higher charged holoprotein is considered to be an intermediate in the refolding process. In addition, the refolding from the higher charged apoprotein to the lower charged apoprotein without zinc binding may be another pathway. This is in agreement with the previous observation that zinc is not necessary for CA2 refolding.[21]

The two types of apoprotein H_{apo} and L_{apo}, disordered CA2, observed were considered to be the molten globule in CA2 reported in previous studies[23,24] because both apoproteins can bind to the zinc ion and refold to the holoprotein. The presence of the two types of disordered apoprotein found in this study is a novel observation. As reported previously,[24] the first step of the refolding pathway is the binding of the metal ion to the molten globule, thus initiating compaction and formation of the metal-binding site region. An additional pathway initiated from the polypeptide refolding without zinc binding is suggested from the findings of

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this study. Also, the unfolding process from a pH jump from 4.4 to 3.6 is proposed and shown in Scheme 1. Two unfolding pathways, releasing the zinc ion from L_{holo} and the disorder of the polypeptide chain from L_{holo} to H_{holo}, were observed and suggest that the disorder of the polypeptide leads to the rapid release of Zn from H_{holo}. Similar to the abovementioned refolding study, our observations suggest that different folding states of disordered CA2, the apoproteins H_{apo} and L_{apo}, were present in equilibrium.

CONCLUSIONS

The present study has demonstrated that ESI MS coupled with a pH jump is a powerful tool able to probe the time course of the continuous folding change of the metalloprotein CA2 consisting of 259 amino acid residues and a zinc ion. This method can detect the time course of the reversible folding change resulting from a small alteration of the pH environment around the protein, provided the pH shift is less than 1.0, i.e., from 4.4 to 3.6 or vice versa. The ESI mass spectrum at pH 3.6 showed a bimodal charge state distribution suggesting the presence of two folding states in equilibrium for apo-CA2, but at pH 4.4 the spectrum showed a lower charge state distribution corresponding to folded holo-CA2. In both the unfolding and refolding processes caused by pH jumps between 3.6 and 4.4, the existence of four ion types, lower charged apo-CA2 and holo-CA2 ions and higher charged apo-CA2 and holo-CA2 ions, was revealed. The time course of mass spectra and the processing of deconvolution spectra for each type of ion gave information on the folding change. The shift in mass caused by zinc ion binding or releasing and the change in charge state distributions helped clarify the entire molecular folding state. The refolding of the higher charged apo-CA2 resulted in both lower charged apo-CA2 and holo-CA2 without the addition of an extra zinc ion. This suggested that the zinc ion was not required for refolding and that the intrinsic zinc ion could weakly interact with the apoprotein in solution. The higher charged holoprotein observed was considered to be an intermediate in the folding process.

Although circular dichroism (CD) is a method known to examine protein folding, CD only gives the overall folding state of a protein contained in the sample solution. Contrarily, the ESI MS method allows detection of each folding state of the protein contained in the sample solution. In this study we detected four different folding states of CA2, a unique advantage of the ESI MS method applied. With this method, it is also possible to detect conformational change of partial structures in a protein. An additional advantage is that this method is simple to perform and applicable to other kinds of proteins as well as metalloproteins.