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Increasing fragmentation of disulfide-bonded proteins for top–down mass spectrometry by supercharging

Jiang Zhang [a], Rachel R. Ogorzalek Loo [b,c], Joseph A. Loo [a,b,c,*]

[a] Department of Chemistry and Biochemistry, University of California-Los Angeles, Los Angeles, CA 90095, United States
[b] Department of Biological Chemistry, David Geffen School of Medicine at UCLA, University of California-Los Angeles, Los Angeles, CA 90095, United States
[c] UCLA/DOE Institute for Genomics and Proteomics, University of California-Los Angeles, Los Angeles, CA 90095, United States

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ABSTRACT

The disulfide bond is an important post-translational modification to form and maintain the native structure and biological functions of proteins. Characterization of disulfide bond linkages is therefore of essential interest in the structural elucidation of proteins. Top–down mass spectrometry (MS) of disulfide-bonded proteins has been hindered by relatively low sequence coverage due to disulfide cross-linking. In this study, we employed top–down ESI–MS with Fourier-transform ion cyclotron resonance (FT-ICR) MS with electron capture dissociation (ECD) and collisionally activated dissociation (CAD) to study the fragmentation of supercharged proteins with multiple intramolecular disulfide bonds. With charge enhancement upon the addition of sulfolane to the analyte solution, improved protein fragmentation and disulfide bond cleavage efficiency was observed for proteins including bovine β-lactoglobulin, soybean trypsin inhibitor, human proinsulin, and chicken lysozyme. Both the number and relative abundances of product ions representing disulfide cleavage increase with increasing charge states for the proteins studied. Our studies suggest supercharging ESI–MS is a promising tool to aid in the top–down MS analysis of disulfide-bonded proteins, providing potentially useful information for the determination of disulfide bond linkages.

1. Introduction

The formation of disulfide bonds is an important post-translational modification (PTM) to stabilize the folded structures of proteins [1–3], which is often required for protein activity and biological function. It is estimated that disulfide bonds are present in 15% of the human proteome and are associated with a growing list of protein folding related biological processes and diseases [4,5], such as protein globular structure formation [1,6], protein non-covalent interactions, and amyloid fibril formation [7–9]. Therefore, the determination of disulfide bonds is an important aspect for the complete structural elucidation of proteins. However, disulfide bond analysis by mass spectrometry (MS) approaches is challenging, especially for proteins composed of many S—S linkages.

In a conventional "bottom–up" MS approach, disulfide bonds are reductively cleaved, alkylated, and the protein is enzymatically digested prior to identification by MS and liquid chromatography–tandem mass spectrometry (LC–MS/MS), and therefore information on disulfide bond connectivity can be lost. Alternatively, proteins can be digested under non-reducing or partially reducing conditions to generate disulfide-linked peptides for LC–MS/MS measurements [10–12]. Although the latter approach has been applied to peptides and proteins with simple disulfide connections, it has several noticeable drawbacks. Protein sequence coverage can be less than complete, and therefore could result in loss of disulfide linkage information. This becomes more significant for larger proteins with large numbers of disulfide bonds. The experimental conditions also need to be carefully controlled in order to avoid disulfide bond interchange.

Top–down mass spectrometry has become an increasingly important tool for protein characterization. Compared to bottom–up approaches, top–down MS has several distinct advantages. More simplified sample preparation procedures can be employed. The presence of post-translational modifications can be obtained by an intact protein mass spectrum and the modification sites can be further determined by MS/MS with very

* Corresponding author at: University of California-Los Angeles, Department of Chemistry and Biochemistry, 402 Boyer Hall, Molecular Biology Institute, Los Angeles, CA, United States. Tel.: +1 310 794 7023; fax: +1 310 206 4038.
E-mail address: JLoo@chem.ucla.edu (J.A. Loo).

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high sequence coverage. In the past decade, top–down MS has been successfully applied to a wide range of applications including protein PTM characterization [13,14] and determination of protein–protein and protein–ligand non-covalent interactions [15,16]. Top–down MS for protein structure and PTM characterization is not lacking in challenges currently, and these include speed and sensitivity, fragmentation efficiency to achieve complete primary sequence coverage, and efficient software for data interpretation. However, the potential of top–down MS to either compete with and/or complement current bottom–up MS strategies is encouraging.

Several groups previously have used this approach to study disulfide-linked peptides and proteins. McLafferty and co-workers applied top–down MS with electron capture dissociation (ECD), and reported preferential cleavage of disulfide bonds over backbone amide bonds in disulfide-linked peptides [17,18]. Similar preferential cleavage of disulfide bonds was also reported for deprotonated peptides by collisionally activated dissociation (CAD) fragmentation in negative ion MS mode [19–21]. However, the ECD and CAD efficiency of disulfide bond cleavage in proteins is relatively low. To improve disulfide bond cleavage via tandem MS, various approaches have been implemented including metal cationization [22–24], ultraviolet photodissociation [25], reactive electrospray-assisted laser desorption/ionization [26], electrolytic reduction [12], plasma induced oxidation [27], and other electron based fragmentation methods such as ETD [28] and EDD with moderate success [29].

Recently, several groups extended top–down MS to proteins with more complex disulfide bond connections. Chen et al. studied chicken lysozyme, a protein with four intramolecular disulfide bonds, by CAD using an orbitrap mass spectrometer and reported product ions with multiple disulfide bond cleavages [30]. Ge and co-workers studied human salivary α-amylase, a 56 kDa protein with five disulfide bonds, by ECD and CAD and were able to map the disulfide bond connections through a combination of top–down MS and limited digestion [31]. However, thus far, low fragmentation efficiency of disulfide bonds is still a major limiting factor in the top–down MS analysis of proteins.

Previously, our group and others have demonstrated the use of reagents such as sulfolane and *m*-nitrobenzyl alcohol (*m*-NBA) to significantly increase the charge states of proteins and protein complexes in ESI–MS experiments [32–35]. These supercharging reagents can increase the protein charge state [36,37], and therefore can be used for the MS analysis of a wide range of proteins and other biomolecules. We have also shown that supercharging can be applied to protein–ligand complex systems to increase the fragmentation efficiency for top–down MS studies and generate more product ions retaining protein–ligand interactions that provide improved sequencing and non-covalent protein–ligand interaction mapping [34]. In this study, we extended supercharging to top–down MS analysis with ECD and CAD of disulfide-bonded proteins. For several proteins with multiple disulfide-bonded networks, we consistently observed improved cleavage of disulfide bonds and more extensive protein backbone fragmentation for the higher charged proteins generated by supercharging compared to the lower charged proteins.

2. Experimental

2.1. Samples and sample preparation

Bovine β-lactoglobulin, soybean trypsin inhibitor, chicken lysozyme, and human proinsulin were purchased from Sigma–Aldrich (St. Louis, MO). Chemicals were purchased from Aldrich (St. Louis, MO) unless otherwise noted. All protein samples were desalted with 10 mM ammonium acetate using centrifugal filter devices (10,000 molecular weight cutoff, Microcon and Amicon Ultra; Millipore Corporation, Billerica, MA) before analysis. All solutions were prepared in Milli-Q water (Millipore Corporation, Billerica, MA). Glass nanoelectrospray emitters were purchased from Proxeon/Thermo Scientific (West Palm Beach, FL).

2.2. Top–down FT-ICR mass spectrometry

Top–down MS of proteins was performed on an ultrahigh resolution 15-Tesla Bruker SolariX hybrid Qq-FTICR mass spectrometer. Proteins (0.5–5 μM) were prepared in denaturing solution conditions with an acetonitrile (ACN):H_2O:formic acid (FA) ratio of 49.95:49.95:0.1. Supercharging reagent sulfolane was added to a final concentration of 150 mM. The protein solutions were nanoelectrosprayed at flow rates of 20–50 nL/min. MS experiments were performed in the broadband mode from m/z 600–3000 with the following settings: capillary voltage 1000–1200 V; source accumulation time 0.5 s; ion accumulation time 1 s; ion cooling time 0.05 s; time of flight 1 ms. Precursor ions of single charge states were isolated by a quadrupole Q1 with a selection window size of 10–20 m/z. MS/MS experiments were performed with the following settings: ECD, pulse length 0.01 sec; bias 1 V; ECD lens 15 V; CAD, collision energy 8–15 V adjusted for each charge state according to the normalized energy level: CS1 × CE 1 = CS2 × CE 2, where CS denotes the charge state and CE denotes collision energy. 50–100 scans were averaged for each MS experiment. 400 scans were averaged for each ECD experiment in the charge state dependent experiments and 200 scans for each CAD experiment.

2.3. Data analysis

MS/MS data were processed with DataAnalysis and BioTools (Bruker Daltonics). Briefly, monoisotopic masses ($[M+H]^+$) were extracted by DataAnalysis software using a modified Thrash algorithm (SNAP ver 2.0) with the following settings: quality factor threshold 0.5; S/N threshold 2; maximum charge state, ≤ protein precursor charge state. Product ions were assigned using BioTools based on protein sequences determined by accurate mass measurements. Mass accuracy of 15 ppm was used for ECD/CAD product ion assignments. The assigned ions were manually confirmed to ensure the quality of assignments.

Product ion intensities were also extracted from DataAnalysis Software for relative abundance of disulfide bond cleavage calculation. Quantification of disulfide bond cleavage in the MS/MS experiments was calculated as:

$$SS\% = \sum_{I_{total}}^{I_d} \times 100\%$$

where I_d denotes the ion abundance of product ions with disulfide cleavage, and I_{total} is the total ion abundance.

3. Results and discussion

3.1. Supercharging and top–down MS of β-lactoglobulin

β-Lactoglobulin is an 18 kDa protein present as a major milk component in many mammalian species. Fig. 1 shows the high resolution ESI–MS mass spectra of β-lactoglobulin collected under both conventional and supercharging conditions. The addition of sulfolane to 150 mM did not significantly decrease the signal intensity for the proteins studied. However, adding sulfolane significantly increased both the average and maximum charge states of β-lactoglobulin; the most abundant charge state was increased to 15+ with sulfolane, compared to 12+ in the solution without sulfolane. The maximum observed charge state observed