Visualizing Protein "Functional Structures" in Aqueous Solutions via Mass Spectrometry

Toshifumi Takao*,**

Abstract

To fully understand the functional structures of proteins, it is crucial to investigate their states under physiological conditions, which are often difficult to capture using conventional X-ray crystallography or cryo-electron microscopy (cryo-EM). This article introduces two examples (SOD1 and RNase H) in which electrospray ionization mass spectrometry (ESI-MS) was utilized to analyze the reversible formation and structural states of protein-metal ion-substrate complexes in aqueous solutions. Although challenges remain regarding instrumental constraints and the preservation of weakly bound noncovalent complexes in the gas phase, ESI-MS has emerged as a powerful tool capable of visualizing in-solution structural states and binding modes at the molecular level, potentially playing a vital complementary role in the future of structural biology.

1. Introduction

How much of a protein's "structure" are we truly seeing, and what might we be missing? Recent advances in structural analysis technologies, such as X-ray crystallography and cryo-EM, have revealed numerous high-resolution structures. These findings have contributed significantly to our understanding of the functions of proteins and their complexes. However, the static images obtained through these methods do not always reflect the actual "functioning state" of the proteins.

Proteins functioning in vivo are thought to dynamically alter their structures in response to post-translational modifications, the binding of metal ions or ligands, and changes in the surrounding environment. In other words, under physiological conditions, proteins undergo reversible and transient structural changes in solution. Such structural "fluctuations" and "intermediate states" are difficult to capture with conventional crystallography or electron microscopy, representing a true "blind spot" in structural analysis.

While solution NMR is a powerful technique that offers high spatial resolution and dynamic information, it faces technical limitations when applied to large macromolecular complexes or low-concentration samples. Fluorescence microscopy and similar techniques can visualize molecular behavior in environments closely resembling in vivo conditions; however, they typically require the attachment of bulky fluorophores, which can sometimes introduce structural artifacts and are less suited for obtaining high-resolution structural details.

In this context, electrospray ionization mass spectrometry (ESI-MS) has garnered attention as a method capable of detecting molecules in their "native states" within aqueous solutions. By utilizing the physical quantity of molecular mass, ESI-MS can indirectly visualize structural states and complex formation, offering great potential to complement other structural analysis methods.

This note introduces two case studies (SOD1 and ribonuclease H) where reversible complexes formed between proteins, metal ions, and substrates were observed in aqueous solutions using ESI-MS, and discusses the new possibilities and limitations of this structural analysis approach.

2. ESI-MS Method

Mass spectrometry utilizes different types of analyzers depending on the analytical objective. When measuring noncovalent complexes such as protein-ligand interactions, the sample dissolved in an aqueous solution (like a buffer) is directly introduced into the ion source via ESI (Fig. 1). In ESI, the sample solution is sprayed

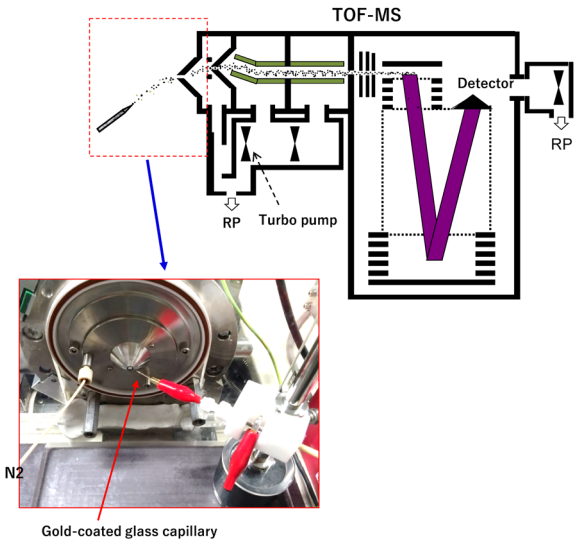

Fig. 1. Electrospray ion source and time-of-flight mass spectrometer.

* Life Science Product Division, Rigaku Corporation.
** Institute for Protein Research, Osaka University.

as microscopic droplets of a few nanoliters or less.
Desolvation of the generated droplets proceeds under the heated and reduced-pressure conditions within the ion source, ultimately ionizing the intact complex particles for mass analysis. Therefore, the volume of the sample solution introduced into the ion source is strictly limited, and the so-called nano-electrospray (nanoESI) technique is generally employed.
On the other hand, under these conditions, ions travel at near the speed of sound and undergo numerous collisions with gas molecules. Consequently, the stability of the complex becomes a critical parameter determining the success of the measurement. Specifically, weakly bound complexes or those encompassing numerous solvent molecules (like water) are prone to dissociate during the measurement process, often making analysis difficult.
The analyzer shown in Fig. 1 is a time-of-flight (TOF) mass spectrometer. Continuously introduced ions are orthogonally accelerated and deflected by a pulse voltage at the top of the analyzer, and their mass is determined by measuring the flight time to the detector. Generally, high-resolution analyzers are advantageous for measuring high-mass ions like complexes. However, instruments with long ion residence times or those employing complex trajectory controls may cause the dissociation of complexes during analysis.
Furthermore, while it is necessary to use samples prepared in buffer solutions to maintain the structure of the complex, ESI-MS fundamentally requires the use of volatile aqueous buffers.
Despite these constraints, ESI-MS remains a highly effective method for identifying the constituents of complexes present in aqueous solutions and directly evaluating their stoichiometry.

3. Metal Ion Binding and Enzyme Activity in Cu,Zn-Superoxide Dismutase (SOD1)

Cu,Zn-superoxide dismutase (SOD1) is a crucial enzyme that eliminates reactive oxygen species *in vivo*, forming a stable noncovalent homodimer containing two different metal ions per subunit[1]. While many enzymes utilize metal ions to express activity, in SOD1 the zinc (Zn) ion plays a key role in stabilizing the structure, whereas the copper (Cu) ion is essential for catalytic activity.
We hypothesized that measuring this enzyme via ESI-MS could clarify the following points: 1) Can we identify the type and number of bound metal ions? 2) Is it observed as a homodimer? 3) Is the metal ion selectivity an inherent property of the protein itself?
Positive results were obtained for both points 1 and 2. The type of metal ions can be clearly distinguished from mass differences, and whether the species is a dimer or monomer can be identified from the charge states of the observed multiply charged ions (Fig. 2).
The most intriguing results were obtained regarding point 3. First, various divalent metal ions were mixed with metal-depleted apo-SOD1 at molar ratios ranging

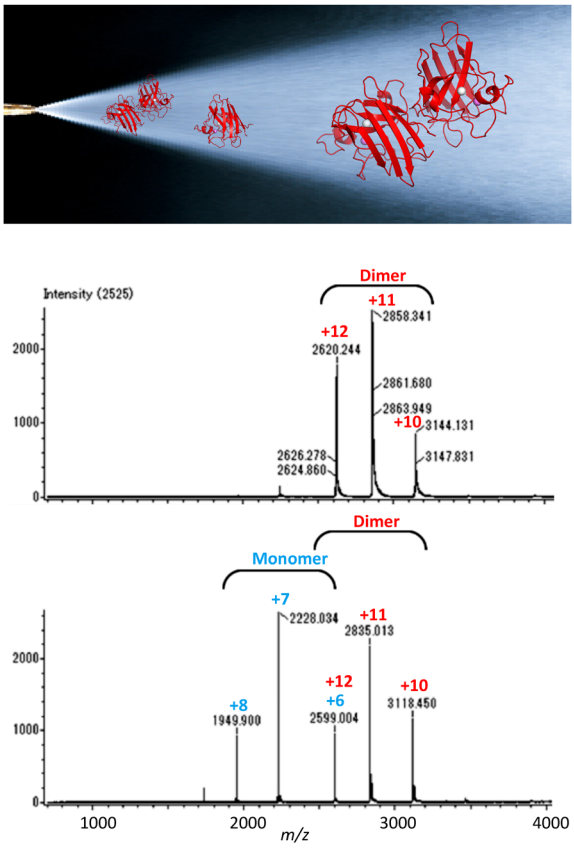

Fig. 2. ESI-MS of Bovine SOD1[2]. 1 μL of SOD1 aqueous solution (5 pmol/μL, 10 mM ammonium acetate) was introduced into the nanoESI tip shown in Fig. 1 for measurement. The Cu_2^{2+},Zn_2^{2+}-SOD1 homodimer is observed as the main component (middle panel). Increasing the potential difference between Orifice 1 and 2 (Fig. 1) promotes collisions with gas molecules, causing the dimer to partially dissociate and the monomer to be observed (lower panel). The upper panel shows an illustration of the SOD1 dimer (PDB ID: 2SOD) and monomer being sprayed by electrospray.

from 1 : 2 to 1 : 12 to investigate binding specificity (for detailed results, see Ref. (2)). Furthermore, we verified whether Cu,Zn-SOD1 would correctly reconstitute when Zn^{2+} and Cu^{2+} were added simultaneously (Fig. 3).
The results showed that the enzymatic activity of SOD1 reconstituted by simultaneously adding Zn^{2+} and Cu^{2+} remained at about 80% of the native form. ESI-MS analysis of this sample revealed that a Cu_2-form (bound only to Cu) and a Cu,Zn-form were generated in an approximately 1 : 1 ratio (Fig. 3, upper right).
Separate experiments have shown that a state where all sites are occupied by Zn^{2+} is difficult to generate unless Zn^{2+} is added in large excess. This is likely because the Zn-binding sites of SOD1 are easily occupied by Cu^{2+}, whereas Zn^{2+} does not easily coordinate to the Cu-binding sites. Interestingly, when both ions are added simultaneously, the Cu_4-form and Cu_2Zn_2-form are generated to a similar extent immediately after mixing. However, when the solution is left to stand for a while, a new Cu_3Zn-form emerges,

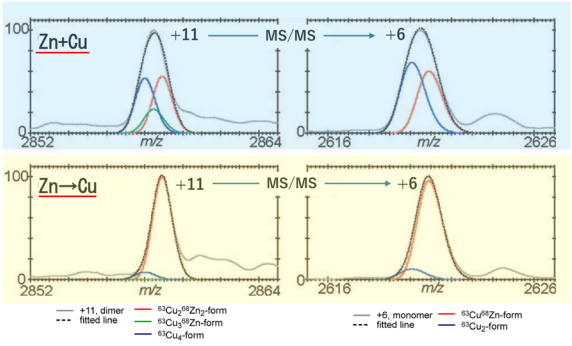

Fig. 3. Reconstitution experiment of SOD1. Zn^{2+} and Cu^{2+} were added simultaneously to apo-SOD1 (upper row), or Zn^{2+} was added first followed by incubation and subsequent addition of Cu^{2+} (lower row). To clearly distinguish Zn (average mass 65.4) and Cu (63.5), the stable isotopes ^{68}Zn and ^{63}Cu were used, respectively. The observed peaks for the three components—blue: $^{63}Cu_4$-form, green: $^{63}Cu_3{}^{68}Zn$-form, and red: $^{63}Cu_2{}^{68}Zn_2$-form—were deconvoluted using “Isotopica”[3, 4]. Note that the Zn_4-form was not included in the analysis as it has already been confirmed that it is rarely generated under these conditions. Adapted with permission from Ref. (2). Copyright 2008 American Chemical Society.

ultimately resulting in an approximate distribution ratio of $Cu_4 : Cu_3Zn : Cu_2Zn_2 \approx 2 : 1 : 2$ (Fig. 3, upper left). This result suggests that while two types of subunits with different metal-binding states (Cu_2-form and CuZn-form) preferentially form homodimers in the initial stage, they undergo subunit exchange (shuffling) between dimers in aqueous solution over time.

Conversely, when Zn^{2+} was added first, followed by the introduction of Cu^{2+} for reconstitution, nearly native Cu,Zn-SOD1 was generated (Fig. 3, lower row). These results strongly suggest that SOD1 acquires its correct metal configuration *in vivo* through the stepwise introduction of metal ions. The observation that SOD1 prepared by simultaneous addition of Zn^{2+} and Cu^{2+} exhibited only ~80% activity is presumed to be due to the partial formation of Cu_4 and Cu_3 forms, which possess lower activity compared to the native form.

By directly detecting dimers with multiple metal-binding states in solution while maintaining their noncovalent interactions, ESI-MS successfully visualized the process of metal selectivity and structural reconstitution in SOD1, providing insights unattainable by other structural analysis methods.

4. Observation of the Ternary Complex of Ribonuclease H (RNase H)/Metal Ion/Substrate

Enzymes are central molecules in biological reactions, and numerous structural studies have been conducted on them to date. Recently, attempts to capture parts of the reaction process using time-resolved structural analysis with high-brilliance X-rays have advanced. While understanding the catalytic reaction mechanism is particularly important, substrate complex structures have often been analyzed under conditions where the enzyme is inactivated, making it difficult to observe the native enzyme-substrate complex directly.

To address the ongoing debate regarding the actual types and numbers of metal ions under physiological conditions, we probed the functional state of Ribonuclease H (RNase H), an RNA-cleaving enzyme, using ESI-MS (Fig. 4).

RNase H is an enzyme that cleaves the RNA strand in a complementarily bound DNA/RNA hybrid and is widely conserved from microorganisms to higher organisms. The reaction strictly requires Mg^{2+} or Mn^{2+}, and it is thought to proceed via the formation of a ternary complex consisting of the enzyme, substrate, and metal ions, followed by rapid dissociation of the complex after cleavage. However, capturing this ternary complex in its intact state has historically been challenging.

We hypothesized that ESI-MS could directly identify which metal ions and how many bind to RNase H in aqueous solution. Thus, we attempted to detect the ternary complex in the presence of various divalent metal ions. Figure 5 shows the ESI-MS results of a binary complex consisting of RNase H and a substrate DNA (8-mer)/RNA (8-mer) hybrid in a buffer at pH 6.0. A highly stable complex was observed as multiply charged ions ranging from +7 to +9.

Next, Mn^{2+} was added to this solution at 4 molar equivalents relative to the enzyme, and measurements were taken. However, when the sample was introduced into the nanoESI tip using standard procedures after mixing, predominantly only the free enzyme protein was observed. This was attributed to the fact that it typically takes several minutes from sample mixing to the start of the measurement, during which time the enzymatic

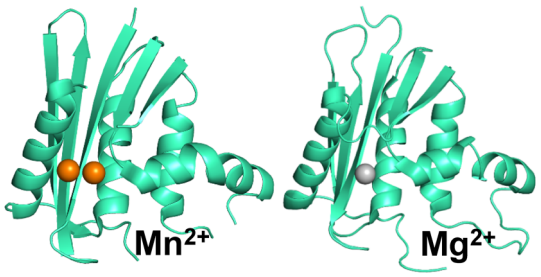

Fig. 4. Crystal structures of *E. coli* RNase H containing divalent metal ions. Left: Structure containing two Mn^{2+} ions (PDB ID: 1G15)[5]. Right: Structure containing one Mg^{2+} ion (PDB ID: 1RDD) [6].

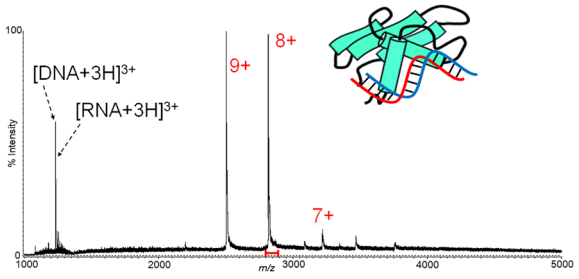

Fig. 5. ESI-MS observation of a binary complex consisting of RNase H and a DNA/RNA 8-mer.

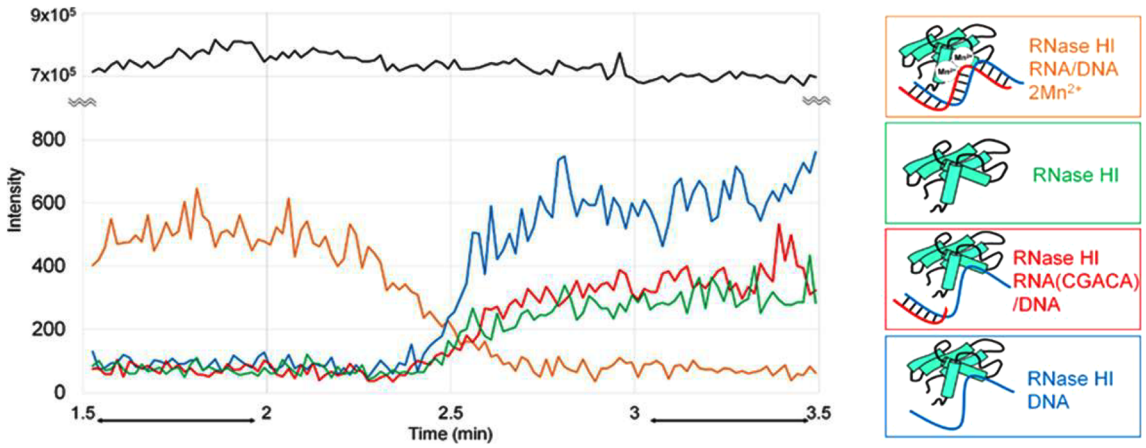

Fig. 6. ESI-MS observation of the RNase H ternary complex after the addition of Mn^{2+} [7]. The reaction solution was measured 1.5–3.5 minutes after mixing the enzyme, substrate (DNA-8 mer/RNA-8 mer), and Mn^{2+}. As the reaction proceeds, the RNA is cleaved (RNA-8 mer→RNA-6 mer), and the dissociation of Mn^{2+} is observed. Orange: Enzyme-DNA/RNA-Mn^{2+} ternary complex; Green: Free enzyme; Red: Enzyme-DNA/RNA-6 mer complex; Blue: Enzyme-DNA complex. The black line represents the total ion current.

reaction had already proceeded to completion.

To begin measurements as quickly as possible after initiating the reaction, we custom-built a movable mount that can fix a glass capillary nanoESI tip (gold-coated at the tip for applying 1–2 kV) at an arbitrary angle and distance relative to the ion inlet (Orifice 1) (Fig. 1). This device enabled us to start measurements approximately 1.5 minutes after sample mixing.

Figure 6 shows the results of continuous ESI-MS monitoring of the reaction solution 1.5 to 3.5 minutes after initiation. The black trace represents the total ion current, indicating stable ionization during the measurement. At the start of the measurement, a ternary complex comprising the enzyme, substrate, and Mn^{2+} was observed as the main component (orange trace, upper right model). Its molecular mass revealed that two Mn^{2+} ions transiently bind to the active complex.

It should be noted that in the cytoplasm of *E. coli* (the source of the RNase H used here), given the relatively high physiological concentration of Mg^{2+}, Mg^{2+} is widely considered the primary natural cofactor. However, due to its moderate affinity for the enzyme and extremely fast reaction turnover, directly capturing the dynamic Mg^{2+}-bound complex in solution remains analytically challenging. Mn^{2+}, which shares similar coordination properties and is often utilized in structural studies to capture functional states, serves as an excellent in vitro surrogate in this context. By utilizing Mn^{2+}, we were able to sufficiently stabilize and visualize the transient structural state of the “two-metal-ion catalytic mechanism”[5], providing direct in-solution evidence that strongly complements previous structural findings.

Looking at the time-course changes in the above measurements, interestingly, as the reaction progresses, the signal of the ternary complex decreases. Concurrently, ions corresponding to the free enzyme (green), the enzyme/DNA-8 mer/RNA-6 mer complex (red), and the enzyme/DNA-8 mer complex (blue) increase. In other words, the results demonstrated that upon cleavage of the RNA strand, the ternary complex dissociates and transitions into the free enzyme and binary complexes. Furthermore, none of these products retained Mn^{2+}, suggesting that the divalent metal ions bind transiently during the reaction process and undergo turnover.

By directly capturing the formation and dissociation of transient ternary complexes during the enzymatic reaction process in aqueous solution using the “ruler” of molecular mass, ESI-MS provided dynamic information inaccessible through conventional structural analysis methods.

5. Conclusion

The two enzymes introduced in this article both suggested that dynamic and reversible intermolecular interactions play crucial roles during their functional processes under physiological conditions. Such transient complexes and reaction intermediates are difficult to capture with static structural analysis methods like crystallography or electron microscopy, reaffirming the necessity of interrogating structural states under conditions that closely mimic physiological environments.

ESI-MS serves as a complementary method providing this information, as it can directly observe complexes existing in solution using molecular mass as a clear indicator. In particular, the ability to quantitatively capture selective metal ion binding states and the evolving nature of complexes as a reaction progresses is a unique feature of ESI-MS.

In the future, with improvements in measurement systems and refinement of ionization conditions, the capability to analyze increasingly complex intermolecular interactions and real-time reaction dynamics under near-physiological conditions will undoubtedly expand. To understand protein functions from a structural perspective,

incorporating a “time axis” into the analysis will undoubtedly become increasingly important.

References

(1) J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson: *J. Mol. Biol.*, **160** (1982), 181–217.

(2) Y. Yamazaki and T. Takao: *Anal. Chem.*, **80** (2008), 8246–8252.

(3) J. Fernández-de-Cossio, L.J. Gonzalez, Y. Satomi, L. Betancourt, Y. Ramos, V. Huerta, V. Besada, G. Pad ron, N. Minamino, and T. Takao: *Rapid Commun. Mass Spectrom.*, **18** (2004), 2465–2472.

(4) J. Fernandez-de-Cossio, L. J. Gonzalez, Y. Satomi, L. Betancourt, Y. Ramos, V. Huerta, A. Amaro, V. Besa da, G. Padron, N. Minamino, and T. Takao: *Nucleic Acids Res.*, **32** (2004), 674–678.

(5) W. Yang, W. A. Hendrickson, R. J. Crouch, and Y. Satow: *Science*, **249** (1990), 1398–1405.

(6) K. Katayanagi, M. Miyagawa, M. Matsushima, M. Ishikawa, S. Kanaya, M. Ikehara, T. Matsuzaki, and K. Morikawa: *Nature*, **347** (1990), 306–310.

(7) T. Ando, N. Jongruja, N. Okumura, K. Morikawa, S. Kanaya, and T. Takao: *J. Biol. Chem.*, **296** (2021), doi: 10.1016/j.jbc.2021.100462