Characterization of Acid-Induced Protein Conformational Changes and Noncovalent Complexes in Solution by Using Coldspray Ionization Mass Spectrometry

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Coldspray ionization (CSI) mass spectrometry, a variant of electrospray ionization (ESI) operating at low temperature (20 to -80°C), has been used to characterize protein conformation and noncovalent complexes. A comparison of CSI and ESI was presented for the investigation of the equilibrium acid-induced unfolding of cytochrome c, ubiquitin, myoglobin, and cyclophilin A (CypA) over a wide range of pH values in aqueous solutions. CSI and nanoelectrospray ionization (nanoESI) were also compared in their performance to characterize the conformational changes of cytochrome c and myoglobin. Significant differences were observed, with narrower charged-state distribution and a shift to lower charge state in the CSI mass spectra compared with those in ESI and nanoESI mass spectra. The results suggest that CSI is more prone to preserving folded protein conformations in solution than the ESI and nanoESI methods. Moreover, the CSI-MS data are comparable with those obtained by other established biophysical methods, which are generally acknowledged to be the suitable techniques for monitoring protein conformation in solution. Noncovalent complexes of holomyoglobin and the protein-ligand complex between CypA and cyclosporin A (CsA) were also investigated at a neutral pH using the CSI-MS method. The results of this study suggest the ability of CSI-MS in retaining of protein conformation and noncovalent interactions in solution and probing subtle protein conformational changes. Additionally, the CSI-MS method is capable of analyzing quantitatively equilibrium unfolding transitions of proteins. CSI-MS may become one of the promising techniques for investigating protein conformation and noncovalent protein-ligand interactions in solution. (J Am Soc Mass Spectrom 2009, 20, 845–851) © 2009 American Society for Mass Spectrometry

Characterization of native conformations and noncovalent complexes of proteins is of great significance to understanding a variety of biological processes at the molecular level. Electrospray ionization mass spectrometry (ESI-MS) [1, 2] has grown into a powerful technique in this field. Besides its capability of handling large biomolecules, the advantages of ESI-MS over other spectroscopic and biophysical methods include high analysis speed, minimal sample consumption, and the characterization of individual conformational states that may coexist in solution at equilibrium [3–5]. The charge-state distribution (CSD) in the ESI mass spectra represents a specific conformational state of protein [6–9]. Broad CSDs at high charge states are generally associated with un-

folded proteins, whereas narrower distributions centered on lower charge states are treated as characteristics of folded proteins [6, 10–15]. However, the harsh conditions during the ionization process in MS are often detrimental to the preservation of protein conformation and the survival of noncovalent complexes. Many studies have examined the influence of the ionization process and the operating settings of ESI, including the curtain gas [16, 17], the pressure in the ion source [18–20], the temperature [21–26], the cone voltage [3, 27], the spray mode [28], and the solvent composition [29, 30]. To preserve the native protein conformation, a significant amount of effort has also been made on modifications to the ESI source. The electrosonic spray ionization (ESSI) [31, 32] method, using a traditional micro ESI source with supersonic nebulizing gas, allows for the characterization of protein complexes in solution. In addition, the ESI source combined with an air amplifier (ESI-AA) [33] also showed improvement in

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preserving folded conformations. Nanospray [34–36] is often a gentle ionization method of allowing the use of less harsh conditions including a lower heated capillary temperature, the absence of heater gas, and a lower spray voltage while also providing high ionization efficiency for small-sample consumption. These advantages favor the application of nanospray to study protein conformation and noncovalent complexes [37–39].

Recently, coldspray ionization (CSI) mass spectrometry was developed. This method is a variant of ESI operating at low temperature (20 to -80°C) and allows for the facile and precise characterization of labile organometallic complexes and organic and biomolecular noncovalent interactions in solution [40–47]. In the present study, the equilibrium acid-induced conformational transitions of proteins, including cytochrome *c*, ubiquitin, myoglobin, and cyclophilin A (CypA), were investigated using CSI-TOFMS, a coldspray ion source combined with a time-of-flight (TOF) mass analyzer, over a wide range of pH values in aqueous solutions. Furthermore, noncovalent complexes were observed in the case of holomyoglobin (the heme group is noncovalently bound in a hydrophobic pocket of myoglobin [48]) and the protein–ligand complex between CypA and cyclosporin A (CsA). CypA is a ubiquitously distributed intracellular protein belonging to the immunophilin family and is recognized as the host cell receptor for the potent immunosuppressive drug CsA [49–51]. Compared with conventional ESI and nanoESI, CSI has clear advantages in investigating acid-induced conformational transitions and protein–ligand complexes. In this study, we demonstrated for the first time that the CSI-MS method has the capacity of characterizing protein conformations and protein complex structures in solution.

Experimental

Materials and Sample Preparation

Ubiquitin, cytochrome *c*, and horse heart myoglobin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sample solutions were prepared by dissolving the proteins in water to a concentration of 5 mg mL^{-1} and then diluting to $1\text{ }\mu\text{mol L}^{-1}$ using aqueous ammonium hydroxide or acetic acid. The pH of the solution was adjusted by adding acetic acid or ammonium hydroxide and was measured with an Accumet meter (AB 15, Fisher Scientific, Pittsburgh, PA, USA) before the addition of protein. The DNA fragment encoding human CypA was cloned into the pET32a vector (Novagen, San Diego, CA, USA) with NdeI/Hind III restriction enzymes and CypA was expressed as a fusion protein with a C-terminal histidine tag. The fusion proteins were affinity-purified with a Ni-NTA Agarose column (Invitrogen, Carlsbad, CA, USA) according to standard methods. CsA was purchased from Ruibio (Darmstadt, Germany).

Mass Spectrometry

The CSI/ESI-TOF mass spectra were acquired using an AccuTOF CS mass spectrometer (JMS-T100CS, JEOL, Tokyo, Japan). The needle voltage was set at 2.2–2.4 kV; the orifice 1 voltage was set at 40 V and the orifice 2 voltage was set at 6 V. The flow rate of nebulizer gas was set at 1.0 to 2.0 L/min. The spray temperature was set between -20 and 20°C for CSI measurements, whereas the desolvation chamber temperature was set between 200 and 250°C for conventional ESI measurements.

The positive-ion nanoelectrospray ionization experiments were performed using an Applied Biosystems QSTAR Elite system, equipped with a nanoelectrospray ion source (Concord, ON, Canada). The MS conditions were as follows: curtain gas, 20 (arbitrary units); ion-spray voltage, 1900 V; nebulizer gas, 23 (arbitrary units); declustering potential, 40 V; heater gas (gas 2) and interface heater temperature, no use.

Results and Discussion

To obtain optimal operating conditions for the characterization of folded protein conformations in solution, the investigation of the effect of spray temperature on the ESI and CSI mass spectra of the model protein, cytochrome *c*, was carried out at pH 2.5. All other instrumental parameters were also optimized. In addition, a nanoESI mass spectrum was obtained under the same solution conditions. It was anticipated that a bimodal CSD would be present in the mass spectra corresponding to the coexistence of the folded and unfolded states of cytochrome *c* at pH 2.5 [52, 53]. The mass spectra obtained at varying temperatures are shown in Figure 1 and Supplementary Figure S1, which can be found in the electronic version of this article. As illustrated in Figure 1a, in the ESI mass spectrum obtained at 250°C only a single-mode CSD centered at +16, corresponding to the unfolded protein, was observed. As the temperature was decreased, Figure 1b–e clearly demonstrate that in addition to the high charge-state distribution, another distribution centered at +8 corresponding to the folded protein can be observed. These results illustrate that a relatively high abundance of the folded form can be obtained using CSI-MS at spray temperatures ranging from 20 to -20°C . A single-mode CSD centered at +16 corresponding to the unfolded protein was observed in the nanoESI mass spectrum (Figure 1f) similar to the one observed in a conventional ESI mass spectrum. The advantage observed with nanoESI was an increase in sensitivity compared with that of conventional ESI. However, there were evident differences between the nanoESI mass spectrum (Figure 1f) and the CSI mass spectrum (Figure 1c) obtained at almost the same temperature, which can be traced to the different acid-induced effects associated with the differences in designs of ion source and ionization processes. The conformational transition of cytochrome *c* is very sensitive to the change of pH value around 2.5 [53]; the

variation in pH values of the droplets under nanoESI ionization conditions probably cause evident acid-induced denaturation. These results suggest that CSI is more suited to the preservation of solution-phase conformations than either the ESI or nanoESI methods. It is noted that the relative amount of folded cytochrome c characterized using CSI-MS at 10 °C is consistent with that obtained by spectroscopic techniques [52]. Thus, a spray temperature of 10 °C was adopted in this study.

For purposes of comparison, CSI and ESI mass spectra were collected using cytochrome c at solution pH values ranging from 11.0 to 3.0. Among these spectra, those collected at pH values of 3.0, 7.0, and 11.0 are shown in Figure 2 and those collected at pH values of 5.2 are shown in Supplementary Figure S2. As shown in Figure 2a–c and Supplementary Figure S2a, single-mode and narrow CSDs with maximum at +7 or +8 were recorded for cytochrome c in CSI mass spectra obtained at pH values between 11.0 and 3.0, indicating a tightly folded conformation. In the ESI mass spectra (Figure 2d–f and Supplementary Figure S2b), broadening CSDs associated with an unfolded protein were observed in this pH range. Along with further acidification of the solution, the distribution and the maximum charge state are gradually shifted to higher charge states and extend up to +16 at pH 3.0 in the ESI mass spectra.

In addition, the cooperative effects of pH and temperature on the unfolding of cytochrome c were inves-

Figure 2. CSI (a, b, and c) and ESI (d, e, and f) mass spectra for cytochrome c at pH 11.0, 7.0, and 3.0.

tigated. The tightly folded conformation relative to the total in both tightly and loosely folded conformations is plotted against temperature for pH values of 5.2, 3.4, and 3.0 in Figure 3a. The fraction of folded protein for cytochrome c was calculated as the sum of intensities of the peaks corresponding to the +6, +7, +8, +9, and +10 ions, divided by the sum over all the peaks.

$$\text{Folded \%} = \frac{\sum_{i=6-10} I_i}{\sum_{i=1-n} I_i} \quad (1)$$

As can be seen from Figure 3a, the results obtained at the temperature of CSI reflect the folded state of cyto-

Figure 1. ESI (a, conventional temperature of ESI-MS, b, different temperature of ESI-MS), CSI (c–e) and nanoESI (f, room temperature) mass spectra of cytochrome c in water and acetic acid (pH 2.5).

Figure 3. (a) Relative amounts of folded cytochrome c versus temperature at three different values of solution pH using CSI/ESI-MS detection. (b) Relative amounts of folded cytochrome c against pH values using CSI-MS. The error bars for CSI-MS data represent the standard deviation.

chrome c in solution. Under the same temperature, the lower the pH, the more difficult it is to maintain a folded conformation, which demonstrates that the actual pH values of the droplets are much lower than those of the native solution under ESI ionization conditions [54, 55], and thus result in additional acid-induced unfolding. Under the same pH, the higher the temperature, the more difficult it is to maintain a folded conformation because of heat-induced unfolding. These results reveal that the pH and temperature cooperatively influence the ESI ionization process and thus induce alterations to the CSD. Moreover, we used CSI-MS to quantitatively analyze the equilibrium of the unfolding transitions. Figure 3b shows the relative amount of folded cytochrome c in CSI mass spectra at solution pH values from 2.0 to 3.0 according to eq 1. As illustrated in Figure 3b, the relative amount of the folded form obtained by CSI-MS matches the values obtained using tryptophan fluorescence spectrometry [52]. These results indicate that the CSI-MS method preserves the native protein structure, especially under low pH values, and has the advantage of being able to accurately characterize acid-induced equilibrium unfolding transitions.

Series of spectra for ubiquitin were also obtained for solution pH values ranging from 11.0 to 1.2 by using ESI-MS and CSI-MS. Among these spectra, those collected at pH values of 7.0, 2.3, and 1.7 are shown in Figure 4 and those collected at pH values of 2.0 and 1.2 are shown in Supplementary Figure S3. Single-mode

Figure 4. CSI (a, b, c) and ESI (d, e, f) mass spectra for ubiquitin at pH 7.0, 2.3, and 1.7.

CSDs centered at +6 or +7 charge states corresponding to the folded ubiquitin were observed for solution pH values 7.0 to 2.3 in CSI-MS spectra (Figure 4a and b). No pronounced changes of the CSD in CSI mass spectra between pH 11.0 and 2.3 were observed (some of the data are not shown), which are in accordance with the results of a previous study performed by CD spectroscopy [56]. Below pH 2.3, another distribution centered at +11 appeared in the CSI mass spectra (Supplementary Figure S3a). Notably, a new distribution with main peaks at +8 and +9 was observed when the pH values decrease to 1.7 (Figure 4c), indicating the presence of an acid-induced unfolded ubiquitin intermediate [22, 57, 58]. Figure 4d shows that the maximum at pH 7.0 in the ESI mass spectrum remains at +7 but the charge-state distribution is broadened. A further decrease in the solution pH values results in the distribution and the maximum charge state to be gradually shifted to higher values (Figure 4e and f and Supplementary Figure S3c). As shown in Supplementary Figure S3b and d, the high charge states completely dominate the spectra at pH 1.2, and the results of CSI-MS and ESI-MS spectra are almost the same. The above-cited results further emphasize that the prevention of thermal-induced and additional acid-induced effects during the CSI process enables the CSI method to be used for precisely monitoring conformational changes.

An analogous set of experiments was performed for horse heart myoglobin at pH values ranging from 11.0 to 2.2 by using CSI-MS, ESI-MS, and nanoESI-MS. The spectra obtained at pH 7.6, 4.0, and 3.4 are shown in Figure 5. In the CSI mass spectra, the intact folded holomyoglobin (hMb) was observed at pH 7.6 (Figure 5a), pH 4.0 (Figure 5b), and above (data not shown). Figure 5d shows that hMb and aMb ions in higher and lower charge states were observed in the ESI mass spectra. The dominant peaks in the ESI mass spectrum (Figure 5e) at pH 4.0 are attributed to aMb in high charge states. Supplementary Figure S4a shows that the intact folded hMb was obtained at pH 7.6 in the nanoESI spectrum. In the nanoESI mass spectrum (Supplementary Figure S4b) distributions of hMb ions with main peaks at +10 were observed and aMb ions in higher charge states (+16) appear with low intensity when pH values decrease to 4.0. Furthermore, as can be seen from Figure 5g obtained at pH 3.4 (a magnified portion of Figure 5c), in addition to the high CSD associated with the unfolded aMb, the magnified CSI mass spectrum exhibits a bimodal distribution of hMb with one distribution with a maximum at 10+ and a second maximum around 14+. Previous studies [59–61] showed that the kinetics of hMb unfolding under acidic conditions follow a two-step mechanism. In the first step a short-lived intermediate that is unfolded becomes populated, but still retains its heme group. In the second step the unfolded heme-protein dissociates and unfolded aMb and free heme are formed. The coexistence of three CSDs corresponding to folded hMb, unfolded hMb, and unfolded aMb in the CSI mass

Figure 5. CSI (a, b, c) and ESI (d, e, f) mass spectra for myoglobin at pH 7.6, 4.0, and 3.4. (g) A magnified CSI mass spectrum at pH 3.4. Biomodal CSDs of hMb with maximum at hMb10⁺ corresponding to the folded myoglobin (labeled by ellipse) and hMb14⁺ identified with the folded intermediate (labeled by ball).

spectrum provides evidence for the kinetics of hMb unfolding. Moreover, the pH value of 3.4, the lowest at which hMb peaks were observed in equilibrium with CSI-MS, is lower than values reported using ESI-MS, even when gentle conditions were used [33]. The ESI (Figure 5f) and nanoESI (Supplementary Figure S4c) mass spectra show the distributions of aMb in higher charge states corresponding to unfolded proteins. The above-cited results clearly demonstrate the ability of the CSI-MS method to preserve native protein conformation, noncovalent complex structures and its ability to monitor the transient unfolding of intermediates.

Based on the preceding investigations, the protein–ligand complex between CypA and CsA was further studied by using the CSI-MS method. The conformational transitions of CypA were observed by CSI-MS and ESI-MS at solution pH values ranging from 11 to 3.2. Once again, significant differences between CSI and ESI mass spectra were obtained. Figure 6a and b show that the folded conformation of CypA was retained in

the CSI mass spectra from pH 7.0 to 4.3. No significant changes in the CSI mass spectra were found between pH values 11.0 to 4.3 (some data are not shown). Below pH 4.0 another distribution centered at +19 appeared (see Supplementary Figure S5a). When the solution pH value was decreased to 3.2 the high charge states completely dominated the spectra (Supplementary Figure S5b). The single-mode distributions in the ESI mass spectra (Figure 6d and Supplementary Figure S5c and d) under the same pH range are interpreted as representing unfolded proteins, whereas the bimodal distribution (Figure 6c) indicates the coexistence of folded and unfolded protein. It is worth noting that intact CypA–CsA noncovalent complex ions can be observed in the CSI mass spectrum under nearly physiological conditions (pH 7.0). Figure 6e shows that native CypA interacted selectively with CsA at +8, +9, and +10 charged states. However, the intact folded CypA–CsA complex ion was absent in the ESI mass spectrum (Figure 6f). These results suggest that the CSI-MS method can provide specific binding information for the CypA–CsA complex and can be used to search for ligand candidates with a specific affinity for a target protein.

The results reported in this study demonstrate the advantage of the CSI-MS method in characterizing

Figure 6. CSI (a, b) and ESI (c, d) mass spectra for CypA at pH 7.0 and 4.3. CSI (e) and ESI mass spectrum (f) of CypA in a mixture with CsA at pH 7.0 (1:1 molecular ratio at concentration level of 100 μ M). 8⁺, 9⁺, and 10⁺ indicate [MCypA + 2H + 6Na]⁸⁺, [MCypA + 3H + 6Na]⁹⁺, and [MCypA + 4H + 6Na]¹⁰⁺, respectively; D²¹⁺ indicates [2MCypA + 21H]²¹⁺.

native protein conformation and noncovalent complexes. Notably, CSI ionization has the capability of preventing heat-induced denaturation and acid-induced conformational transitions at low pH values. On the basis of the fact that CSI enables the precise characterization of labile organic species and noncovalent interactions of biomolecules in solution and affords multiply charged molecular ions attached with multiple solvent molecules, it is believed that CSI possesses an unusual ionization mechanism [42, 43]. It is supposed that in addition to providing gentle ionization conditions the CSI ionization process results in the formation of droplets that form cluster ions similarly to an aerosol and that this, in turn, preserves the solution structures.

Conclusions

We compared the performance of CSI, ESI, and nanoESI for characterization of acid-induced protein conformational changes and noncovalent complexes. The results demonstrated that the CSI technique is not merely a low-temperature ESI because it uses a unique process of ion formation and ion transformation. CSI has clear advantages over conventional ESI and nanoESI in the characterization of acid-induced conformational transitions. The noncovalent complexes of holomyoglobin and the protein–ligand complex between CypA and CsA were also characterized using CSI-MS at a neutral pH. The CSI-MS data are in good agreement with solution phase results from the literature. We found that the CSI method is a reliable and facile analytical method that can directly characterize different conformational states for proteins, which are not easily separated by spectroscopic and biophysical methods. CSI-MS may become one of the promising techniques for investigating native protein conformation and noncovalent protein–ligand interactions.

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Appendix A. Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2008.12.024](https://doi.org/10.1016/j.jasms.2008.12.024).

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