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Top-down mass spectrometry of supercharged native protein–ligand complexes

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This article is dedicated to John Fenn, who pioneered the virtues of multiple charging for protein MS analysis. He inspired us "to continue stumbling for a while along the road ahead, kicking over stones here and there, driven by curiosity to find out what may be hidden under the next one [36]." We should all strive to be as curious as John Fenn, as one can't always predict what hidden jewels can be discovered by turning over stones on the road of scientific exploration.

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ABSTRACT

Tandem mass spectrometry (MS/MS) of intact, noncovalently bound protein–ligand complexes can yield structural information on the site of ligand binding. Fourier transform ion cyclotron resonance (FT-ICR) top-down MS of the 29 kDa carbonic anhydrase-zinc complex and adenylate kinase bound to adenosine triphosphate (ATP) with collisionally activated dissociation (CAD) and/or electron capture dissociation (ECD) generates product ions that retain the ligand and their identities are consistent with the solution phase structure. Increasing gas phase protein charging from electrospray ionization (ESI) by the addition of supercharging reagents, such as *m*-nitrobenzyl alcohol and sulfolane, to the protein analyte solution improves the capability of MS/MS to generate holo-product ions. Top-down proteomics for protein sequencing can be enhanced by increasing analyte charging.

1. Introduction

Although most proteomic approaches currently incorporate a "bottom-up" strategy in which proteins are digested into smaller-sized peptides, and protein identifications are derived from the mass spectrometry (MS)-based analysis of the enzymatically cleaved peptides, there is value in the direct measurement and fragmentation of intact proteins, or "top-down" sequencing [1]. The molecular mass of an intact protein defines the native covalent state of a gene's product including the effects of post-transcriptional/translational modifications, and associated heterogeneity that are modulated by the actions of other gene products. Moreover, the fragmentation pattern of large gas phase proteins can generate sufficient information for identification from sequence databases, particularly when combined with accurate mass measurements of both the intact molecule and its product ions [2].

The development of electrospray ionization (ESI) and the ready generation of multiply charged molecules opened up the prospects for tandem mass spectrometry and top-down MS [3] (but this was predicted by Fenn, as he speculated that the ability of ESI to produce multiply charged ions "could make a most important contribution to the practice of tandem mass spectrometry of MS–MS in sequencing biopolymers [4]"). Soon after Fenn's development of ESI, it was shown that the enhanced efficiency for collisionally activated dissociation (CAD) of multiply charged molecules could generate sequence-informative product ions for biomolecules as large as 66 kDa serum albumin proteins [5], 80 kDa transferrin [6] and to beyond 200 kDa [7]. CAD-MS/MS of multiply charged proteins yield multiply charged products [8]. Although the multiply

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charged products could be interpreted with data from low resolving power analyzers, the determination of precursor and product charge state is much more amenable with higher resolving power instruments (e.g., Fourier transform ion cyclotron resonance (FT-ICR) and orbitraps). Further, data from instruments armed with electron capture dissociation (ECD) [9–12] or electron transfer dissociation (ETD) [13] can yield much more substantial sequence coverage for larger proteins. *Nature Methods* recently listed top-down proteomics among "methods to watch" and an emerging method essential for characterizing various protein variants and posttranslational modifications, with potentially high impact in biomedical research [14].

An additional potential application of top-down MS is the elucidation of noncovalent ligand binding sites to targeted proteins. Ligands are represented by a variety of molecules including metal ions, small molecules, DNA/RNA, or other proteins that interact specifically with a host protein (or other type of biomolecule) to form functional complexes. These complexes are the fundamental machines for almost all cellular activities and processes. Understanding such complexes on a molecular level is important for knowing how they are function on a biological level. It also aids efforts in drug design and drug discovery for the development of more effective therapeutics.

ESI-MS has been used to measure complex stoichiometry for a variety of protein–ligand complexes and to determine the relative or absolute solution binding affinity of an association [15–17], even for weakly bound complexes with solution dissociation constants (k_d) in the millimolar range [18,19].

For noncovalently bound protein–ligand complexes, it was previously thought that noncovalent ligand binding would not survive the MS/MS process. For example, because of the weak interactions between a protein and its ligand, CAD or even infrared multiphoton dissociation (IRMPD) of the complex usually results in simply the separation of the ligand from the protein, revealing little new structural information. However, we have demonstrated previously that top-down MS with ECD and/or CAD can be used to determine the ligand binding sites for specific protein–ligand complexes [19,20]. Earlier work had suggested that weak, noncovalent intermolecular bonds could be preserved upon ECD [21]. We used ECD to localize the binding site of a polyamine compound, spermine, to the 13 kDa α-synuclein protein that has been implicated in Parkinson's disease. Spermine was retained by the c-/z•-products to localize spermine binding to the C-terminal region of the protein. Thus, although the solution binding association for the α-synuclein/spermine complex is relatively weak ($k_d \sim 10^{-3}$ M), ligand binding is retained in the gas phase and even upon ECD [19].

A factor that governs whether tandem MS of noncovalent protein–ligand complexes can yield any structural information is the relative stability of the *gas phase* complex. For example, the strength of electrostatic interactions is significantly *enhanced* in the absence of solvent. Recently, we reported the unusually stable gas phase complex formed between proteins and di- and triphosphate nucleotides, such as the ribonuclease A-cytidine triphosphate (RNase A-CTP) complex [22]. With covalent-like strength, enhanced gas phase electrostatic interactions can be sustained in CAD-MS/MS experiments.

This report demonstrates the enhanced efficiency of top-down MS of noncovalent protein–ligand complexes upon increasing the multiple charging of the gas phase complex. John Fenn's pioneering work paved the path to exploit the benefits of multiple charging by mass spectrometry. Enhanced multiple charging of native proteins and protein complexes can be induced by the addition of "supercharging" reagents, such as *m*-nitrobenzyl alcohol (*m*-NBA) [23,24] and sulfolane [25]. Because electron capture cross-sections increase quadratically with charge [26], addition of one more charge can dramatically enhance the efficiency of ECD/ETD. We show that increasing the multiple charging of protein–ligand complexes by the addition of supercharging reagents improves the prospects for gaining ligand binding site information.

2. Experimental

2.1. Materials

ATP, adenylate kinase (AK; myokinase, from chicken muscle, product number M5520), carbonic anhydrase II (bovine), *m*-NBA, and sulfolane were purchased from Sigma–Aldrich (St. Louis, MO). All protein samples were desalted and concentrated with 20 mM ammonium acetate buffer (pH 6.8) using centrifugal filter devices (10 kDa MWCO, Amicon Ultra; Millipore Corporation, Billerica, MA). After desalting, ATP was added to a 5 μM AK solution in 20 mM ammonium acetate. The solution protein and ligand ratio was kept at 1:1.

2.2. Top-down MS experiments

A nanoESI source and Au/Pd coated borosilicate glass capillaries (Proxeon Biosystems, Odense, Denmark), with flow rate around 50 nL/min, were coupled to an 7-T LTQ-FT Ultra mass spectrometry (Thermo Fisher Scientific, San Jose, CA) to acquire positive ionization mode ESI-MS spectra. Instrument parameters for data acquisition have been described previously [20]. In the source region of the LTQ-FT Ultra, the capillary temperature was set to 210 °C, the capillary voltage was +45 V, and the tube lens was set to +225 V. The resolution of the FT-ICR measurements was established to be 200,000 at 400 mass-to-charge ratio (m/z). Top-down MS/MS were accomplished with CAD or activated ECD (aiECD) in which ECD is coupled with infrared laser-heating of the product ions to dissociate hydrogen bonds retained upon ECD and thereby enhance product ion yield. Product ion mass measurement accuracy was generally better than 7 ppm.

3. Results and discussion

The multiple charging properties of ESI were exploited for MS/MS of intact proteins relatively early in the recent history of electrospray ionization, as protein ions can be dissociated effectively to generate sequence-informative products. This "top-down" strategy works well for proteins in denaturing, acidic solutions that promote high analyte charge. However, ESI charging for proteins and noncovalently bound protein complexes in physiological pH solutions is generally lower, and this characteristic conspires to lower the efficiency for generating sequence-bearing product ions in tandem MS experiments.

We have demonstrated that the addition of charge-promoting agents (i.e., supercharging) can increase the ESI multiple charging of native proteins and protein complexes, and the supercharged protein complexes retain noncovalent binding with its ligand partners. Although the mechanism of supercharging of native proteins is not well understood [23–25], it is clear that the protein complexes are not sufficiently denatured upon supercharging that noncovalent ligand binding is disrupted. Also, this increased charging results in more effective MS/MS for determining ligand binding sites.

3.1. CAD of Zn-bound carbonic anhydrase

Carbonic anhydrase II (CA-II) is a 29 kDa zinc metalloenzyme that catalyzes the hydration of carbon dioxide to carbonic acid. A divalent zinc ion is an essential cofactor for CA-II [27]. The high