Observation of Water Molecules Bound to a Protein Using Cold-Spray Ionization Mass Spectrometry

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The characterization of water molecules bound to ribonuclease T₁ (RNase T₁) was carried out using cold-spray ionization mass spectrometry (CSI-MS). CSI-MS is a variant of electrospray ionization mass spectrometry (ESI-MS) operating at low temperature, and is particularly suitable for investigating the weaker molecular associations, since the temperature at the spray interface is much lower than that in the conventional ESI-MS. In this approach, ion peaks due to the addition of nine water molecules were identified at a spray temperature of 48°C. This result showed good agreement with that inferred by the combinational analysis of NMR and X-ray crystallography, indicating that CSI-MS is capable of rapidly providing reliable information to characterize the number of water molecules bound to a macromolecule.

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Introduction

Internal and ordered surface water molecules of biological molecules often play significant roles in stabilizing through the formation of bridging hydrogen-bonds in protein structures and in enzymatic mechanisms.^{1,2} Much effort has been made concerning the kinetic and structural characterization of these water molecules. Although large numbers of water molecules are often identified in high-resolution X-ray crystal structures of proteins and nucleic acids,^{3,4} it is not possible to determine by X-ray crystallography whether these water molecules arise from the crystal packing forces, or are intrinsic components of the macromolecules. Recent NMR methods to study the location of macromolecular bound water molecules in solution have taken advantage of the selective excitation of the water resonance; 2D ROESY-HMQC and ROESY-HSQC have been presented for ¹⁵N- or ¹³C/¹⁵N-enriched protein.⁵⁻⁸ However, these NMR methods are available only for proteins that can be largely expressed by using a recombinant system of isotopical enrichment. Herein, we present the characterization of macromolecular bound water molecules using cold-spray ionization (CSI)^{9,10} mass spectrometry, a variant of electrospray ionization¹¹⁻¹³ mass spectrometry (ESI-MS) operating at low temperature. Although ESI-MS and other mass spectrometry have been used for the detection of noncovalent macromolecular associations¹⁴⁻¹⁶ and the hydration of peptides

and proteins,¹⁷⁻¹⁹ it has been shown that CSI-MS is more sensitive for observing weaker associations among proteins, since the temperature at the spray interface is much lower. The efficiency of this approach was demonstrated in a study of the water molecules bound to ribonuclease T₁ (RNase T₁). RNase T₁ has been a target of extensive biochemical and biophysical studies, and several approaches have been used to study water molecules bound to RNase T₁, including, protein engineering, X-ray crystallography and NMR.^{20,21} Taking advantage of past studies concerning RNase T₁, the reliability of the approach in a study of bound water molecules using CSI-MS was investigated.

Experimental

Expression and purification of RNase T₁

Recombinant RNase T₁ was expressed in *E. coli* strain HMS174(DE3) grown at 37°C in M9 medium using the expression vector pET25b (Novagen). Overexpression of RNase T₁ was induced at an OD₆₀₀ of 0.5 by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. The fermentor was then thermostated to 30°C for an additional 14–16 h. The cells were harvested by centrifugation. The recombinant RNase T₁ was released from the periplasmic space by an osmotic shock treatment, and purified using a Sephadex-75 column (1.6 × 60 cm) (Amersham Biosciences).

CSI-MS

CSI-MS measurements were performed using a time-of-flight

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Fig. 1 CSI-MS spectra of RNase T₁ with 10 mM of NH₄OAc (pH 7.5) at spray temperatures of (a) 48 and (b) 115 °C.

mass spectrometer (JEOL JMS-T100) equipped with an orthogonal CSI source. An orthogonal cold-spray apparatus equipped with a spray- and ion-source temperature control system using liquid N₂ was used. A thermocouple was equipped at the inlet of the extraction orifice, which is considered to be an ion-generating point in this orthogonal cold-spray instrument. The temperatures at this point were defined as the spray temperature. A schematic diagram of the cold spray ionization source was described previously.¹⁰ The measurement conditions and the sample-preparation procedures were as follows: acceleration voltage, 2.0 kV; needle voltage, 1.4 kV; orifice voltage, 193 V; resolution, 6000; spray temperature, 48 – 115 °C; sample flow rate, 8.5 µL/min; 0.1 mM RNase T₁ dissolved in 10 mM NH₄OAc (pH 7.5). The spectra were obtained *via* the infusion of RNase T₁ through the cold-spray interface in the positive ionization mode.

Results and Discussion

The CSI mass spectra of RNase T₁ at spray temperatures of 48 and 115 °C are shown in Fig. 1. Under these conditions, RNase T₁ generated envelopes of ions between *m/z* 1500 and 3000, corresponding to 7+ through 4+ charge states. Expansions of the 6+ ion peaks measured at spray temperatures of 48 and 115 °C are shown in Fig. 2. Most of the ion peaks were observed as sodium adducts. Interestingly, two envelopes of ions, which were more evident at a higher spray temperature of 115 °C, were observed. At a spray temperature of 115 °C, one of the envelopes of ions between *m/z* 1855 and 1870 at the 6+ charge state corresponded to RNase T₁ bound with one to two water molecules. The other envelope between *m/z* 1873 and 1893 corresponded to seven to eight water molecules, and peaks due to the addition of up to eight water molecules were clearly identified (Fig. 2b). At a spray temperature of 48 °C, one of the envelopes of ions between *m/z* 1855 and 1873 corresponded to RNase T₁ bound to two water molecules. This observation was slightly different from that at a spray temperature of 115 °C. Peaks due to the addition of just one water molecule were present only at a high spray temperature, which is considered to be the result of desolvation. The other envelope between *m/z* 1873 and 1908 corresponds to seven to nine water molecules (Fig. 2a). A measurement at the lower spray temperature of 48 °C could detect one additional bound water molecule.

Solvation in the CSI-MS measurement was investigated for a small ionic molecule, cesium iodide (CsI), in an acetonitrile solution.⁹ The molecular ions of Cs⁺ and [Cs+CH₃CN]⁺ formed

Fig. 2 Expansions of the 6+ ion peak of RNase T₁ measured at spray temperatures of (a) 48 and (b) 115 °C. The number of water molecules correspond to [RNase T₁ + *m*Na + (6–*m*)H + (H₂O)_{*n*}]⁶⁺. The numbers of sodium atom and water molecules are indicated at each ion peak. Peaks due to the addition of water molecules are apparent.

by heat and solvation, respectively, were almost equally observed in the ESI-MS measurement, while the molecular ion of [Cs+CH₃CN]⁺ was dominantly observed in the CSI-MS measurement. The solvation of adamantanoid type Pt(II) complexes was also compared in the ESI-MS and CSI-MS measurements.⁹ Multiple charged molecular ions of [M–(PF₆)_{*n*} + (CH₃CN)_{*m*}]^{*n*+} (*n* = 3 – 10, *m* = 0 – 21) (M; monomeric molecule) were clearly observed without decomposition only in the CSI-MS measurement. Considering the above results, multiply charged molecular ions of a protein with bound waters are highly expected to be observed in the CSI-MS, which uses new ion sources operating at low temperature.

The hydration of RNase T₁ was fairly investigated by NMR using ¹³C/¹⁵N-enriched proteins.²⁰ In this research, 2D heteronuclear NOESY-HSQC and ROESY-HSQC experiments were performed with 2.0 mM RNase T₁ in 92% H₂O/8% D₂O at pH 5.5 to determine the spatial proximity of water molecules; the backbone carbonyl oxygens involved in the intermolecular hydrogen bonds to water molecules were identified based on the ¹J_{NC} coupling constants. Ten amide protons and five aliphatic proton groups cross-relaxing with bound water protons were identified. An examination of the high-resolution (1.5 Å) crystal structure of RNase T₁²² revealed that all of these protein protons donate hydrogen bonds, or are in close proximity (~3.5 Å) to twelve water molecules that must reside for at least several hundred picoseconds, indicating that these amide/aliphatic protons are highly correlated with the bound water molecules. Besides these twelve bound water molecules, twenty-nine water molecules were classified as ambiguous bound waters, since the possibility of ROE (rotating frame NOE) interactions between protein protons and rapidly exchanging protons could not be excluded. The numbers of water molecules obtained by CSI-MS measurements were nine and eight at spray temperatures of 48 and 115 °C, respectively. Considering the difference of the sample solutions used in NMR and CSI-MS measurements, the results of CSI-MS are in good agreement with that inferred by the combinational analysis of NMR and X-ray crystallography.

Although the location of water molecules at the binding sites cannot be determined by mass spectrometry, it would be worth

noting the potential bound water molecules of RNase T₁ observed in the CSI-MS measurements. In a combinational analysis of NMR and X-ray crystallography, twelve water molecules were classified as bound water molecules.²⁰ Six of twelve water molecules (W106, W107, W108, W110, W135, W166) are positioned in close proximity to the hairpin-like loop 3 (L3) comprising residues 62 to 76.²² Two strands of the loop are connected together by a chain formation of water molecules (W107, W108 and W110) with low-temperature factors, anchored at Trp59(N^εH). Two of these (W107 and W108) are considered to bind tightly owing to their multiple contacts to the protein. Water molecule W107 accepts two hydrogen bonds from Trp59(N^εH) and Tyr68(NH), and donates a hydrogen bond to Pro60(O). W108 accepts a hydrogen bond from Leu62(NH) and donates it to Asp66(O). Two bound water molecules identified in the envelope of ions between *m/z* 1855 and 1873 (Fig. 2a) could correspond to W107 and W108.

Since the detection of water molecules by NMR depends on observing the correlation between protons of protein and those of water molecules, complete assignments of the backbone and the side-chain are inevitable. Any ambiguity of the assignments or an overlapping of the cross peaks in the NMR spectra could lead to a misinterpretation, which would make a detailed analysis significantly difficult for larger proteins and nucleic acids. The present results indicate that CSI-MS is capable of rapidly providing reliable information to characterize the number of water molecules bound to a macromolecule. Although the location of hydration sites cannot be determined by CSI-MS, this method is highly amenable to the quick interpretation of bound water molecules associated to proteins of which the expression level and solubility are low, and to a better understand the functional role of water in macromolecules. MS measurements at low solution temperature will be favored for observing biomolecules under physiological conditions.

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