International Journal of Mass Spectrometry 354–355 (2013) 288–291

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Native electrospray ionization and electron-capture dissociation for comparison of protein structure in solution and the gas phase

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ARTICLE INFO

Article history:
Received 30 April 2013
Received in revised form 19 June 2013
Accepted 24 June 2013
Available online 3 July 2013

Keywords:
Protein structure
Gas-phase vs. solution structure
Native ESI
Electron-capture dissociation (ECD)
Top-down mass spectrometry

ABSTRACT

The importance of protein and protein-complex structure motivates improvements in speed and sensitivity of structure determination in the gas phase and comparison with that in solution or solid state. An opportunity for the gas-phase measurement is mass spectrometry (MS) combined with native electrospray ionization (ESI), which delivers large proteins and protein complexes in their near-native states to the gas phase. In this communication, we describe the combination of native ESI, electron-capture dissociation (ECD), and top-down MS for exploring the structures of ubiquitin and cytochrome *c* in the gas phase and their relation to those in the solid-state and solution. We probe structure by comparing the protein's flexible regions, as predicted by the B-factor in X-ray crystallography, with the ECD fragments. The underlying hypothesis is that maintenance of structure gives fragments that can be predicted from B-factors. This strategy may be applicable in general when X-ray structures are available and extendable to the study of intrinsically disordered proteins.

1. Introduction

Mass spectrometry (MS) has undergone dramatic evolution in two decades ranging from routine analysis of small molecules to challenging measurements of mega-dalton protein assemblies [1,2]. To keep large protein assemblies or complexes nearly intact, native ESI can be employed to introduce them to the gas phase. This is done by spraying the protein from an aqueous solution of ammonium acetate or other volatile salts [3]. Native ESI provides a quick and relatively simple approach for measuring protein assemblies or protein–ligand complexes [4] and determining their stoichiometry and topology [5]. Thus, MS-based protein characterization is now being extended from single proteins to large protein complexes. Some recent review articles provide appropriate references for native ESI [1,6–9].

ECD, as a new dissociation approach, was introduced by the McLafferty group [10,11] in 1998 to fragment intact proteins in top-down sequencing [12]. The electrons used in the ECD experiment can also be viewed as a probe for protein structure in the gas phase. The positively charged residues (e.g., K and R) serve as focal points or destinations for the electrons. Thus, fragment ions from a native structure should be principally from the surface of the protein and serve as readout for locating those surface regions. The McLafferty group [13] first demonstrated this ECD application in structural studies of unfolded proteins in the gas phase. Although many previous studies [14–16] used organic or acidic solutions in their ESI experiment to increase the charging and improve signal intensity to obtain excellent coverage and intermediate state information, we are using native spray to maintain the media as close as possible to the native environment.

We expect that native ESI and ECD top-down MS will now evolve into an approach in structural MS [17]. Although a deficit of native spray is that the limited charging makes fragment generation difficult, as illustrated by early experiments [18], we recently found that top-down ECD of protein assemblies introduced by native ESI is effective and produces results quite different than those from collisional activation [19]. For example, the tetrameric yeast alcohol dehydrogenase (ADH) complex, when introduced to the gas phase by native ESI and submitted to ECD, affords small *m*/*z* fragment ions in addition to the expected charge-reduced ions. These small fragments reveal subunit sequence and post-translational modifications (PTMs) [20].

More importantly, the cleavage sites for their formation correlate with the B-factor from X-ray crystallography. The B-factor is a parameter that represents atomic displacement and is large for protein regions having flexibility and high dynamics [21,22]. We hypothesized that those regions of a protein that have large B-factors should fragment readily by ECD *provided solid-state structure is maintained in the gas phase*. We previously investigated three large protein complexes, ADH complex (147 kDa), concanavalin A (ConA, 103 kDa) and photosynthetic Fenna–Matthews–Olson antenna protein complex (FMO, 140 kDa), as was reported

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http://dx.doi.org/10.1016/j.ijms.2013.06.019

previously [20]. ECD fragmentation occurs in flexible regions (high B-factors) of the complexes: N-terminus for ADH and ConA and the loop close to C-terminus for FMO. The data show that top-down ECD can probe the near native state of large protein complexes. If the gas-phase protein finds a new conformation to expose new flexible regions, the top-down ECD will show fragmentation that does *not* correlate with B-factors.

Protein complexes in the gas phase seem to retain major structural features seen in the crystal form for at least the timescale of the ECD experiment. Ultimately, as McLafferty and coworkers [23] pointed out, gas-phase proteins collapse into new structures via several unfolding and folding steps. To enable further testing of this hypothesis, we introduce in this communication another structural approach that employs correlations between fragment cleavage sites and B-factors. We apply the approach to ubiquitin (8.6 kDa) and cytochrome *c* (12 kDa) and test whether it can be used to compare gas-phase and solution-phase structures.

2. Experimental

2.1. Chemicals, proteins and sample preparation

Ammonium acetate, water, ubiquitin (bovine 8.6 kDa), cytochrome *c* (horse, 12 kDa) were purchased from Sigma–Aldrich (St. Louis, MO). Lyophilized protein powders were dissolved in 100 mM ammonium acetate (pH = 6.5–7) to afford a final concentration of 1.0 μM.

2.2. Native ESI and top-down MS

The protein sample was delivered by syringe pump (Harvard PHD Ultra syringe pump, Instech Laboratories, Inc., Plymouth Meeting, PA) at flow rate 5–300 nL/min to a nano spray source, which was comprised of a custom-pulled nano spray tip (Sutter Instrument Co., Novato, CA) of silica capillary tubing (360 μm od, 150 μm id, Polymicro Technologies, Phoenix, AZ). Mass spectra were acquired with a Bruker Solarix 12 T FTICR mass spectrometer (Bruker Daltonics, Bremen, Germany). The detailed front-end instrument parameters were previously reported [20,24].

Mass spectra were recorded at 60,000 mass resolving power (at $m/z = 1000$) with ion accumulation times of 0.001 s in source, 0.5 s in the collision/accumulation cell for full spectra, and 2 s in the collision/accumulation cell prior to ECD of isolated ions. The time-of-flight was ~1.2 ms for the protein ions. The trapping voltages of the infinity ICR cell were 0.7 V (front plate) and 0.8 V (back plate). Excitation was 25% full power. Electrons were generated by passing 1.6 A through the hollow-cathode dispenser, with 10 V on the grid, −0.6 V for electron extraction, 60 ms of extraction time, as well as slight adjustments of the latter two to optimize the ECD fragmentation of each protein. Up to 200 scans were averaged with 1 M data points for each scan. External calibration was done by using the ECD fragments from all the charge states of ubiquitin.

2.3. Data analysis

Peak picking and spectra deconvolution were performed with Bruker Data Analysis software (Bruker Daltonics, Bremen, Germany) and checked manually. Bruker Biotools software was used for mapping the measured peak mass list with calculated fragment mass list from the protein sequences.

3. Results and discussion

Nano native ESI of ubiquitin from 100 mM ammonium-acetate aqueous solution produces mainly 6^+ and 5^+ charge states (Fig. 1 top). The narrow charge-state distribution with relatively fewer protons compared to a conventional ESI spectrum is consistent with the folded ubiquitin structure [25]. Previously, McLafferty and coworkers [13,25] reported that only the 6^+ to 9^+ ubiquitin ions show tertiary noncovalent bonding. Bowers and coworkers [14] also measured the native-structure size of this protein on the 0.1 s time scale by using ion mobility-mass spectrometry (IM-MS). These results show that folded ubiquitin can be transferred to the gas phase. To test further whether this folded ubiquitin structure is consistent with the solid-state phase structure, we introduced the protein by native spray and submitted it to the ECD top-down experiment. ECD of these two charge states (6^+ and 5^+) generated both charge-reduced and sequence ions (Fig. 2 bottom). Unlike the result from large protein assemblies [19], the charge-reduced ions are overlapped with sequence ions in the range from m/z 1000 to 2000. Nevertheless, successful assignment of the sequence ions can be achieved (Fig. 2). In the traditional ECD top-down experiment of unfolded ubiquitin (data not shown), full sequence coverage (except for the three proline sites not cleavable by ECD) from both *c* and *z* ions can be obtained. In the native ESI ECD top-down experiment, ECD fragments also arise from cleavages on both termini, indicating that both termini are flexible and available for fragmentation in the gas phase. Both solution NMR (pdb id: 1D3Z) [26] and X-ray crystallography (pdb id: 1UBQ)[27] structural models of ubiquitin show that it has an extremely flexible C-terminus whereas the N-terminus is among the most compact regions. This is reflected by the B-factor scale: the C-terminus has the highest B-factors whereas the N-terminus has the lowest. The N-terminus must be less accessible to electrons in native folded state, which is clearly not the case from our experimental results (Fig. 2).

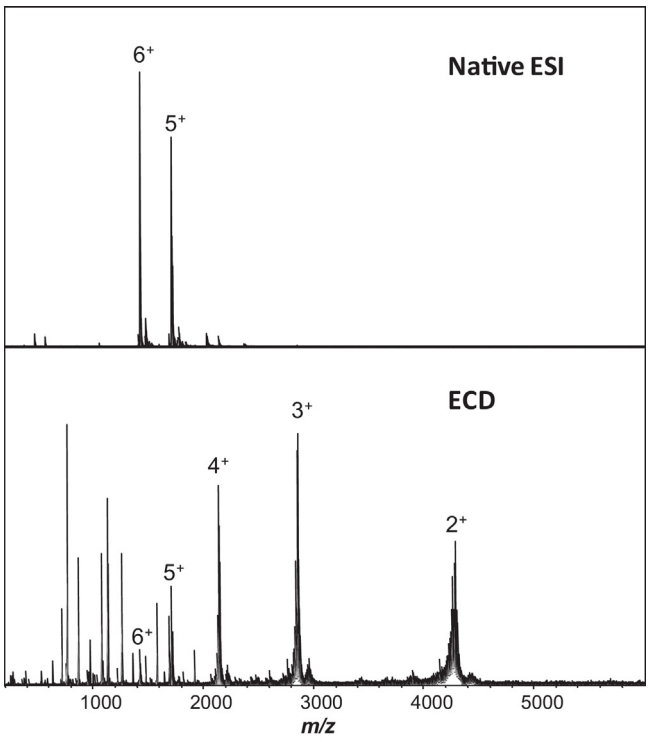

Fig. 1. Native ESI spectrum of ubiquitin (top) and ECD product ion spectrum from both the 6^+ and 5^+ charge states (bottom).

The inconsistency provides evidence that the gas-phase structure of ubiquitin under the conditions of our experiment is *not* the same as in solution. The unfolding of ubiquitin happens during the transfer from solution phase to the gas phase. This result is in agreement with previous studies of gas-phase ubiquitin. Both IM and ECD