Native mass spectrometry protein structural characterization via surface induced dissociation: instrumentation and applications

DISSertation

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By

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Abstract

Protein complexes are essential in cellular processes, and protein complex characterization plays a key role in the understanding of biological processes. Native mass spectrometry has become a useful tool for the study of protein complexes. Surface induced dissociation (SID) has been shown to provide folded noncovalent products of protein complexes, reflecting the native structure of protein complexes. This dissertation focuses on characterizing the structure of protein complexes by native mass spectrometry coupled to an SID device, developing methods to solve structural biology questions.

In Chapter 2, correlations between SID fragmentation patterns and the subunit interactions of globular homotetramers with known structure is determined. This work uses ion mobility (IM) and SID in a quadrupole time of flight (Q-TOF) instrument. SID provides insight into the interface of homotetramers studied with the smallest interfaces initially cleaved. Different SID fragmentation patterns of protein complex ions with different conformations further show that SID is capable of monitoring conformational changes of protein complexes.

Chapter 3 describes the application of SID to three uncharacterized protein complex systems: a protein oligomer (lambda exonuclease), a DNA-protein complex (nucleosome) and an enzyme-substrate complex (methyltransferase substrates), providing structural information to answer different biological questions. Subunit exchange in the mixture of lambda exonuclease wild-type and mutant is confirmed by using SID to provide the
stoichiometry information. Subunit information and nucleosome stability information are gained from the SID of wild-type and H2A.Z variant nucleosomes. Both SID and CID of methyltransferase substrates can identify a substrate from the enzyme-substrate complex in a cellular milieu, providing a native MS method to use in broad screening for enzyme-subtract pairs.

In the work described in Chapter 4, conformations of globular protein complex ions generated in gas phase ion-ion reactions and solution additive charge reduction are compared. When the conformation of the precursor ions is disrupted by the supercharging or cone activation, the difference in SID fragmentation patterns of ions generated from gas phase ion-ion reactions can be observed, indicating that the conformation of protein complex ions can be preserved in gas phase ion-ion reactions.

Chapter 5 describes the study of protein conformational changes during gas phase trapping in a Q-TOF instrument. No significant conformational changes were observed during the trapping time studied, based on similar SID fragmentation patterns that were observed from streptavidin ions trapped for various amount of time in the gas phase.

Chapter 6 describes the implementation of an SID device in a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, which provides ultrahigh resolution m/z measurements, to pursue more detailed structural information on protein complexes in SID. SID of several protein complex systems in the FT-ICR provides similar SID fragmentation patterns to those obtained previously from Q-TOF platforms. Also, the ultrahigh resolution and mass accuracy measurements make it possible to assign modifications and metal adduct to specific protein subunits.
In summary, SID can not only provide connectivity, stoichiometry and ligand/adduct binding information on various protein complex systems, but also reveals conformational changes of globular protein complexes in the gas phase.
Dedication

This document is dedicated to my parents, Heping Yan and Hanhua Zhou.
Acknowledgments

I would like to thank everyone who has helped me in the last five years. Firstly, I would like to thank my advisor, Dr. Vicki Wysocki, for her help in various aspects, such as critical thinking, writing, public presentation and communication with collaborators. Her patience and enthusiasm for science have encouraged me to learn more about different projects. She kindly provides opportunities for me to go to various conferences, making it possible for me to broaden my knowledge by communicating with people from different backgrounds.

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Publications


Royston S. Quintyn, Jing Yan, and Vicki H. Wysocki, “Surface-Induced Dissociation of Homotetramers with D2 Symmetry Yields their Assembly Pathways and Characterizes the Effect of Ligand Binding”, Chemistry & Biology 2015, 22 (5), 583-592. (Co-first author with Royston S. Quintyn)

Sophie R. Harvey, Jing Yan, Jeffery M. Brown, Emmy Hoyes, and Vicki H. Wysocki, “Extended Gas-Phase Trapping Followed by Surface-Induced Dissociation of Noncovalent Protein Complexes”, *Analytical Chemistry* **2016**, 88 (2), 1218-1221.


Fields of Study

Major Field: Chemistry
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>ammonium acetate</td>
</tr>
<tr>
<td>AdoVin</td>
<td>S-adenosylvinthionine</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>AMBA</td>
<td>2-amino-5-mercaptobenzoic acid</td>
</tr>
<tr>
<td>CCS</td>
<td>collisional cross section</td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
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<tr>
<td>ConA</td>
<td>concanavanlin A</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTB</td>
<td>cholera toxin B</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>cryo-electron microscopy</td>
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<td>Cyro-ET</td>
<td>cryo-electron tomography</td>
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<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture dissociation</td>
</tr>
<tr>
<td>EDDA</td>
<td>ethylenediamine diacetate</td>
</tr>
<tr>
<td>EHSS</td>
<td>exact hard-sphere scattering</td>
</tr>
<tr>
<td>EID</td>
<td>electron induced dissociation</td>
</tr>
<tr>
<td>ERMS</td>
<td>energy-resolved mass spectrometry</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ETD</td>
<td>electron transfer dissociation</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform ion cyclotron resonance</td>
</tr>
<tr>
<td>GD</td>
<td>glow discharge</td>
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<td>GDH</td>
<td>glutamate dehydrogenase</td>
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<td>ion mobility</td>
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<td>λ exonuclease</td>
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<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>oaTOF</td>
<td>orthogonal acceleration time-of-flight</td>
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<td>PA</td>
<td>projection approximation</td>
</tr>
<tr>
<td>PSA</td>
<td>projected superposition approximation</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>quadrupole time-of-flight</td>
</tr>
<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>SAP</td>
<td>serum amyloid P</td>
</tr>
<tr>
<td>SASA</td>
<td>solvent-accessible surface area</td>
</tr>
<tr>
<td>SAXS</td>
<td>small angle X-ray scattering</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SID</td>
<td>surface induced dissociation</td>
</tr>
<tr>
<td>TEAA</td>
<td>triethylammonium acetate</td>
</tr>
<tr>
<td>TJM</td>
<td>trajectory method</td>
</tr>
<tr>
<td>TNB</td>
<td>2-nitro-5-thiobenzoic acid</td>
</tr>
<tr>
<td>TNH</td>
<td>toyocamycin nitrile hydratase</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>TPMT</td>
<td>thiopurine S-methyltransferase</td>
</tr>
<tr>
<td>TWIG</td>
<td>traveling wave ion guide</td>
</tr>
<tr>
<td>TWIM</td>
<td>traveling wave ion mobility</td>
</tr>
<tr>
<td>PDCH</td>
<td>perfluoro-1,3-dimethylcyclohexane</td>
</tr>
<tr>
<td>PFHA</td>
<td>perfluoroheptanoic acid</td>
</tr>
<tr>
<td>PTR</td>
<td>proton transfer reaction</td>
</tr>
<tr>
<td>UHV</td>
<td>ultra-high vacuum</td>
</tr>
<tr>
<td>UVPD</td>
<td>ultraviolet photodissociation</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1. Protein structural biology

Protein complexes are essential in cellular processes such as cell cycle, signaling, transcription, RNA metabolism, and protein synthesis.\textsuperscript{1-4} Certain human diseases can be attributed to the protein misfolding and the misassembly of protein complexes.\textsuperscript{5-7} Therefore, protein complex characterization plays an important role in the understanding of the biological process and expediting biomedical development. Various structural biology tools have been applied to protein structural studies.\textsuperscript{8} Among those different methods, nuclear magnetic resonance (NMR), X-ray crystallography and cryo-electron microscopy (cryo-EM) provide high-resolution atomic structure of protein complexes.\textsuperscript{9-14} Small angle X-ray scattering (SAXS) and atomic force microscopy (AFM) provide overall protein topology information.\textsuperscript{15,16} Tandem mass spectrometry coupled with ion mobility can provide stoichiometry, topology and connectivity information on protein complexes.\textsuperscript{17,18}

The techniques discussed above have advantages and disadvantages.\textsuperscript{8} Even though X-ray crystallography can provide high-resolution structures of protein complexes, the structure in the crystal can be significantly different from that in solution.\textsuperscript{12} Also, many proteins are difficult to crystallize.\textsuperscript{19} Solution NMR allows the study of protein dynamics of protein complexes in solution, but it is hard to determine the structure of high molecular
weight protein complexes (>50 kDa).\textsuperscript{8,13} Cyro-EM can be used to study the topology of macromolecular complexes. The development of cryo-electron tomography (cryo-ET) makes it possible to study structural biology in the cell environment, but computational modeling is still needed to refine the structural model.\textsuperscript{20-23} SAXS provides structural information for intrinsically disordered domains, but only low-resolution information can be obtained.\textsuperscript{15} AFM also provides low-resolution structural information; however, it provides useful complementary structural information when combined with other techniques.\textsuperscript{16,24} Even though mass spectrometry cannot provide atomic resolution structural information on protein complexes, it has proven to be a quick method which can provide composition, stoichiometry, interaction and assembly topology information on protein complexes with low sample concentration required.\textsuperscript{17,18} It can provide information that is difficult to obtain with the techniques mentioned above, for example, the number of ligands bound to a protein. In this dissertation, mass spectrometry (MS) was used to study protein complexes in this dissertation and details about mass spectrometry will be introduced in the following sections.

1.2. Native mass spectrometry

In mass spectrometry, the analyte is ionized and transferred into the gas phase, and eventually, the mass-to-charge ratio ($m/z$) of ions is obtained. The molecular weight of the analyte can be calculated based on the $m/z$ if multiple $m/z$ values are measured. In tandem mass spectrometry (MS/MS), ions with specific $m/z$ are isolated and activated for dissociation. The fragments generated from the activation will be detected in the mass analyzer.
Native mass spectrometry studies the quaternary structure of native protein complexes in the gas phase. With the use of buffers near neutral pH (the physiological regime), such as ammonium acetate and ammonium bicarbonate, non-covalent protein complexes can be preserved in solution, making it possible for them to be further analyzed by mass spectrometry.

![Figure 1.1](image_url)

Figure 1.1. Relationships between charge (z), collision cross section (CCS), and solvent accessible surface area (SASA) of protein complexes. Reprinted from Ref 18 with permission from Elsevier, Copyright (2012).

Based on the results from previous studies, mass, charge state, and surface accessible area of globular proteins and protein complexes are correlated, and the
relationships are shown in Figure 1.1.\textsuperscript{18} Charge state distributions can be correlated to different conformational changes in protein.\textsuperscript{28,29} However, conformational changes are more effectively monitored with ion mobility, where the compactness of proteins can be discerned via the corresponding drift time.\textsuperscript{30-32} In addition, collision cross sections (CCSs) of protein complexes in the gas phase calculated based on drift time distributions provide further conformational information on protein complexes when compared with calculated structures.\textsuperscript{33-35} Tandem mass spectrometry, in which protein complexes are dissociated via different activation methods, helps in gaining protein structural information, such as stoichiometry, connectivity, quaternary structure of protein complexes and the protein stability.\textsuperscript{36} With the combination of cross-linking and covalent labeling, detailed interface information can be obtained.\textsuperscript{37,38} The protein-protein association constant can also be determined by MS-based titration experiments.\textsuperscript{39}

However, whether the gas phase structure of individual proteins can represent the solution-phase structure is still under debate. Previous studies have shown that the structure of protein ions can be altered both during and after transfer from solution to gas phase with cytochrome c as an example.\textsuperscript{40,41} The time scale of the MS experiment can be from microseconds to milliseconds.\textsuperscript{42} Yet evidence has shown that large proteins and protein complexes can preserve features of their native structures in the gas phase.\textsuperscript{34,43-45} For example, hydrogen bonds and electrostatic interactions can be retained in the gas phase.\textsuperscript{46-48} The protein quaternary structure, non-covalent protein-protein, protein-RNA and protein-ligand interactions can also be maintained in the gas phase in the absence of water.\textsuperscript{49-51} Therefore, native mass spectrometry can be utilized judiciously to characterize
the stoichiometry, assembly, topology and intermolecular interactions of protein complexes in the gas phase.

1.3. Ionization method

The development of soft ionization methods, such as electrospray ionization (ESI)\textsuperscript{52,53} and matrix-assisted laser desorption/ionization (MALDI),\textsuperscript{54} made it possible for proteins to be introduced to the gas phase for mass analysis. Both methods of ionization are used in proteomics (ESI more so than MALDI) for primary sequence identification.\textsuperscript{55-57} When it comes to the investigation of higher order structure of proteins and protein interactions in native mass spectrometry, ESI is commonly used, even though both ESI and MALDI have been shown to be able to preserve noncovalent interactions.\textsuperscript{27,58-60} Since nanoelectrospray ionization (nESI) was developed, a large number of noncovalent protein complexes have been studied by ESI.\textsuperscript{60,61} A schematic of the ESI process in the positive ion mode is shown in Figure 1.2. In ESI, an electric potential is applied to the liquid within a capillary pulled to a tip, resulting in a high electric field near the tip leading to the enrichment of positive ions in the meniscus.\textsuperscript{62} The distortion of the meniscus induced by the polarization leads to the formation of a Taylor cone and droplets are emitted toward the ionization interface of a mass spectrometer.\textsuperscript{53,64} Each of the droplets are positively charged because of the voltage bias and the presence of ions such as H\textsuperscript{+}, NH\textsubscript{4}\textsuperscript{+}, Na\textsuperscript{+}, and K\textsuperscript{+}.\textsuperscript{65} Among all those cations, H\textsuperscript{+} is the main contributor in a desalted solution. With the evaporation of the solvent, droplets shrink with the increase of surface tension balanced by the Coulombic repulsion at the Rayleigh limit.\textsuperscript{66} Eventually, gas-phase ions are produced for mass spectrometry analysis. The ESI process in negative ion mode follows the same
principle with opposite polarity. Multiply charged ions are generated in ESI such that species with high molecular weight can be detected by mass spectrometers with modest $m/z$ ranges.62

![Figure 1.2. Schematic of ESI process in atmospheric pressure in positive ion mode. Adapted from Ref 62 with permission from John Wiley and Sons.62](image)

Currently, there are three different models for explaining the ESI process, including the charge residue model (CRM), the ion evaporation method (IEM) and the chain ejection model (CEM).65,66 Each of the models are used to describe the formation of ions from different observed systems and the process for each of them is shown in Figure 1.3. The ion evaporation method (IEM), in which the small analyte ion “evaporates” or get ejected from the charged droplet during evaporation, is generally applied to low molecular weight species.67,68 In the charge residue model (CRM), the solvent evaporates from the droplet to the point of “dryness”, and the charges on the surface of the droplet are deposited on the analyte ion. This model is used to explain the ESI process of globular proteins.68-70 Both
Experimental observations and molecular simulations support the CRM for globular proteins.\textsuperscript{68,71} The charge remaining on the protein depends on the size of the protein, which correlates well with the Rayleigh limit of the droplet.\textsuperscript{68,71} The chain ejection model (CEM), in which the end of a charged and unfolded analyte ion protrudes from the droplet surface and eventually ejected from the droplet, is proposed to explain the ESI process of unfolded proteins or polymers.\textsuperscript{65,72} The ESI process can be affected by multiple parameters,\textsuperscript{73} further understanding of the ESI mechanism is essential for the structural study of protein ions in the gas phase.

![Diagram of ESI mechanisms](image)

Figure 1.3. Summarized ESI mechanisms. (a) Ion evaporation model. (b) Charged residue model. (c) Chain ejection model. Adapted from Ref 65 with permission from American Chemical Society, Copyright (2013).\textsuperscript{65}
Since its development, nanoelectrospray ionization (nashi) has been widely used in native mass spectrometry.\textsuperscript{18,74,75} Unlike conventional ESI, nESI requires smaller sample volume and lower flow rate. Microliter volumes of a sample are required in nESI compared with milliliter volumes required in ESI.\textsuperscript{75} nESI uses capillaries with smaller spraying orifice (1 – 10 µm), enabling the production of nanodroplets with a favorable surface-to-volume ratio so that no harsh desolvation conditions are needed.\textsuperscript{18} Lower collision energy is required for desolvation because of the smaller size of droplets generated in nESI. Additionally, nESI has better non-volatile salt tolerance and no addition of volatile organic solvents is required, allowing non-covalent interactions to be preserved in this process for the study of protein complexes under native-like conditions.\textsuperscript{75} Therefore, nESI is ideal for the study of protein complexes under native-like conditions, and nESI will be used to study different protein systems in this dissertation.

1.4. Mass analyzer

In mass spectrometry, ions transferred into the gas phase get separated based on their $m/z$ values in a mass analyzer and are eventually detected by a variant of an electron multiplier or image current detection. The ions in a mass spectrometer can be driven by an electric field (static or dynamic) or magnetic field. The principle, sensitivity, speed, dynamic range and resolving power of the analyzers vary. In this dissertation, three mass analyzers will be used: quadrupole, time-of-flight, and Fourier transform ion cyclotron resonance.
1.4.1. Quadrupole

The quadrupole has been developed to be used as a mass filter, an ion guide or a mass analyzer in mass spectrometry.\textsuperscript{76-78} Ions with specific $m/z$ can be isolated when the quadrupole is used as a mass filter. The mass filter function is realized by the combination of high mass filter and low mass filter.\textsuperscript{79} As a mass analyzer, the ions in a certain $m/z$ range can be selectively destabilized over a set scanning period in a quadrupole. In this dissertation, quadrupole will be used as a mass filter to isolate precursor ions with a specific $m/z$.

A quadrupole consists of four parallel rod electrodes, and both DC voltage ($U$) and RF voltage ($V\cos\omega t$) are applied on each of the electrodes as shown in Figure 1.4. The overall ion path follows the $z$-axis. The opposing rods are held at the same DC potentials with matching RF phases, while adjacent electrodes have opposite polarities of DC applied with RF 180 degrees out of phase.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{quadrupole.png}
\caption{Schematic of a quadrupole. Adapted from Ref 80 with permission from John Wiley and Sons.\textsuperscript{80}}
\end{figure}
The fundamental operating principle of the quadrupole can be explained by the stability diagram of the quadrupole (Figure 1.5) with two parameters (a and q) defined by the Mathieu equations. The equations for parameters a and q are:

\[ a_x = -a_y = \frac{4eU}{m\omega^2 r_0^2} \]
\[ q_x = -q_y = \frac{2eV}{m\omega^2 r_0^2} \]

where \( m \) is the mass of the ion; \( U \) is the DC voltage; \( V \) is the RF voltage; \( \omega \) is the RF frequency; \( r_0 \) is half of the distance between the opposite electrodes.

Figure 1.5. The stability diagram of the two-dimensional quadrupole. Reprinted from Ref 80 with permission from John Wiley and Sons.
As shown in the stability diagram in Figure 1.5, ions with a certain m/z can be stable in x- and y- directions with specific a and q values applied (typically manipulated by varying the voltage magnitude applied in each equation). Region I is normally used for mass selection for a quadrupole, and the diagram is shown in Figure 1.6. As shown in Figure 1.6, with the ratio of U/V set to be a constant (a/q is constant), the ions with a specific m/z range can be stable in both x- and y- directions, thus those ions remain stable during trajectory through the quadrupole. With the decrease of U, the slope of the a/q plot is lower, leading to the transmission of ions with a wider m/z. When U is set to 0, the RF-only quadrupole provides a wide band pass for ions. Thus, the RF-only quadrupole can be used as an ion guide rather than a filter. Higher order multipoles, such as hexapoles and octupoles, can provide better wide band pass. Therefore, they are also used as ion guides, however, they cannot be used as a mass filter.
Figure 1.6. Zoom in on the upper part of Region I in Figure 1.5. Adapted from Ref 81 with permission from American Physical Society, Copyright (1990).\textsuperscript{81}

The maximum $m/z$ that can be transmitted through an RF-only quadrupole is shown in the equation:

$$\left(\frac{m}{z}\right)_{max} = \frac{4eV_{RF_{max}}}{q_{max}r_0^2\omega^2}$$

where $V_{RF_{max}}$ is the maximum RF voltage output (limited by hardware or Pachen’s curve); $q_{max}$ is 0.908 (right-most border of the first order stability diagram in terms of q); $\omega$ is the RF frequency; $r_0$ is half of the distance between the opposite electrodes.\textsuperscript{83}

The quadrupole with conical rods has been shown to provide better focusing for ions transmission.\textsuperscript{84} A similar design of quadrupole will be applied to the design of SID device in Chapter 6.
1.4.2. Time-of-Flight (TOF)

The time-of-flight mass analyzer provides m/z measurements by recording the time needed for accelerated ions to fly through a field-free drift path of predetermined length.\textsuperscript{85} The time (t) for ions to drift through a certain length is proportional to the square root of m/z with an equation:

\[ t = \frac{l}{v} = \frac{l}{\sqrt{2Ue}z/m} = \frac{l}{\sqrt{2Ue}} \sqrt{m/z} \]

where l is the length the ion flies; U is the DC voltage used to accelerate the ion.

Several techniques have been applied to improve the performance of the TOF analyzer. Orthogonal acceleration TOF (oaTOF) analyzer, in which ion pulses are extracted orthogonally from a continuous ion beam, minimizes effects of differences in position and kinetic energy, providing m/z measurements with higher resolution and mass accuracy. Also, the reflectron was used to adjust the time-of-flight of ions with different initial kinetic energies, improving the resolving power of the TOF mass analyzer. In a reflection, which consists of a set of rings with increasingly repulsive potential, ions with higher kinetic energy penetrate deeper into the ring stack than those with lower kinetic energy. The ions are repelled in the opposite direction after reaching zero kinetic energy within the ring stack, thus time-of-flight of ions with different kinetic energies can be corrected.\textsuperscript{82} In this dissertation, both Waters Synapt G2 and G2S HDMS mass spectrometers (Wilmslow, U.K.) used in this dissertation are quadrupole time-of-flight (Q-TOF) instruments with ions selected by the quadrupole and detected by the oaTOF mass analyzer with dual-stage reflectron coupled to provide high-resolution m/z measurements.
1.4.3. Fourier transform ion cyclotron resonance (FT-ICR)

FT-ICR mass spectrometry, which has been shown to provide ultrahigh resolution and mass accuracy, was introduced in 1932. In a uniform magnetic field, the ion cyclotron frequency of ions is related to the \( m/z \) value as shown in the equation:

\[
\gamma_c = \frac{\omega_c}{2\pi} = \frac{1.535611 \times 10^7 B_0}{m/z}
\]

where \( \gamma_c \) is the ion cyclotron frequency in Hz; \( \omega_c \) is the angular velocity in rad/s; \( B_0 \) is the magnetic field strength in tesla. Therefore, the ions with the same \( m/z \) have the identical frequency, which is independent of the velocity of ions.

The \( m/z \) detection process in the ICR cell is shown in Figure 1.7. With the application of a rotating electric field, the ions are excited to an orbital radius which is independent of \( m/z \). The equation of the radius, \( r \), is:

\[
r = \frac{V_{p-p} T_{\text{excite}}}{2dB_0}
\]

where \( V_{p-p} \) is the RF resonant oscillating voltage in volts; \( T_{\text{excite}} \) is the period for oscillating resonant excitation in seconds; \( d \) is the distance between two excitation electrodes in meters. Therefore, the radius of all the ions excited in a given \( m/z \) range has the same orbital radius which is independent of their \( m/z \). Frequency-sweep (chirp) excitation was commonly applied for broadband ion excitation. However, the nonuniform excitation of ions close to both ends of the sweep during the ion isolation with a specific \( m/z \) limits the sensitivity. Stored waveform inverse Fourier transform (SWIFT), which is produced by inverse Fourier transformation of a specific magnitude excitation spectrum from the
resonance frequency of selected ions, is used to excite specifically isolated ions, providing maximum amplitude near two ends of the SWIFT waveform.²⁹,³¹

Figure 1.7. Incoherent ion cyclotron orbital (excite) motion (top left) and coherent (detectable) motion (top right) in a uniform magnetic field. The electronic circuitry diagram of the ICR cell is shown in the bottom. Reprinted from Ref 89 with permission from John Wiley and Sons.⁸⁹
To generate a detectable signal, ion packets are excited to generate spatial coherence. The number of charges in an ICR cell is limited to avoid coalescence, which would ultimately diminish resolution.\textsuperscript{92} The cyclotron motion of the ions induces an image current on a pair detection electrodes, which is proportional to the ion cyclotron radius and the charge state of ions.\textsuperscript{89,93} In addition to the static magnetic field, the electrostatic trapping potential can also generate an axial force, and the ion motion is shown in Figure 1.8.

![Figure 1.8. Ion motion for ions in a Penning trap. Reprinted from Ref 94 with permission from Elsevier.\textsuperscript{94}](image)

The “reduced” cyclotron frequency, which depends on both the magnetic field and the electric field generated from the trapping potential, is the frequency that is observed. The equations of the “reduced” cyclotron frequency ($\omega_+$) and the angular frequency ($\omega_z$) are shown as follows:

$$\omega_+ = \frac{\omega_c}{2} \pm \sqrt{\left(\frac{\omega_c}{2}\right)^2 - \frac{\omega_z^2}{2}}$$
\[ \omega_z = \sqrt{\frac{2qV_{\text{trap}}\alpha}{ma^2}} \]

where \( V_{\text{trap}} \) is the DC voltage applied to the “end cap” electrodes; \( \alpha \) is the trapping scale factor depending on the geometry of the trap; \( a \) is the size of the electrode in meters.\(^99,95\)

The free induction decay (FID) signal was recorded from the detection electrodes. The frequency of excited ions, which is related to the \( m/z \) of ions, is extracted from Fourier transform of the FID signal.

With the development of the “Infinity Cell”, the undesirable ejection of ions along the \( z \)-axis (\( z \)-ejection effect) can be minimized.\(^96\) However, the application of “Infinity Cell” on the detection of the protein complex system is limited because of the low transient preservation. The “ParaCell” was then developed by Eugene Nikolaev \textit{et al.}\(^97,98\) The ParaCell is shown to be able to improve the collection of long transients, which is necessary for protein complex studies.\(^98,99\) The 15 T Bruker SolariX XR FT-ICR used in this dissertation has a ParaCell installed for mass analysis.

In addition to “Infinity Cell” and “ParaCell” which are commercially used in FT-ICR instruments, the narrow-aperture detection electrodes (NADEL) cell, which has a pair of detection electrodes with a narrow aperture, has been shown to be promising in reducing \( \omega_+ \) and \( \omega_c \) can be directly detected.\(^100,101\) Thus the mass accuracy of the FT-ICR technique can be increased in NADEL cell.\(^100,101\)
1.5. Tandem mass spectrometry

In tandem mass spectrometry, ions of interest are activated via various approaches. The activation methods and their corresponding activation time scales are listed in Figure 1.9.¹⁰²

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**Figure 1.9.** Ranges of activation times associated with different activation methods. EID – electron induced dissociation; SID – surface-induced dissociation; CA – collisional activation; (beam) - typical conditions used in a beam-type tandem mass spectrometer; ICR-SORI - ion cyclotron resonance sustained off-resonance irradiation; cw IRMPD - continuous wave infrared multiphoton dissociation. Reprinted from Ref 102 with permission from John Wiley and Sons.¹⁰²
Different ion activation methods lead to different fragmentation behaviors. In native mass spectrometry, both the noncovalent interaction and the covalent interaction of protein complexes can be disrupted during activation. The disruption of noncovalent interaction during activation leads to the disassembly of protein complexes. Collision induced dissociation (CID) and surface induced dissociation (SID) have been used to evaluate the structure of protein complexes via noncovalent fragmentation. In covalent fragmentation, the protein backbone cleaves at different sites, and the ions generated from these cleavages are defined as shown in Figure 1.10. The process of covalently fragmenting intact proteins is commonly referred to top-down, which provides complementary sequence information on proteins. CID, infrared multiphoton dissociation (IRMPD), ultraviolet photodissociation (UVPD), electron capture dissociation (ECD), and electron transfer dissociation (ETD) have been used to conduct top-down analysis of protein complexes. Here, several ion activation methods used in protein complex study are introduced.

![Figure 1.10. Nomenclature of peptide fragmentation.](image-url)
1.5.1. Collision induced dissociation (CID)

Collision induced dissociation (CID), which is also called collision activated dissociation (CAD), is a commonly used activation method in tandem mass spectrometry. In CID, the ions accelerated by an electric field collide with inert gas atoms or molecules. CID has been shown to be able to fragment peptide ions, generating mainly b- and y- ions at the peptide bond, and helps in identifying the sequence of peptides.\textsuperscript{110} CID is the most common and robust activation method used in bottom-up proteomics work.\textsuperscript{111}

CID is also widely applied to the study of noncovalent protein complexes in native mass spectrometry.\textsuperscript{25,104} The CID dissociation behavior of multiple protein complexes with different structures has been well-characterized by Carol Robinson et al.\textsuperscript{112} In the CID of noncovalent protein complexes, an unfolded and highly-charged protein monomer is ejected from the protein complex, leaving behind the complementary (n-1) mer.\textsuperscript{112,113} With the aid of ion mobility, the conformations of the unfolded protein complex as well as the unfolded monomer during the CID process can be monitored by comparing the calculated collision cross section (CCS) values with the theoretical CCS values calculated from known crystal structure.\textsuperscript{35,114} The simulation result of the 14+ transthyretin tetramer showing the ejection of a highly charged unfolded monomer agrees with the previous experimental results.\textsuperscript{112,115}

Various details can be obtained from the gas-phase collision of protein. Collision induced cleaning helps to remove the salt adducts from the protein complex, providing more accurate mass measurements.\textsuperscript{116} While collisional activation with background gas
leads to the unfolding of proteins, the energy required for collision induced unfolding has been shown to be related to the folded domains.\textsuperscript{117} Additionally, the ejection of monomer subunit in CID can also provide stoichiometry information on protein complexes.\textsuperscript{116}

1.5.2. Surface induced dissociation (SID)

Surface induced dissociation (SID) was first proposed by Cooks \textit{et al.} where d\textsubscript{3}-acetophenone is fragmented through a high-energy collision with a stainless-steel surface.\textsuperscript{118} As opposed to CID, which is a “slow heating” process involving multiple collisions, SID is a fast, single-step high energy process. This aspect allows SID to access higher energy pathways not accessible in CID (Figure 1.11).\textsuperscript{119}

![Figure 1.11. Reaction coordinates for SID and CID. Reprinted from Ref 119 with permission from Springer.\textsuperscript{119}}

SID of small molecules, peptides, and proteins has been studied on different mass spectrometry platforms, for example, it has been shown to be able to provide sequencing information on peptides.\textsuperscript{119-124} The energy transfer to peptide ions using different types of
surfaces was also studied. A fluorinated self-assembled monolayer, which is shown to have high translational to vibrational energy transfer efficiency and relatively narrow internal energy distribution, is used in the SID experiments in this dissertation. The ion-surface collision was also studied by the simulation in the Hase group. The SID process of peptides was explained by the Rice-Ramsperger-Kassel-Marcus (RRKM) model, where the peptide ions activated by the surface collision leave the surface, and intramolecular vibrational energy redistribution (IVR) leads to dissociation.

The SID dissociation of protein complexes has been first studied in the Wysocki group via a SID device installed on a Q-TOF instrument. SID capability has been further applied to Waters Q-TOF instruments coupled with ion mobility and Bruker FT-ICR. The study of protein complex structure with SID in Q-IM-TOF platforms will be discussed in Chapter 2 and Chapter 3. The application of SID on the FT-ICR platform will be shown in Chapter 6.

The SID dissociation behavior of protein complexes has shown that SID can provide quaternary structural information on protein complexes. The study of noncovalent protein complexes with known crystal structure has shown that SID of charge reduced species can better represent the native-like structure of protein complexes than those with higher charges. Also, the study of homotetramers with similar structures has shown that the SID dissociation pathway is related to the interface of protein complexes, and it is suggested that the smallest interface is cleaved first in SID of the homotetramers studied. SID has shown the ability to distinguish the conformational differences of protein complex ions with the same collision cross section. Therefore, SID
can also be utilized to monitor the structure of protein complexes; this will be discussed in Chapter 4 and Chapter 5.

1.5.3. Electron capture dissociation (ECD)

The electron capture of multiply charged peptide or protein cations produces a radical ion with a lower charge state. ECD has been shown to be able to cleave the backbone N-Cα bonds of peptides and proteins, largely producing c-, z- ions, while minor a- and y- ions can be generated.138-140 Post-translational modifications (PTMs) and noncovalent binding sites can be preserved in ECD, making it a useful tool to characterize protein interactions.141,142

Electron induced dissociation (EID), which involves the interaction between peptide or protein cations and electrons with more than 10 eV electron energy, leads to more oxidation of cations than reduction, thus higher fragmentation efficiency can be observed in EID and higher sequence yield can be obtained.143-145 The EID of peptides with more than 20 eV electron energy generates a-, b-, c-, x-, y-, z- type ions with 100% efficiency.143 More fragmentation and less generation of charge-reduced species in EID than those in ECD has shown that EID is suitable to study the structure of proteins and protein complexes in native top-down mass spectrometry.144

1.6. Ion mobility

1.6.1. Drift tube ion mobility

Ion mobility (IM) measures the drift time of ions to migrating through a buffer gas which is related to the collision cross section (CCS) of ions. In drift tube ion mobility, which is the most common type of ion mobility, ions are driven through a relatively high-
pressure buffer gas by low electric field.\textsuperscript{146} The equations describing the relationship of the drift time ($t$), ion mobility constant ($K$) and the CCS ($\Omega$) are shown as follows:

\[ t = \frac{L}{v} = \frac{L}{KE} \]

\[ K = \left(\frac{3q}{16N}\right) \left(\frac{2\pi}{kT}\right)^{1/2} \left(\frac{m + M}{mM}\right)^{1/2} \left(\frac{1}{\Omega}\right) \]

where $L$ is the length of the drift tube; $E$ is the electrostatic field strength; $q$ is the charge of the ion; $N$ is the density of the gas; $k$ is the Boltzmann’s constant; $T$ is the temperature in K; $m$ is the mass of the buffer gas and $M$ is the mass of the ion.\textsuperscript{146-149} Drift tube ion mobility has been used to determine the gas phase CCS of lipids, peptides, proteins and protein complexes.\textsuperscript{147,150} Combined with the known crystal structure, the CCS calculated from ion mobility results can be further used to study the gas phase structure of peptides and proteins.\textsuperscript{151}

Ion mobility can separate ions based on their size, charge, and shape. The different conformational changes of cytochrome C have been shown to be able to be separated and the gas phase conformational change can be monitored with the utilization of drift tube ion mobility.\textsuperscript{152,153} The resolving power of the drift time ion mobility is

\[ \frac{t_{DT}}{t_{1/2}} = \frac{1}{4} \left(\frac{q}{kln2}\right)^{1/2} \left(\frac{V}{T}\right)^{1/2} \]

where $t_{DT}$ is the drift time; $t_{1/2}$ is the full width at the half maximum of the peak; $q$ is the charge of the ion; $k$ is the Boltzmann’s constant; $V$ is the voltage difference across the drift tube; $T$ is the temperature in K.\textsuperscript{154} Based on the equation, the resolving power is related to the voltage applied on the drift tube. As low $E/N$ is required to keep the mobility independent of the electric field, higher resolution can be achieved with the increase of
drift tube length and a high-resolution drift tube has been shown to be able to provide more than 170 resolving power.\textsuperscript{154} With circular ion mobility, featuring a looping drift tube to increase the drift length of ion, the resolution of a singly charged C\textsubscript{60} peak can be as high as 400.\textsuperscript{155}

1.6.2. Traveling wave ion mobility

In traveling wave ion mobility (TWIM), a sequence of DC potential waves move along the ion mobility cell, and ions are separated through their interactions with the wave and the buffer gas.\textsuperscript{156} TWIM is commercially available in the Waters Synapt Q-IM-TOF instruments.\textsuperscript{157,158} With the application of RF on the stacked ring electrodes and traveling wave, the sensitivity and resolution are improved.\textsuperscript{157} The ion mobility results shown in this dissertation are obtained from TWIM with nitrogen used as a buffer gas in Waters Synapt instruments.

As opposed to drift tube ion mobility, the CCS of ions obtained from TWIM cannot be directly calculated from the drift time, and the drift time of calibrants with known CCS are required. The drift time values of these calibrants must be obtained under the same conditions as those for the ion of interest. The protocol for CCS calibration on TWIM has been proposed and has been successfully applied to soluble proteins.\textsuperscript{159} In this dissertation, the experimental CCS of protein complexes and their fragments generated from tandem mass spectrometry are calculated based on the calibration curve generated from the calibrants with similar sizes. The experimental CCS is further compared with the theoretical CCS to study the gas phase structure of protein complexes. However, when it comes to the analysis of native-like membrane proteins, the experimental CCS calculation
of low charged membrane proteins with the standard calibration protocol\textsuperscript{159} remains problematic as it underestimates the CCS of membrane proteins.\textsuperscript{160}

The resolution ($R_{TW}$) of TWIM in Waters Synapt instruments can be calculated by the equation shown as follows:

$$R_{TW} = \frac{1}{4} \left( \frac{2q[WH][WL]KE}{[WV]kTln2} \right)^{1/2}$$

where $q$ is the charge of the ion; $K$ is the mobility of the ion; $[WH]$ is the wave amplitude/height in V; $[WL]$ is the wavelength; $E$ is the DC electric field strength between the start and the end of the ion mobility cell; $[WV]$ is the wave velocity in m/s; $k$ is the Boltzmann’s constant; $T$ is the temperature in K.\textsuperscript{161} The maximum resolution of the singly charged 490 Da peptide and the 24+ 141 kDa alcohol dehydrogenase tetramer is 25.\textsuperscript{161} With an increase of the drift length to 13 m, TWIM coupled with Structures for Lossless Manipulations (SLIM) module has shown a resolution of ~ 45 for $m/z$ 622 and 922.\textsuperscript{162}

1.6.3. Theoretical CCS calculation

Gas phase structures of protein complexes are studied by comparing the experimental CCS values calculated from the ion mobility result with the theoretical CCS values calculated from known crystal structures of protein complexes.\textsuperscript{151,163} Projection approximation (PA),\textsuperscript{164,165} exact hard-sphere scattering (EHSS),\textsuperscript{166} trajectory method (TJM)\textsuperscript{167} and projected superposition approximation (PSA)\textsuperscript{168} are methods commonly used to calculate the theoretical CCS of protein complexes. The theoretical CCS can be calculated with PA, EHSS, and TJM via an open source software MOBCAL. Among all the different methods, the trajectory method (TJM) is considered to be the most reliable CCS calculation,\textsuperscript{151} however, it is limited to the calculation of relatively some molecules.
A new script has been developed to calculate the theoretical CCS of large protein complexes with TJM recently. The theoretical CCS calculated from the PA method has been shown to underestimate the actual CCS of protein complexes by 14%. The scaled PA method, in which the CCS calculated from the PA method is scaled with a 1.14 factor, is being used to calculate the theoretical CCS of protein complexes in this dissertation.

1.7. Instruments

1.7.1. Quadrupole time of flight (Q-TOF)

Waters Synapt G2 and G2S (Waters Corp., Wilmslow, U.K.) are used to conduct experiments on Q-IM-TOF instruments in this dissertation. There are two traveling wave ion guides (TWIG) before and after the ion mobility cell, referred to as Trap TWIG and Transfer TWIG, respectively, where the CID experiment can be conducted.

The SID device has been installed in both G2 and G2S, and it has been used to study the structure of non-covalent protein complexes. Different experiments can be conducted with SID installed in various locations (before and/or after the ion mobility cell). With SID installed before the ion mobility cell, the Trap TWIG is truncated so that the SID device sits between the truncated Trap TWIG and the ion mobility cell, and the conformation of the SID products can be studied with ion mobility, thus providing substructure information on protein complexes. With SID installed after the ion mobility cell, the Transfer TWIG is truncated so that the SID device sits between the ion mobility cell and the truncated Transfer TWIG. Therefore, protein complexes with different conformations can be separated by ion mobility and then dissociated by SID. In this
manner, the structure of protein complexes with different conformations is studied.\textsuperscript{137} With two SID devices installed both before and after SID, the protein complexes with different interactions can be sequentially dissociated, providing the assembly information on protein complexes.\textsuperscript{171}

In this dissertation, experiments shown in Chapter 2 – 5 are performed on Synapt instruments with a SID device installed. The results in Chapter 2, Chapter 3 and Chapter 5 are acquired with SID installed before the ion mobility cell. The results in Chapter 4 is acquired with SID installed after the ion mobility cell.

1.7.2. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer

A Bruker SolariX XR 15 T FT-ICR mass spectrometer with a ParaCell\textsuperscript{97,98} (Bruker Daltonics, Bremen, Germany) was used to conduct high-resolution experiments. The ParaCell has been shown to be able to improve the collection of long transients, which is necessary for protein complex studies.\textsuperscript{98,99} The FT-ICR has been shown to be able to provide high mass resolution measurement.\textsuperscript{99,172} With the application of absorption mode, the resolving power can be further improved via phase correction.\textsuperscript{173,174} Therefore, the FT-ICR is used to provide ultra-high resolution \textit{m/z} measurement of protein complexes in this dissertation.

Several different activations can be performed to study protein structure in the FT-ICR instrument: in-source CID is in the source region after the second ion funnel; both CID and ETD experiment are performed in the QCID collision cell between the quadrupole and the ICR cell; both SORI-CID and ECD experiments are conducted in the ICR cell, and the
energy of electrons can be set to as high as 30 eV so that EID experiment can also be performed.\textsuperscript{99,144,175,176}

The QCID collision cell has been modified so that both CID and SID experiment can be conducted, and the fragments can be sent to the ICR cell m/z detection.\textsuperscript{132} The experiment shown in Chapter 6 is conducted on the Bruker SolariX XR 15 T FT-ICR mass spectrometer with SID installed.
Chapter 2. Application of SID on Q-TOF platform: fundamental structural analysis

Some content in this chapter is derived from the paper Quintyn, Royston S.; Yan, J.; Wysocki, Vicki H., Surface-Induced Dissociation of Homotetramers with D2 Symmetry Yields their Assembly Pathways and Characterizes the Effect of Ligand Binding. *Chemistry & Biology* 2015, 22 (5), 583-592. (Co-first author with Royston S. Quintyn)

2.1. Introduction

Studying the interaction of protein complexes is essential for better understanding their functions.\(^2\) In the cellular process, the majority of proteins function as oligomers with symmetric structures.\(^{177}\) The misassembly of both membrane proteins and soluble protein complexes can contribute to diseases.\(^5,^{178}\) A previous study has shown that the assembly/disassembly pathways of protein complexes can be used to mimic their evolutionary pathways,\(^{179}\) thus studying disassembly pathway of protein complexes can help in gaining an understanding of the protein assembly pathway.

Native mass spectrometry has made it possible for the study of intact protein complexes in the gas phase,\(^{25}\) and the dissociation pathway of protein complexes and be further studied by tandem mass spectrometry.\(^{104}\) In protein complex studies, collision induced activation (CID) is the most commonly used among various activation methods in tandem mass spectrometry.\(^{103,104}\) However, because of the fact that one highly charged
monomer subunit typically gets ejected from the protein complex in CID dissociation, only limited connectivity information can be obtained by the protein assembly with CID.\textsuperscript{112} Previous studies have shown that the high charge states of protein monomers correlates with unfolding, indicating that a conformational rearrangement occurs during the CID process.\textsuperscript{35,115,180} Alternatively, surface induced dissociation (SID) has been shown to be able to produce more informative noncovalent fragments by dissociating protein complexes and provide folded dissociation products, which better reflect the native structure of protein complexes.\textsuperscript{114,131,133} For example, the SID of the tetradecamer, GroEL with a double-ring structure, produces efficient fragmentation into oligomers with different oligomeric states and the production of heptamer directly reflecting its native quaternary structure, while in CID, only highly charged monomers and their complementary 13mers were generated.\textsuperscript{134} In addition to the separation based on $m/z$ scale, coupling ion mobility (IM) with mass spectrometry provides additional separation based on the charge, size and shape of protein complexes, helping in monitoring the conformational change of protein complexes.\textsuperscript{31,181} The collision cross sections (CCS) of protein complexes in the gas phase can be calculated from the drift time obtained from ion mobility.\textsuperscript{150} Comparing the experimental CCS with their theoretical CCS calculated from the corresponding crystal structure and NMR structure provide insight into the conformation of protein complexes in the gas phase.\textsuperscript{35,159} The combination of SID and IM makes it possible to better separate different oligomers generated by SID fragmentation with a different size but similar $m/z$, and study the conformation of each of the fragment ions.
Streptavidin is a homotetramer with $D_2$ symmetry, which is a dimer of dimers.\textsuperscript{182} However, the production of highly charged streptavidin monomers from the tetramer in a previous CID study\textsuperscript{176}, which is consistent with typical CID, does not match its known connectivity information. The streptavidin – biotin complex has been shown to have a high binding affinity in the solution phase with a dissociation constant ($K_d$) of $4 \times 10^{-14}$ M.\textsuperscript{183,184} The biotin binding has also been shown to be able to increase the thermal stability of streptavidin.\textsuperscript{185}

In this chapter, streptavidin was used as a model system to explore the relationship between SID fragmentation patterns and the subunit interactions of protein complexes with known structure. An SID-IM-TOF instrument was used to study the SID fragmentation of protein complexes in three aspects: i) Study the dissociation pathway of streptavidin in SID. Streptavidin and neutravidin, which are two protein complex systems with similar known structures, were used to study the correlations between SID dissociation pathway and the protein subunit interactions. ii) The streptavidin – biotin system was also studied by using SID to study the effect of biotin on streptavidin tetramer in the gas phase. iii) The SID fragmentation pattern of streptavidin with different conformations induced by collisional activation in the ion source.

2.2. Method

2.2.1. Mass spectrometry

The MS and MS/MS experiments were conducted on a modified Waters Synapt G2S instrument (Waters Corp., Wilmslow, U.K.) with a SID device incorporated between a truncated Trap TWIG and the ion mobility cell (Figure 2.1).\textsuperscript{114,131} The schematic
presentation of the SID device is shown in Figure A.1. A nanoelectrospray ionization (nESI) source was used to generate protein complex ions. Each of the samples was filled into a 0.8 – 1.1 x 90 mm glass capillary (Kimble, Vineland, NJ, U.S.A.) pulled by a Sutter Instruments P-97 micropipette puller (Novato, CA, U.S.A.). The capillary voltage was 1.0 – 1.2 kV. The cone voltage and cone offset were set to 20 V. The Ar gas flow rate of the trap and transfer was set to 2 mL/min for MS and SID experiments, and was set to be 4 mL/min for CID experiments. The He flow rate was set to 120 mL/min and the N₂ flow rate in the ion mobility cell was 60 mL/min for all experiments.

Figure 2.1. A schematic of a modified Waters Synapt G2S with a SID device installed between the truncated Trap TWIG and the ion mobility cell. Reprinted from Ref 186 with permission American Chemical Society, Copyright (2016).¹⁸⁶

In MS experiments, all ions were detected in the TOF analyzer. In MSMS experiments, the ions were isolated in the quadrupole, dissociated by CID or SID, the fragment ions were then separated in the ion mobility cell and detected in the TOF analyzer.
CID experiments were conducted with the SID device set in transmission mode. CID-IM experiments were achieved by accelerating ions before getting into the Trap TWIG by increasing voltages on all the lenses before the entrance lens of the Trap TWIG. SID experiments were conducted with the SID device tuned to allow the ions collide with the surface and the other electrodes were tuned to maximize the transmission of fragment ions. The spectra were acquired in IM mode. The detailed tuning parameters for the SID device in transmission mode and SID mode are shown in Table A.1. The collision energy in eV is calculated by multiplying the acceleration voltage by the charge state.

The CCS calibration was conducted under the same experimental conditions described above by using standard proteins (transthyretin, avidin, concanavalin A, and serum amyloid P) as calibrants. The calibration curve was generated by following the protocol published previously. The $R^2$ value is higher than 0.998.

2.2.2. Surface preparation

The 12 mm × 18 mm gold coated surfaces (Evaporated Metal Films Corp, Ithaca, NY, U.S.A.) used in the experiment were with 100 nm gold coated on 1 nm titanium via glass support. The protocol for surface preparation was developed based on the previous study. The surface is rinsed with methanol and dried. After that, the surface is placed in a UV cleaner for 15 minutes. The UV cleaned surface is then immersed in 1 mM FC12 (fluorinated hydrocarbon thiol, CF$_3$(CF$_2$)$_6$CH$_2$CH$_2$SH) solution in ethanol for 24 - 48 h for the coating via gold-thiol reaction. After that, the coated surface is sonicated in ethanol six times (1 minute for each sonication). The modified surface is stored in ethanol before use. The surface was freshly prepared and replaced every time when the instrument was vented.
2.2.3. Sample preparation

Streptavidin and neutravidin were both purchased from Thermo Scientific Pierce Biotechnology (Rockford, IL, U.S.A.). The protein samples stock solutions were prepared by dissolving protein samples in water at concentrations of 205 µM for streptavidin tetramer and 143 µM for neutravidin tetramer. The aliquots of stock solutions were stored in – 20 °C freezer. The stock sample solutions were thawed and buffer exchanged into 100 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, U.S.A.) with 6-kDa cut-off size exclusion chromatography spin columns (Bio-Rad, Hercules, CA, U.S.A.). The protein solutions were then diluted to a protein complex concentration of 10 µM with 100 mM ammonium acetate (AA). Streptavidin – biotin complex was generated by adding 800 µM biotin (Sigma-Aldrich, St. Louis, MO, U.S.A.) into 10 µM streptavidin solution and the final biotin concentration was 60 µM (1.5 biotins per one streptavidin subunit). Charge reduced protein complexes were generated by mixing the sample solution with 100 mM triethylammonium acetate (TEAA, Sigma-Aldrich, St. Louis, MO, U.S.A.) in a ratio of 4:1 (v : v). \textsuperscript{188,189} Supercharged protein complexes were generated by adding 1% m-nitrobenzyl alcohol (m-NBA, St. Louis, MO, U.S.A.) into the sample solution. \textsuperscript{190,191}

2.2.4. Theoretical CCS calculation

Crystal structures of streptavidin (PDB: 1SWB) and neutravidin (PDB: 1VYO) from the Protein Data Bank were used for theoretical CCS calculation. Water and other adducts were removed from the PDB file. Hydrogen atoms were then added to the crystal structures by using the open source Open Babel 2.3.2 software with pH set to be 7. The theoretical CCS values of the protein complexes were calculated using the Projection Approximation
(PA) algorithm\textsuperscript{192} implemented in MOBAL.\textsuperscript{164,193} The CCS values were then corrected as previously described\textsuperscript{35} by multiplying the PA value by 1.14.\textsuperscript{26}

2.3. Buffer condition optimization for SID of streptavidin

Ammonium acetate (AA) is the most commonly used buffer for protein complex studies.\textsuperscript{27} Protein complexes dissolved in ammonium acetate solution generates “normal” charged ions in nESI. By adding solution additives, the charge state distribution of protein complexes shifts.

![Figure 2.2](image)

Figure 2.2. Mass spectra of supercharged, normal charged and charge reduced streptavidin. A) MS of streptavidin in 100 mM ammonium acetate with 1% m-NBA added. B) MS of streptavidin in 100 mM ammonium acetate. C) MS of streptavidin in 80 mM ammonium acetate and 20 mM TEAA.
With the addition of TEAA, charge-reduced ions are generated,\textsuperscript{188} and ions with higher charge state (supercharging) are generated with the addition of m-NBA.\textsuperscript{190,191} Streptavidin dissolved in AA, AA + TEAA, and AA + m-NBA were sprayed, and their corresponding mass spectra are shown in Figure 2.2.

The streptavidin tetramer has already partially dissociated into dimers when sprayed from AA + m-NBA as shown in Figure 2.2A. When dissolved in 100 mM ammonium acetate, the average charge state of the predominant streptavidin tetramer peak is 15+ (Figure 2.2B). With the addition of m-NBA and TEAA, the charge states of predominant streptavidin tetramer peak observed are 18+ and 11+, respectively (Figure 2.2A and C). The CCS results of streptavidin tetramers with different charge states (Figure 2.3) further confirmed that the CCS values of ions generated from supercharged conditions (green dots) is higher than theoretical CCS values calculated from the crystal structure of streptavidin (dash line), indicating that streptavidin tetramers ions generated from AA + m-NBA solution are unfolded. The unfolding of streptavidin tetramer with higher charge state is expected because of the chemical and/or thermal denaturation from a higher concentration of supercharging reagent (m-NBA) during the ionization process.\textsuperscript{194} Based on the CCS results shown in Figure 2.3, the CCS values of normal charge and charge reduced streptavidin tetramer ions generated from AA and AA + TEAA solution is similar to the theoretical CCS. Therefore, streptavidin dissolved AA and AA + TEAA were further studied by tandem mass spectrometry.
Figure 2.3. CCS of streptavidin tetramers under different buffer conditions. Streptavidin tetramers generated from AA + TEAA, AA and AA + m-NBA are represented by blue, red and green dots, respectively. The dashed line represents the theoretical CCS calculated from the crystal structure (PDB: 1SWB) by using corrected PA method. The error bars were calculated from the results from three repeats.

The 15+ streptavidin tetramer (Figure 2.2B) from normal charge streptavidin was selected and dissociated by SID. The relative intensities of fragments generated under different SID energies are shown in Figure 2.4.
Figure 2.4. Plots of the relative intensity of SID fragments from the 15+ streptavidin tetramer. A) Energy-resolved plots showing the relative abundance of SID fragments produced from the normal charged 15+ streptavidin tetramer. B) Stack column plot of the relative intensity of the various charged SID fragments produced from the normal charged 15+ streptavidin tetramer.
As shown in the energy-resolved mass spectrometry (ERMS) plots in Figure 2.4A, the majority of the tetramer has already dissociated into dimers at the lowest SID collision energy studied (300 eV), matching well with the known structure of streptavidin as a dimer of dimers with relatively weak interaction between dimers.\(^{182}\) With the increase of SID collision energy, the relative intensity of monomers increases while the relative intensity of dimers decreases, indicating the secondary fragmentation from dimers to monomers when higher SID collision energy is involved. The relative intensity of the SID fragments with different charge states was further analyzed and shown Figure 2.4B. Within the SID collision energy range studied (300 eV – 2250 eV), the major fragments were monomer and dimers, and low intensity of tetramers and trimers was observed.

With the increase of SID collision energy, the relative intensity of 9+, 8+ and 7+ dimers decreases, while the relative intensity of 3+, 4+ and 5+ monomers increases. With the decrease of the intensity of 8+ and 9+ dimers, the increase of 5+ monomer gets slower, indicating the dissociation from 9+ dimer to 5+ and 4+ monomers, as well as the dissociation from 8+ dimer to 5+, 4+ and 3+ monomers. When the SID collision energy is higher than 1800 eV, the decrease of the relative intensity of 7+ dimer with the increase of the relative intensity of 3+ and 4+ monomer suggests the dissociation from 7+ dimer to 4+ and 3+ monomers. Therefore, within the SID collision energy range of 300 eV – 2250 eV, dimers dissociates into monomers with charges symmetrically distributed on the monomers (7+ → 4+ and 3+).
The CCS distributions of dimers generated from the 15+ streptavidin tetramer were calculated by using the drift time distribution obtained from ion mobility. As the majority of 15+ streptavidin tetramer has already been dissociated into dimers at the lowest SID collision energy studied, the CCS values of dimers generated from 15+ streptavidin at the SID collision energy of 300 eV was calculated to minimize the further activation of dimers during SID, and the CCS distribution of dimers with different charge states are shown in Figure 2.5.

![Figure 2.5. CCS distributions of streptavidin dimers produced from SID of 15+ streptavidin tetramer at a collision energy of 300 eV. The dashed line represents the theoretical CCS calculated from the dimer clipped from the crystal structure.](image-url)
Figure 2.5 shows that with the increase of charge state, the dimers are more unfolded. The 6+ dimer has a compact structure with CCS similar to the theoretical CCS, while the 7+ dimer starts to unfold and low intensity of unfolded conformation is observed. The 8+ dimer, however, is a combination of multiple conformations. The relative intensity of the distinct unfolded conformation is similar to that of the more compact conformation, which has the CCS close to the theoretical CCS. 9+ dimer is fully unfolded with wide CCS distribution and no compact conformation remaining. The unfolding of highly charged dimers might be because of the Coulombic repulsion. Therefore, in order to study the quaternary structure of protein complexes, precursors with lower charge state is needed and the SID of charge reduced protein complexes are studied.

2.4. SID dissociation pathway of streptavidin and neutravidin

As discussed above, charge reduced protein complex ions can better preserve the compact structure, thus charge reduced streptavidin and neutravidin were studied using SID and CID. Both streptavidin and neutravidin are homotetramers with similar structures as a dimer of dimers.\textsuperscript{182,185,195} Their crystal structures are shown in Figure 2.6A and D. 11+ is the predominant charge state when proteins dissolved in 80 mM AA and 20 mM TEAA were sprayed via nESI source (Figure 2.2). 11+ streptavidin and neutravidin tetramer ions were therefore selected in the quadrupole and further dissociation by SID and CID. The fragment ions were separated by ion mobility before TOF detection. The SID and CID spectra acquired under different collision energies are shown in Figure 2.6. In SID, both streptavidin and neutravidin dissociate into monomer, dimer and trimers at an SID collision energy of 770 eV, while highly charged monomer (6+ and 5+), which contains around half
of the precursor charge state, and their complementary trimers can be observed in CID spectra at a CID collision energy of 1650 eV.

Figure 2.6. SID and CID spectra of 11+ streptavidin and neutravidin tetramers under different collision energies. A) Crystal structure of streptavidin (PDB: 1SWB). B) SID and C) CID of 11+ streptavidin tetramer under different collision energies. D) Crystal structure of neutravidin (PDB: 1VYO). E) SID and F) CID of 11+ neutravidin tetramer under different collision energies. Both streptavidin and neutravidin are homotetramers. Adapted from Ref 136 with permission from Elsevier.136

Different from the observation of the production of monomers, dimers and trimers at high SID collision energy (770 eV), at low SID collision energy (330 eV), both 11+ streptavidin and neutravidin tetramer mainly fragments into dimers (Figure 2.6B) and the charge states of the dimers (mainly 5+ and 6+) are around half of the precursor charge state. The relative intensity of the tetramer precursor is higher in the neutravidin spectrum than that in the streptavidin spectrum at an SID collision energy of 330 eV (Figure 2.6B and E).

To analyze the relationship between SID fragmentation pattern and protein interface information, the interface interactions of streptavidin and neutravidin were
analyzed. Because the interface strength has been shown to be proportional to the interface area when the interface area is larger than 1000 Å², it is valid to use interface area to represent the interface strength between subunits when more than 1000 Å² interface area is involved. The streptavidin (PDB ID: 1SWB) and neutravidin (PDB ID: 1VYO) crystal structures were used for the analysis using PISA analysis. The crystal structure of streptavidin and neutravidin are shown in Figure 2.6A. The interfacial area, number of hydrogen bonds and the number of salt bridges were obtained from PISA and are shown in Figure 2.7.

![Figure 2.7](image.png)

Figure 2.7. Relative interactions between subunits in streptavidin and neutravidin tetramers. The interactions for A) streptavidin and B) neutravidin tetramer indicated by interaction surface areas, the number of hydrogen bonds (HB), and the number of salt bridges (SB).

Different combinations of possible noncovalent fragments from the tetramer are listed and the total interface areas involved to generate those fragments were calculated and shown in Table 2.1.
For both streptavidin and neutravidin, the production of I-IV and II-III dimers (1176 Å² for streptavidin and 1388 Å² for neutravidin) has the lowest interface area cleaved comparing with the interface areas required to produce one monomer and one trimer (2139 Å² for streptavidin and 2583 Å² for neutravidin), or four monomers (4278 Å² for streptavidin and 5166 Å² for neutravidin). The SID result that dimers are the main products at low SID collision energy (330 eV), suggests the cleavage of the smallest interface at low SID collision energy for both streptavidin and neutravidin is favorable. The statement is further validated by comparing the experimental CCS of dimers generated from 11+ streptavidin and neutravidin with the theoretical CCS generated from the crystal structure of dimers (I-II, I-III and I-IV dimers) in Figure 2.8. The theoretical CCS values for I-II (or III-IV) dimer and I-III (or II-IV) dimer are like each other and larger than that of and I-IV (or II-III) dimer. The experimental CCS of dimers generated from 11+ tetramers are
consistent with the theoretical CCS of I-IV (or II-III) dimers, which are dimers generated with the lowest interface area involved in the cleavage of tetramers, assuming there was no collapse before the ion mobility measurement (Figure 2.8).

Figure 2.8. CCS distributions of dimers generated from streptavidin and neutravidin tetramers and the corresponding theoretical CCS values. CCS distributions of dimers generated from 11+ (A) streptavidin and (B) neutravidin tetramers. The red dotted line, the black dotted line and the black solid line represents the theoretical CCS calculated from I-II (or III-IV) dimer, I-III (or II-IV) dimer and I-IV (or II-III) dimer clipped from the crystal structure, respectively. Adapted from Ref 136 with permission from Elsevier.

The narrow CCS distributions of dimers show that the dimers generated from the SID of 11+ streptavidin and neutravidin are compact. Therefore, at the collision energy of 330 eV, the smallest interface of the tetramer (1176 Å² for streptavidin and 1388 Å² for neutravidin) was cleaved, and I-IV and II-III dimers were generated in SID. The fact that dimer-dimer interface area (1388 Å²) in neutravidin is higher than that in streptavidin (1176 Å²)
Å²) also matches with the observation that the relative intensity of the 11+ neutravidin tetramer precursor is higher than that of the 11+ streptavidin tetramer precursor at the same collision energy (Figure 2.6B and E). As shown in Figure 2.6B and E, with the increase of SID collision energy from 330 eV to 770 eV, the production of monomers and trimers can also be observed in the SID spectra of the 11+ streptavidin and neutravidin tetramers, indicating the existence of other pathways in SID fragmentation.

Figure 2.9. Energy-resolved mass spectrometry plots of the relative intensities of SID fragments produced from streptavidin and neutravidin. Plots of the relative abundance of SID fragments produced from 11+ (A) streptavidin and (C) neutravidin as a function of SID collision energy. $E_{D,\text{max}}$ is the collision energy where the relative intensity of dimer reaches the maximum. $E_{D,1/2\text{max}}$ is the collision energy where the relative intensity of dimers reaches half of the maximum. Plots of the relative abundance of the SID fragments with different charge states produced from 11+ (B) streptavidin and (D) neutravidin tetramers as a function of SID collision energy. Adapted from Ref 136 with permission from Elsevier.136 Two repeats of neutravidin results courtesy of Royston Quintyn.
The SID dissociation pathways of tetramers were studied by analyzing the relative intensities of SID fragments generated under different SID collision energies (Figure 2.9). The energy-resolved mass spectrometry (ERMS) plots were generated by calculating the relative intensity of each of the SID fragments divided by the sum of the intensities of all the SID fragments. The ERMS plot of the SID products of 11+ streptavidin tetramer (Figure 2.9A) shows that when the SID collision energy is lower than 330 eV, the relative intensity of dimers increases with the decrease of the relative intensity of tetramers, suggesting the dissociation of tetramer into dimers. When the SID collision energy is 330 eV – 770 eV, low intensity of monomers and trimers were generated showing a minor CID like dissociation pathway. When the SID collision energy is higher than 770 eV, the relative intensity of dimers decreases while the relative intensity of monomers increases, indicating the secondary dissociation from dimers to monomers. The statement can be further confirmed by the ERMS plot of SID fragments from the 11+ streptavidin tetramer with different charge states (Figure 2.9B). In the ERMS plot in Figure 2.9B, with the increase of SID collision energy, the relative intensity of 3+ and 4+ monomers increase with the decrease of the relative intensity of 7+ and 6+ dimers. The relative intensity of 5+ & 4+ dimers, and 6+ & 5+ monomers are relatively constant. The trend in the plot indicates that when higher SID collision energy is applied, the 11+ streptavidin tetramer symmetrically dissociates into 6+ and 5+ dimers. With the increase of SID collision energy, 7+ dimers further dissociate into 4+ and 3+ monomers, 6+ dimers dissociate into two 3+ dimers. The secondary fragmentation results are also confirmed by SID-IM-SID experiments. Therefore, in the SID of the 11+ streptavidin tetramer, the dimer-dimer
interface with the lowest total interface area (1176 Å²) is cleaved. The major pathway is tetramer → dimer → monomer. There is a minor pathway in which tetramers dissociate into monomers and trimers.

The SID ERMS plots of the 11+ neutravidin tetramer show similar trend to streptavidin (Figure 2.9C). Neutravidin is a dimer of dimers, hence dimers are produced from tetramers at low SID collision energy (330 eV) and the experimental CCS of dimers matches with the corresponding theoretical CCS calculated from the crystal structure of the I-IV (or II-III) dimer (Figure 2.8B), indicating the cleavage of the smallest dimer-dimer interface (1388 Å²) as shown in Table 2.1. With the increase of collision energy, the relative intensities of 6+ and 7+ dimers decrease while the relative intensities of 3+ and 4+ monomers increase indicating the dissociation of dimers into monomers. (Figure 2.9D) Therefore, the major pathway is tetramer → dimer → monomer. There is a minor pathway in which tetramers dissociate into monomers and trimers.

The collision energies at which the half of the maximum relative abundance of streptavidin and neutravidin dimers (E_{D,1/2max}) is observed are labeled in Figure 2.9A and C. The E_{D,1/2max} is 210 eV for streptavidin and 360 eV for neutravidin. The higher SID collision energy required for cleaving the dimer-dimer interface of neutravidin matches with the fact that the dimer-dimer interface area (1388 Å²) in neutravidin is higher than that in streptavidin (1176 Å²). The relationship between the energy required for protein interface cleavage and protein interface area has been studied with more systems with similar structure.¹³⁶
In contrast to SID, unfolded monomer subunits were ejected from the tetramer in CID, and only highly charge monomers and their complementary trimers were observed (Figure 2.6C and F).

Figure 2.10. Energy-resolved plots of the relative abundance of CID fragments produced 11+ (A) streptavidin and (B) neutravidin tetramers. Adapted from Ref 136 with permission from Elsevier. As two repeats of neutravidin results courtesy of Royston Quintyn.

As shown in Figure 2.6C and F, the 6+ streptavidin monomer and 5+ neutravidin monomer are the main charge states for the monomers generated by CID, which are around
half of the precursor charge state. The behavior matches with previous studies showing that one subunit unfolds during the multistep collision process in CID and gets ejected from the protein complex. The charge state distributions of the fragments generated does not change with the increase of collision energy (Figure 2.6C and F). The generation of monomer and trimers as the major fragments in CID (Figure 2.10) is consistent at different collision energies. Therefore, CID fails in showing the interactions between different interfaces these protein complex assemblies.

In SID of streptavidin and neutravidin, the smallest interface (dimer-dimer interface) gets cleaved first and the major SID fragmentation pathway is tetramer → dimer → monomer, which is the reverse of their assembly pathway. Combing with the result from SID of transthyretin, which is a homotetramer with similar size and structure, the collision energy required for the cleavage of the dimer-dimer interface is well correlated with the dimer-dimer interface area. However, CID does not provide the relative interface information on streptavidin and neutravidin.

2.5. Effect of biotin binding on streptavidin

The effect of ligand binding on protein complexes was further studied using SID. Streptavidin-biotin system was chosen as a model system to study the protein-ligand binding. Biotin has high binding affinity to streptavidin in the solution phase (K_d = 4 x 10^{-14} M), and the binding of has been previously shown to be able to increase the thermal stability of streptavidin. The 11+ streptavidin - biotin tetramer was selected and dissociated with SID, and the result is shown in Figure 2.11. Similar to the SID fragmentation pattern of the 11+ apo form of streptavidin tetramer (Figure 2.6B), the 11+
streptavidin - biotin also produces dimers 6+ and 5+ dimers as major SID fragments at an SID collision energy of 330 eV (Figure 2.11A). There are 0 – 2 biotin binding to the dimers generated by SID with the apo form as the major products.

At SID collision energy of 330 eV, CCS distributions of the 11+ streptavidin tetramer remaining from the dissociation of the apo form of streptavidin tetramer and the 11+ streptavidin - biotin tetramer remaining from the dissociation of the holo form of the streptavidin-biotin tetramer are shown in Figure 2.11B.

Figure 2.11. Biotin binding stabilizes the streptavidin tetramer. (A) SID of 11+ streptavidin - biotin complex at a collision energy of 330 eV. (B) CCS distribution of apo- (left) and holo- (right) streptavidin tetramer under an SID collision energy of 330 eV. Inset: the binding pockets of streptavidin - biotin (PDB: 3RY2). The theoretical CCS of calculated from the crystal structure of the streptavidin tetramer is indicated by the dotted line. Adapted from Ref 136 with permission from Elsevier.136
As shown in Figure 2.11B, under the same SID activation energy, the *holo* form of streptavidin is compact and the CCS matches with the theoretical CCS, indicating the native-like conformation, while the *apo* form of streptavidin is more unfolded with two distinct conformations, suggesting a stabilization of the streptavidin tetramer upon biotin binding.

Figure 2.12. Energy resolved plots of the relative intensity of different fragments generated by SID activation of the apo (red) and holo (blue) forms of the 11+ streptavidin. Adapted from Ref 136 with permission from Elsevier.136
The comparison of the relative intensity of each SID fragments generated from apo and holo forms of streptavidin shows (Figure 2.12) that the fragmentation pattern is similar for the apo and holo forms of streptavidin. At the SID energies when complete biotin loss has not occurred, the relative intensity of undissociated tetramer generated from the holo form of streptavidin (blue) is less than 10% lower than that from the apo form streptavidin (red), suggesting slightly more fragmentation with the holo form of streptavidin. The more rigid structure of the holo form of streptavidin with a lower number of degrees of freedom might be the reason that leads to more internal energy contributes to dissociation, while the internal energy of apo form of streptavidin was partially contributed to unfolding.136

2.6. SID fragmentation of different conformations of streptavidin

Streptavidin was used as a model system to further test the ability of SID to distinguish protein complexes with different conformations. The experiment was conducted by increasing the sampling cone voltage from 20 V to 100 V, 120 V, and 150 V. The streptavidin ions activated in the source region were then isolated in the quadrupole and dissociated by SID or CID. The 11+ streptavidin tetramer was selected as the precursor in the cone activation experiment. The CCS distributions of 11+ streptavidin tetramer activated by different cone voltages are shown in Figure 2.13A. When the cone voltage is lower than 120 V, the CCS distributions of 11+ streptavidin tetramer are similar and they match with the theoretical CCS calculated from the crystal structure of streptavidin (PDB: 1SWB). As the cone voltage gets to 150 V, the CCS distribution shifts to lower values, indicating a collapse of the 11+ streptavidin tetramer. In order to explore whether the
conformational change observed in ion mobility can also be monitored by SID and whether SID can provide more information, the 11+ streptavidin tetramer ions activated by cone activation voltages were then fragmented by SID, and the SID spectra are shown in Figure 2.13B.

Figure 2.13. CCS distributions and SID fragmentation pattern of 11+ streptavidin tetramer obtained under different cone voltages. (A) CCS distribution of 11+ streptavidin tetramer and (B) their corresponding 550 eV SID spectrum under 20 V, 100 V, 120 V, and 150 V cone activation voltages. The dashed line represents the CCS calculated from the crystal structure of streptavidin (PDB: 1SWB).
As shown in Figure 2.13B, the 11+ streptavidin tetramer dissociates into monomers, dimers and trimers at SID collision energy of 550 eV when the cone voltage is set to 20 V, which is considered as “gentle” condition to minimize the activation in the source. At cone voltage of 20 V, the 6+ and 5+ dimers are the dominant species generated from 11+ streptavidin and the relative intensity of the precursor peak is approximately 50%. The low intensity of 5+ streptavidin monomer indicates a minor CID like pathway. When the cone voltage is 120 V, the relative intensity of 11+ streptavidin tetramer and 5+ monomer increases dramatically, suggesting the more unfolded structure of the precursor even though there is no significant shift in the CCS distribution. When the cone activation is 150 V, the 11+ streptavidin ions collapses, and the SID spectrum shows more undissociated 11+ streptavidin tetramer and 5+ monomer, and less dimer production, which is a more CID like fragmentation pattern, suggesting a significant conformational change of the precursor ions during cone activation. SID fragmentation pattern helps in distinguishing the precursor ions with different conformations. The subtle conformational change, which cannot be well monitored by ion mobility, can also be distinguished by SID. Therefore, the combination of ion mobility and SID helps in better understanding the conformational change of protein complexes.

The cone activated precursor ions were also dissociated by CID, and the results are shown in Figure 2.14. At CID collision energy of 880 eV, 6+ monomer was ejected from 11+ streptavidin tetramer. The CID fragmentation patterns are similar for the precursors activated by different cone voltages. The conformational change of the streptavidin
tetramer ions activated in the source region (Figure 2.13A), does not affect the CID fragmentation pattern (Figure 2.14).

Figure 2.14. 880 eV CID spectra of 11+ streptavidin tetramer under different cone activation voltages.

SID is, therefore, capable of monitoring the conformational change of 11+ streptavidin tetramers during cone activation, while CID fragmentation pattern is not
affected by the conformational change of the precursor ion during the cone activation. In order to explore how different conformations affect the SID fragmentation pattern, more systems have been studied by using IM-SID, in which precursor ions with different conformations can be separated in ion mobility and dissociated by SID, to show that SID is capable of distinguishing different conformations with similar CCS and the results have been published.137

2.7. Conclusions

Charge reduced streptavidin ions were studied by SID in a Q-IM-TOF instrument because more compact SID fragments can be generated from the charge reduced streptavidin tetramer comparing those with higher charge states. The combination of ion mobility and SID has shown to be capable of providing quandary structural information on globular protein complexes with monomer structures that show no intertwining/domain swapping. For streptavidin and neutravidin tetramers, which are dimer of dimers, the smallest interfaces (dimer-dimer interface) of the tetramers are cleaved first in SID. The major gas phase SID fragmentation pathways for both streptavidin and neutravidin are shown to be tetramer → dimer → monomer, which are the reverse of their assembly pathways in solution phase. Also, combined with the result from SID of transthyretin,136 the good correlation between the SID collision energy required for the cleavage of the dimer-dimer interface and the dimer-dimer interface area of those three tetramers indicates that SID can provide insight into the interface of globular protein complexes with monomer structures that show no intertwining/domain swapping.
The SID has also been successfully applied to the study of ligand binding protein complex. In the conformational study of streptavidin tetramer generated from the SID of biotin binding streptavidin, the observation of more compact conformation in the *holo* form of the streptavidin tetramer than that in *apo* form, demonstrate that the biotin binding stabilizes the native-like conformation of streptavidin during SID activation. Therefore, SID has shown the potential to study the stability of ligand binding protein complexes.

When activated in the source region, the protein complex experiences a conformational change. Different SID fragmentation patterns of protein complex ions were observed from precursor ions with different conformations generated from in-source activation, thus the SID fragmentation pattern has been shown to be sensitive to conformational change. Therefore, SID is capable of monitoring conformational changes of protein complexes.
Chapter 3. Application of SID on Q-TOF platform: different previously uncharacterized systems

3.1. Protein complex: lambda exonuclease

Some content in this chapter is derived from the paper Xinlei Pan, Jing Yan, Aalapi Patel, Vicki H. Wysocki, and Charles E. Bell, Mutant Poisoning Demonstrates a Nonsequential Mechanism for Digestion of Double-Stranded DNA by λ Exonuclease Trimers, *Biochemistry* 2015, 54 (3), 942-951.

3.1.1. Introduction

λ exonuclease (λexo) is an enzyme in phage λ recombinant system that binds to double-stranded DNA (dsDNA) end and releases 5’-mononucleotides. The crystal structure of λ exonuclease revealed that it is a ring-shaped trimer with DNA binding to its center channel. To better understand the active sites of λ exonuclease, the inactive mutant (K131A) was mixed and incubated with wild-type (WT), and the activity was studied. It was observed that the incubation of λexo wild type (WT) with K131A mutant results in no loss of activity (Figure 3.1). Understanding whether subunit exchange has happened during the incubation is essential to understanding the contributor to the observed
activity of λ exonuclease. In this chapter, the combination of native mass spectrometry and surface induced dissociation (SID) was used to obtain the stoichiometry information on heterooligomer λexo. The stoichiometry of various oligomers in the λexo wild-type (WT) and K131A mutant mixture was studied by using native mass spectrometry. SID was used to dissociate the complexes into subunits for confirming the subunit exchange behavior. The results have already been published, and some content in this chapter is derived from the paper from X. Pan et al. 201

![Graph showing exonuclease reactions with WT λexo with 1 h incubation of equal amounts (5 μM) of WT and 6xHis-K131A at 37 °C shows no loss of activity. Adapted from Ref 201 with permission from American Chemical Society, Copyright (2015).](image)

Figure 3.1. Exonuclease reactions with WT λexo with 1 h incubation of equal amounts (5 μM) of WT and 6xHis-K131A at 37 °C shows no loss of activity. Adapted from Ref 201 with permission from American Chemical Society, Copyright (2015). 201

3.1.2. Method

The samples were prepared by Xinlei Pan from Dr. Charles Bell’s lab in the Department of Biological Chemistry and Pharmacology at The Ohio State University. A 1:1 mixture of K131A mutant and wild-type (WT) λexo proteins was analyzed in the
present study. The samples with 100 μM protein concentration were buffer exchanged into 100 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, U.S.A.) by using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA, U.S.A.), and analyzed by a modified Waters Synapt G2-S HDMS mass spectrometer (Waters Corp., Wilmslow, U.K.) with an SID device installed before the ion mobility cell as previously described in Chapter 2.2.1.114,131 A nanoelectrospray ionization (nESI) source was used in the present study. The capillary voltage was 1.0 – 1.5 kV and the sampling cone voltage was 20 V. The source pressure was $5 \times 10^{-3}$ mbar, the gas flow rate to the helium cell and ion mobility cell was 120 mL/min and 60 mL/min, separately. The pressure in the TOF was $7 \times 10^{-7}$ mbar. The spectra were acquired in IM mode. The detailed tuning parameters for the SID device in transmission mode and SID mode are shown in Table A.1. Each of the peaks in the MS was isolated by the quadrupole and further dissociated by SID at the acceleration voltage of 50 V.

3.1.3. Results

The molecular weight of the WT λexo subunit is 26,190 Da. The molecular weight of K131A mutant subunit is 57 Da lower than that of WT λexo subunit. The hetero and homo trimers in the mixture of untagged K131A mutant and WT cannot be distinguished in the MS in the Q-TOF instrument because the peaks are broad. Thus 6xHis-tag (sequence: MGSSHHHHHHHSSGLVPR) was added to the N-terminus of one of the species, which added to 1,882 Da, to obtain distinguishable peaks for different subunits by tandem mass spectrometry. Both the mixture of untagged K131A and 6xHis-WT and the mixture of 6xHis K131A and untagged WT λexo proteins were prepared for the present study. However, the mixture of 6xHis K131A and untagged WT λexo protein precipitates during
buffer exchange, indicating that it was not stable in 100 mM ammonium acetate. The mixture of untagged K131A and 6xHis-WT is relatively more stable in 100 mM ammonium acetate than the mixture of 6xHis K131A and untagged WT λexo. Therefore, the mixture of untagged K131A and 6xHis-WT was used to analyze the stoichiometry of the subunits in the mixture. The untagged K131A and 6xHis-WT were also studied individually. The theoretical molecular weight difference for untagged K131A and 6xHis-WT is 1,939 Da. The mass spectra of K131A mutant, 6xHis-WT, and the mixture of K131A mutant and 6xHis-WT are shown in Figure 3.2.

![Figure 3.2. Native nanoelectrospray mass spectra of hybrid λexo trimers. (A, B) Spectra for untagged K131A mutant (A) and 6xHis-WT (B) show the expected peaks for trimers. (C) Spectrum for a mixture of untagged K131A and 6xHis-WT shows the expected peaks for hybrid trimers. Reprinted from Ref 201 with permission from American Chemical Society, Copyright (2015).](image-url)

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Figure 3.2A and B show a clear mass shift of untagged K131A trimer and 6xHis-WT trimer. In the MS of the mixture shown in Figure 3.2C, the observation of additional peaks that do not correspond to those in Figure 3.2A and B indicate the production of new species. Because the 6xHis-WT protein (not the untagged K131A protein) partially precipitates in the ammonium acetate buffer, the intensities of peaks for species containing 6xHis-WT subunits were lower than expected in both Figure 3.2B and C. The theoretical mass of K131A and 6His-WT are 26132.75 Da and 28071.90 Da, respectively. The theoretical mass of each of the trimers with different ratios of subunits was compared with the experimental mass calculated from the MS of the K131A and 6His-WT mixture (Table 3.1). The experimental mass of each species is higher than the corresponding theoretical mass indicating salt adducts binding to each of the species.

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Table 3.1. Mass of trimers in the mixture of 6xHis-WT and K131A λexo trimers
Based on the results shown in Table 3.1, the molecular weights of the species obtained from the MS match the theoretical molecular weights of (K131A)$_3$, (K131A)$_2$(6xHis-WT)$_1$ and (K131A)$_1$(6xHis-WT)$_2$. To further verify the stoichiometry of each of the species shown in Figure 3.2C, each of the trimer peaks was isolated by the quadrupole and dissociated by SID. The SID of 6xHis-WT and K131A at 50 V acceleration voltage are shown in Figure 3.3 and Figure 3.4.

Figure 3.3 is a representative SID spectrum of the $16 + (K131A)_2$(6xHis-WT)$_1$ peak selected from Figure 3.2C. From Figure 3.3, four different species were generated by SID, untagged K131A monomer, 6xHis-WT monomer, (K131A)$_1$(6xHis-WT)$_1$ and (K131A)$_2$. The charge state distributions of each of the fragment species are highlighted in different colors. The higher intensity of the untagged K131A (highlighted in red) than that of the 6xHis-WT monomer (highlighted in blue), and the higher intensity of (K131A)$_1$(6xHis-WT)$_1$ (highlighted in grey) than that of (K131A)$_2$ (highlighted in green) indicates that (K131A)$_1$(6xHis-WT)$_1$ is the complementary fragment of untagged K131A monomer and (K131A)$_2$ is the complementary fragment of the 6xHis-WT monomer. The SID results further confirm that the precursor is (K131A)$_2$(6xHis-WT)$_1$ and there are two dissociation pathways in the SID process.
Figure 3.3. Surface induced dissociation (SID) tandem mass spectrum of 16 + (K131A)₂(6xHis-WT)₁ precursor from the Lambda Exonuclease mixture of K131A and HisWT (50 V acceleration voltage). Peaks for K131A and HisWT monomers, (K131A)₃(HisWT)₁ and (K131A)₂ dimers are highlighted with red, blue, grey and green separately. Reprinted from Ref 201 with permission from American Chemical Society, Copyright (2015)²⁰¹

Similarly, the other trimers peaks shown in Figure 3.2C were also fragmented by SID and the SID spectra of the other trimers are shown in Figure 3.4. The SID results match with the stoichiometry information obtained from the mass spectra.
Figure 3.4. Surface induced dissociation (SID) tandem mass spectra of (A)16 + (K131A)$_1$(HisWT)$_2$ and (B) 15+ (K131A)$_3$ precursors from the Lambda Exonuclease mixture of untagged K131A and 6xHis-WT (50 V acceleration voltage).

3.1.4. Conclusions

The mixture of K131A mutant and 6xHis-WT was studied by native mass spectrometry and tandem mass spectrometry. Based on the molecular weights calculated from the charge state distribution in the mass spectrum and the SID fragmentation pattern in the MS/MS spectra, the existence of (K131A)$_3$, (K131A)$_2$(6xHis-WT)$_1$ and (K131A)$_1$(6xHis-WT)$_2$ have been confirmed, demonstrating subunit exchange during incubation. The combination of results from the activity study and the mass spectrometry analysis lead to the conclusion that trimers containing only one or two WT subunits
contribute significantly to the observed activity.\textsuperscript{201} Therefore, SID is capable of verifying the stoichiometry of different species in the λexo trimer.

3.2. DNA-protein complex: nucleosome

3.2.1. Introduction

Surface induced dissociation (SID) can provide quaternary structural information on protein complexes and stoichiometry information on RNA-protein complexes.\textsuperscript{105,202,203} However, collision induced dissociation (CID), a commercially available activation method, fails to provide enough energy for the dissociation of large complexes, or ejects an unfolded subunit when the energy is sufficient for complex dissociation.\textsuperscript{105,202,203} Here, the protein-DNA complexes, nucleosomes, are studied to explore the application of SID.

The nucleosome is a DNA-protein complex with a double stranded DNA wrapped around a histone octamer.\textsuperscript{204} The histone octamer consists of two H2A-H2B dimers and one (H3)\textsubscript{2}(H4)\textsubscript{2} heterotetramer.\textsuperscript{205-207} Therefore, there are two copies of 4 different histones, H2A, H2B, H3 and H4 in one histone octamer.\textsuperscript{207} Two different X-ray crystal structures of human nucleosomes are shown in Figure 3.5.
Figure 3.5. Nucleosome crystal structures from the protein data bank. The X-ray crystal structures shown on the left (PDB ID: 2AFA)\textsuperscript{208} and right (PDB ID: 1KX5)\textsuperscript{209} are from the human nucleosome structures with different histone sequences.

Previous studies have shown that histone modifications affect nucleosome dynamics.\textsuperscript{210-212} The nucleosome core particles containing H2A.Z have proved to be less stable than those containing H2A.\textsuperscript{213,214} Mass spectrometry has been applied to the study of histones.\textsuperscript{215-217} Nucleosomes have been reported to be successfully observed in native mass spectrometry,\textsuperscript{218} consistent with work in the Wysocki lab. Also, the hexasome and the octasome can be distinguished with the aid of ion mobility.\textsuperscript{218} However, the published papers did not report tandem mass spectrometry of nucleosomes. In the present study, wild type and H2A.Z variant nucleosomes were studied to be by native mass spectrometry and activated by CID and SID. The result obtained from wild type and H2A.Z variant
nucleosomes were compared to explore the relationship between SID fragmentation and nucleosome stability.

3.2.2. Method

The nucleosome samples were prepared as described previously\textsuperscript{219} by Cecil J. Howard from Dr. Jennifer Ottesen’s lab in the Department of Chemistry & Biochemistry at The Ohio State University. The samples were dialyzed into 75 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, U.S.A.) with a concentration lower than 5 µM for mass spectrometric analysis.

All the MS and MS/MS experiments were performed on a modified Waters Synapt G2 HDMS mass spectrometer (Waters Corp., Wilmslow, U.K.) with an SID device installed before the ion mobility cell as described previously\textsuperscript{114} The SID device is shown in Figure A.1. Nanoelectrospray ionization was used to spray nucleosome samples. The capillary voltage was set to 1.0 kV. The source temperature was set to room temperature. The sample cone voltage was set to 50 V, and the source offset was 20 V. The instrument backing pressure was set to approximately 6 mbar for better high m/z transmission. The trap gas flow rate was set to 4 mL/min for MS and SID spectra acquisition and 10 mL/min for CID spectra acquisition. The spectra were acquired in IM mode. The detailed tuning parameters for the SID device in transmission mode and SID mode are shown in Table A.2. “Quadrupole profile”, which suppresses the low m/z transmission, was used to isolate nucleosome ions via the quadrupole. CID and SID spectra were obtained by setting “quadrupole profile” to 8,000. Therefore, ions with higher m/z (m/z > 6,400) were transmitted through the quadrupole before further activation by SID. The experimental
conditions used for the work described here were optimized based on settings initially provided by Dr. Yun Zhang from Dr. Vicki Wysocki’s group in the Ohio State University.

The CCS measurements for nucleosome samples were performed under the same experimental conditions described above by using standard proteins (avidin, concanavalin A, and serum amyloid P) as calibrants. The calibration curve was generated by following the protocol published previously. The R² value is higher than 0.997.

3.2.3. Results

Both wild-type (WT) and H2A.Z variant nucleosomes were buffer exchanged into 75 mM ammonium acetate and sprayed via nanoelectrospray ionization. The mass spectra are shown in Figure 3.6. From the mass spectra shown in Figure 3.6A and B, high intensities of low m/z species can be observed, including sucrose ions, free DNA ions, and highly charged nucleosome ions. Because of the low concentration of nucleosome in the sample as well as the signal suppression from the existence of low m/z species, the signal of nucleosome peaks in the mass spectra was relatively low for single charge state isolation. Therefore, quadrupole profile was set to m/z 8,000 to suppress the transmission of ions with m/z lower than 6,400. After quadrupole suppression of low m/z species, the charge state distributions of WT and H2A.Z variant nucleosomes can be observed (Figure 3.6C and D). The predominant charge states observed from WT and H2A.Z variant nucleosomes are similar, 28+ - 29+ for WT nucleosome and 28+ for H2A.Z variant nucleosome. The experimental molecular weights for WT and H2A.Z nucleosomes are 202.0 ± 0.3 kDa and 201.0 ± 0.1 kDa, respectively. These values are larger than their theoretical molecular
weights (199.3 kDa for WT nucleosome and 198.2 kDa for H2A.Z variant nucleosome), because of the nonspecific binding of adducts (salt and solvent).

Figure 3.6. Typical mass spectra of wild-type (WT) and H2A.Z variant nucleosomes in 75 mM ammonium acetate acquired with and without quadrupole profile. High intensity of low-mass species can be observed in the mass spectra of (A) wild-type nucleosome and (B) H2A.Z variant nucleosome. The mass spectra of (C) wild-type nucleosome and (D) H2A.Z variant nucleosome were acquired with quadrupole profile to suppress the transmission of low m/z ions.
In the wild-type nucleosome sample, there are two copies of H2A, H2B, H3.1 C110A, H4 and a 147-base pair 601 DNA. The H2A.Z variant nucleosome sample contains H2A.Z instead of H2A. The sequences of the histones and DNA in the nucleosome samples studied are listed in Table 3.2.

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<td>AGGKAGKDSGKAKTKAVSQRAGLQFPVGRHRLKSRRTTSHGRVGAATAVUYSAAILEYLTAEVLELAGNASKDLKVKRITPRHLQLAIRGDEELDSLIKATIAGG</td>
</tr>
<tr>
<td>GVPHIHKLGGGGQKQKT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human H2B:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDPAKASAPAPKKGSKKAVTKAQKKDGKKKRKRSRKSYESIYVYKVLKQVHPDTSIISAMGGIMNSFVENDIFERIAGEASRLAHYNKRSTITSREIQTAVERNLLPGELAKHASEGTVKTYTSSK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human H3.1 C110A:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARTKQTARKSTGKAPRQLATKAARKSAPATGGVKKPHRYPGTVALREIRRYQKSTEMLRKLPQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVAFLDVTNLAAIHAKRVTIHKDQIALARRIRGERA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human H4:</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGRGKGGKGLGKKGAKHRKVLRDNIQGITKPAIRRLARRGVKRSGLIYEETRGVKLKFLENVIRDAVTYEHAHKRTVTTAMDVVYALKRQGRRTLYGFGG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>601 DNA:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCGGAGGAATCCCGGTGCGGAGGCCGCTCAATTGTGTCGTAGACAGCTCTAGCACCCTAAACCGCAGTACGTGCTGCTCCCCCGCGTTTAAACCAGCCAGAGGG</td>
</tr>
<tr>
<td>ATTACTCCCTGCTCCAGGCACGTGTCAGATATATACATCCTGT</td>
</tr>
</tbody>
</table>

Table 3.2. The sequences of H2A, H2A.Z, H2B, H3.1 C110A, H4 and 601 DNA in the nucleosome.
The sequences of histone samples were confirmed by spraying 40 μM histone samples dissolved into 75 mM ammonium acetate to the modified Waters Synapt G2 HDMS mass spectrometer in sensitivity mode. The experimental molecular weights and the corresponding theoretical molecular weights are listed in Table 3.3.

<table>
<thead>
<tr>
<th>Histone</th>
<th>Experimental average molecular weight (Da)</th>
<th>Theoretical average molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A</td>
<td>13973.98 ± 0.95</td>
<td>13974.28</td>
</tr>
<tr>
<td>H2A.Z</td>
<td>13421.71 ± 1.40</td>
<td>13421.55</td>
</tr>
<tr>
<td>H2B</td>
<td>13774.83 ± 0.96</td>
<td>13774.95</td>
</tr>
<tr>
<td>H3 C110A</td>
<td>15238.71 ± 1.60</td>
<td>15238.80</td>
</tr>
<tr>
<td>H4</td>
<td>11235.71 ± 1.02</td>
<td>11236.15</td>
</tr>
</tbody>
</table>

Table 3.3. Experimental and theoretical molecular weights of histones.

Based on the results shown in Table 3.3, the experimental molecular weights match well with the corresponding theoretical molecular weights for histones. The X-ray crystal structure from PDB 3AFA was used to calculate the theoretical collision cross section (CCS) of a nucleosome based on the method described in Chapter 2.2.4. The experimental CCS of nucleosome ions is 80.1 ± 1.5 nm², which is lower than 86 nm², which is the theoretical CCS calculated from scaled PA method. A previous study has shown that a dramatic decrease of the CCS (approximately 25%) was observed from nucleosome ions generated from a buffer with the charge reducing reagent (triethylammonium acetate)
added. Therefore, unlike protein complexes studied previously, where charge reducing reagent can better preserve the native-like structure of protein complexes,\textsuperscript{114,203} gas-phase ions generated from ammonium acetate solution have CCS values that better match the CCS values calculated from known crystal structures.

The WT nucleosome ions generated by spraying samples dissolved in ammonium acetate were isolated with quadrupole profile and further fragmented by CID and SID, and the results have been reported previously.\textsuperscript{219} Based on previous observation, histones can be generated from the SID of the nucleosome, while covalent fragmentation from H3.1 C110A was observed in CID spectrum of the nucleosome. In the present study, the SID fragmentation behavior of WT and H2A.Z variant nucleosomes were compared by dissociation of nucleosome ions isolated from quadrupole at different SID collision energies as shown in Figure 3.7. At an SID acceleration voltage of 80 V (Figure 3.7A and B), nucleosome ions were dissociated with histone ions generated in the low m/z region (m/z < 3,000). Charge stripping peaks from nucleosome precursors can be observed in m/z range 8,000 – 14,000. The predominant charge state of nucleosome ions shifted from 29+ - 28+ (Figure 3.6C and D) to 22+ (Figure 3.7A and B). Based on the type of products generated in SID, the SID fragmentation patterns for WT and H2A.Z variant nucleosomes at the same SID acceleration voltage are similar with histones observed in low m/z region and charge reduced nucleosome ions found in the high m/z region.
Figure 3.7. SID spectra of the nucleosome at different SID acceleration voltages. The wild-type (WT) and H2A.Z variant nucleosomes isolated by quadrupole profile were dissociated by SID at acceleration voltages of 80 V ((A) for WT, (B) for H2A.Z) and 140 V ((C) for WT, (D) for H2A.Z). The histones ions generated by SID are highlighted with a blue box.

When comparing the relative intensities of SID products, differences can be observed as shown in Figure 3.7A and B. The relative intensities of the remaining nucleosome observed in the SID dissociation of the WT nucleosome is significantly higher than that observed in the SID dissociation of the H2A.Z variant nucleosome. Therefore, a higher acceleration voltage is required to dissociate the WT nucleosome, which may
indicate the more stable structure of WT nucleosome and it may match the fact that the nucleosome core particles containing H2A.Z are less stable than those containing H2A.\textsuperscript{213,214} However, the nucleosome precursor ions sent for SID study were not single charge isolated, making it difficult to determine the SID collision energy directly. In addition, the charge state distributions of nucleosome ions isolated from quadrupole profile vary from each other even though the predominant charge states are similar (Figure 3.6 C and D), for example, more 32+ nucleosome ions were isolated from WT nucleosome than that isolated from H2A.Z variant nucleosome. Therefore, to better compare the SID collision energy required to dissociate the two nucleosome samples, single charge isolation in the quadrupole is needed, which is currently limited by the amount of low m/z species and the sample concentration.

When the SID acceleration voltage increased to 140 V, nucleosome ions were fully dissociated and histone ions were the main observed product (Figure 3.7C and D). To further analyze the histones generated by SID, the m/z 500 – 2,600 regions of the SID spectra were further zoomed in and shown in Figure 3.8.
Figure 3.8. Zoom in spectra of SID wild-type (WT) and H2A.Z variant nucleosomes of under different SID acceleration voltages. The zoom in of m/z 500 – 2,600 regions from Figure 3.7.

Based on the zoomed in region shown in Figure 3.8, four histone ions from their corresponding nucleosome precursor ions can be released from SID under both 80 V and 140 V SID acceleration voltages. With the increase of the SID acceleration voltage, the charge state distributions of histones generated from nucleosome ions (both WT and
H2A.Z variant) were shifted to lower (higher m/z), which matches with previous observation on monomer ions generated in the SID of protein complexes.\textsuperscript{136}

Different from the charge conservation observed in the SID of protein complexes,\textsuperscript{136,171} the sum of charge states of histone ions generated by SID is higher than the charge state of the nucleosome precursor ions. For example, as shown in Figure 3.8 D, with high SID acceleration voltage, the charge state distribution observed from histone ions generated by SID has been decreased, with the predominant charge states observed as 9+ (H2A.Z), 8+ (H2B), 11+ (H3) and 10+ (H4). The sum of the predominant charge states is 38+, which is much higher than the predominant charge state of the precursor ion, 28+. It might be because of the retention of anions on DNA during SID fragmentation, leading to the high charge states observed from histones products.

3.2.4. Conclusions

CCS of nucleosome ions generated from samples dissolved in ammonium acetate solution correspond better to the known crystal structure while the structure of ions with low charge state collapse. The production of four histones can be observed from the SID spectra of both WT and H2A.Z variant nucleosomes, while the relative intensities of the remaining nucleosome observed in the SID dissociation of the WT nucleosome is significantly higher than that observed in the SID dissociation of the H2A.Z variant nucleosome at an SID acceleration voltage of 80 V, which may indicate the more stable structure of WT nucleosome. Single charge state isolation is needed to directly compare the energy required for the dissociation of different nucleosome samples, which can be
achieved by spraying nucleosome samples with less low $m/z$ species and higher concentration.

The decrease charge state of histones products generated with higher SID acceleration voltage matches with the observation from the SID of protein complexes. However, the sum of charge states of histone ions generated from the SID of nucleosome ions is much higher than the charge state of nucleosome precursor ions, which is a unique SID dissociation behavior observed in the SID of the nucleosome. Therefore, the study of the SID dissociation pattern of more protein-DNA complex samples is needed to help explaining the observation.

3.3. Enzyme-substrate complex: methyltransferase substrates


3.3.1. Introduction

Studying the catalytic capability of enzymes plays an important role in understanding cellular biology processes.\textsuperscript{220} Identifying substrate-binding to an enzyme is essential because there are still many of enzymes with unknown functions.\textsuperscript{221} High-throughput screening has become a commonly used method for identifying substrates of
enzymes.\textsuperscript{222,223} The method has also been applied in the study of methyltransferase substrates,\textsuperscript{224,225} which catalyze the methylation of substrates and are related to various diseases.\textsuperscript{226-228} However, the method failed to analyze methyltransferases in cellular context due to the transient nature of this enzyme-substrate interaction and due to promiscuity.\textsuperscript{229,230} Along these lines, mass spectrometry is limited to the study enzyme-substrate complexes with relatively strong interactions.\textsuperscript{231,232} However, a previous publication from Dr. Zhaohui Sunny Zhou’s group has shown that the covalent bisubstrate adduct formed by the addition reaction between the nucleophilic substrate and S-adenosylvinthionine (AdoVin) can tightly bind to the thiopurine methyltransferase (TPMT), which makes it possible for substrates from cell lysate to be directly detected by mass spectrometry.\textsuperscript{233} The schematics are shown in Figure 3.9.

![Figure 3.9](image_url)

**Figure 3.9.** Schematics of methyltransferase-catalyzed transfer of S-adenosylvinthionine (AdoVin) and persistent interaction between an enzyme and the bisubstrate adduct formed \textit{in situ}. Adapted from Ref 233 with permission from American Chemical Society, Copyright (2016).\textsuperscript{233}
Here, the TPMT with different substrates prepared *in vitro* and *ex vivo* were studied by native mass spectrometry. Enzyme-substrate complexes were further analyzed by tandem mass spectrometry. The results have been published, and some content in this chapter is derived from the paper from K. C. Catcott *et al.*. 234

3.3.2. Method

The samples from *in vitro* and *ex vivo* reactions were prepared as previously described by Wanlu Qu from Dr. Zhaohui Sunny Zhou’s group at Northeastern University. 233, 234 The AdoVin sample was prepared by using both labeled (+15 Da) and unlabeled ATP so that the substrates with and without +15 Da mass shift can be observed in mass spectrometry. 233, 234 The samples were buffer exchanged via diafiltration into 20 mM ammonium acetate with pH adjusted to 8.0 with ammonium hydroxide.

The MS and MS/MS experiments were conducted on a modified Waters Synapt G2S HDMS mass spectrometer (Waters Corp., Wilmslow, U.K.) with an SID device installed before the ion mobility cell as described in Chapter 2.2.1. 114, 131 The data were acquired with SID tuning parameters shown in Table A.1. The samples were sprayed by nESI with the capillary voltage set 1.2 – 1.5 kV. 234 The sampling cone voltage and the source offset voltage were set to 20 V. The trap gas flow rate was set to 2 mL/min for MS and SID experiment and 4 mL/min for CID experiment. The helium cell and ion mobility cell gas flow rate were set to 120 mL/min and 60 mL/min, respectively. The source pressure was $5 \times 10^{-3}$ mbar, and the pressure in the TOF was $1.2 \times 10^{-6}$ mbar. The enzyme-substrate
complex ions with a specific charge state were selected by the quadrupole and fragmented by both CID and SID as described previously.\(^{136}\)

3.3.3. Results

Thiopurine S-methyltransferase (TPMT, EC:2.1.1.67) is a 30,343.6 Da protein based on the calculation from the sequence obtained from UniProtKB - P51580 with His-tag added. The mass spectrometry measurement has shown that the TMPT sample has a molecular weight of 30,212.6 ± 0.9 Da, which is 131.0 ± 0.9 Da lower than the theoretical mass, indicating the loss of N-terminal methionine in the protein studied.\(^{235}\) A previous study has shown that TPMT releases the product and the byproduct S-adenosylhomocysteine (AdoHcy) when reacting with S-adenosylmethionine (AdoMet) and the substrate, while tightly bound to the bisubstrate adduct (AdoVin-substrate adduct) when reacting with AdoVin and substrates.\(^{233}\) TNB (2-nitro-5-mercaptobenzoic acid) and AMBA (2-amine-5-mercaptobenzoic acid) were used as substrates in the study. The structures and corresponding molecular weight of bisubstrates and AdoHcy are shown in Figure 3.10.
Figure 3.10. The structures and molecular weight of AdoVin-TNB adduct, AdoVin-AMBA adduct and AdoHcy.

The binding of AdoHcy was tested with the sample prepared with TPMT mixed with AdoHcy ($K_d \approx 1 \mu M$ in aqueous solution). The sample was analyzed by mass spectrometry with the concentration of TPMT diluted to 5 µM in 20 mM ammonium acetate (pH 8.0). The MS and MS/MS results are shown in Figure 3.11.
Figure 3.11. MS and MS/MS spectra of [TPMT•AdoHcy] complex. (A) Mass spectrum of [TPMT•AdoHcy] complex with 5 µM TPMT in 20 mM ammonium acetate (pH 8.0). The 10+ [TPMT•AdoHcy] complex (*) was selected by the quadrupole and (B) dissociated in CID with a collision energy of 300 eV and (C) the zoom in of m/z lower than 400. Reprinted from Ref 234 with permission from John Wiley and Sons. 234

Based on the mass spectrum shown in Figure 3.11A, the [TPMT•AdoHcy] complex was observed as the predominant species. The production of apo TPMT (Figure 3.11B) and AdoHcy (Figure 3.11C) in the CID of 10+ [TPMT•AdoHcy] complex confirms the presence of [TPMT•AdoHcy] complex as the main species in the sample. AdoHcy was removed in the study of TPMT binding to AdoVin-TNB adduct and AdoVin-AMBA adduct, which are much stronger binding than the binding of TPMT•AdoHcy.
Figure 3.12. MS and MS/MS spectra of *in vitro* TPMT-[AdoVin-TNB] adduct complex. (A) Mass spectrum of TPMT-[AdoVin-TNB] adduct complex with 10 μM TPMT concentration in 20 mM ammonium acetate (pH 8.0). The 10+ charge (*) TPMT-[AdoVin-TNB] adduct complex was dissociated by (B) CID and (C) SID with a collision energy of 500eV. The free AdoVin-TNB adduct (highlighted and zoomed in in the insets). The fragmentation pattern of the adduct can be observed at higher energy activation shown in the spectra from (D) CID at 1200 eV and (E) SID at 1000eV confirms its identity. Adapted from Ref 234 with permission from John Wiley and Sons.234

The TPMT sample with AdoVin-TNB adduct with the AdoVin containing a 1:1 ratio of heavy and light label, was also studied. The results are shown in Figure 3.12. Both *apo-* and *holo-* forms of TPMT-[AdoVin-TNB] adduct complex with a wide charge state distribution can be observed in the mass spectrum of TPMT-[AdoVin-TNB] adduct complex with 10 μM TPMT concentration in 20 mM ammonium acetate in Figure 3.12A. The TPMT-[AdoVin-TNB] adduct complex with a charge state 10+ was shown to be
compact in ion mobility. The 10+ TPMT-[AdoVin-TNB] adduct complex was selected by the quadrupole and dissociated by CID and SID with a collision energy of 500 eV (Figure 3.12B and C) to identify the ligand binding. Both the free TPMT and the [AdoVin-TNB] adduct can be observed in CID and SID spectra. The lower precursor intensity observed in Figure 3.12C reflects the more favorable dissociation in the SID. Charge stripping of the 10+ TPMT-[AdoVin-TNB] adduct complex precursor can also be observed in the SID spectrum. The peaks at m/z 610 and 625 can be observed corresponding to the mass of 1:1 labeled [AdoVin-TNB] adduct. In addition to the information provided by the molecular weight of the adduct, the higher energy fragmentation was also conducted to further confirmed the structure of [AdoVin-TNB] adduct (Figure 3.12D and E). Similar covalent fragmentation patterns can be observed from 1200 eV CID and 1000 eV SID. Figure 3.12D with detailed structure of fragments labeled is shown in Figure 3.13. The peaks with Δm/z of 15 and similar intensities were fragments generated from 1:1 labeled [AdoVin-TNB] adduct. The 15 Da mass difference provided from light and heavy labeling makes it easier to assign the fragment ions, and the structure of fragments shown in Figure 3.13 further confirmed the identification of the [AdoVin-TNB] adduct bound to TPMT. Therefore, by analyzing the in vitro TPMT-adduct complexes with native mass spectrometry combining with tandem mass spectrometry, adducts bound to TPMT can be easily identified. CID and SID provide similar fragmentation patterns of the [AdoVin-TNB] adduct.
Figure 3.13. Zoom in of 1200 eV CID of 10+ charge TPMT-[AdoVin-TNB] adduct complex. The labeled positions were highlighted with blue dots. The fragments from stable isotope labeling species generate peaks with $m/z$ difference of 15. Reprinted from Ref 234 with permission from John Wiley and Sons.234

The ex vivo sample was prepared with 2 µM TPMT concentration in 20 mM ammonium acetate (pH 8.0). The mass spectrum is shown in Figure 3.14A. The 11+ and 10+ TPMT-adduct complex peaks can be observed from MS. The 10+ TPMT-adduct complex peak was selected by the quadrupole and dissociated by CID and SID, respectively (Figure 3.14B and C). Similar to dissociation pattern observed previously from in vitro TPMT-[AdoVin-TNB] adduct complex sample, the free TPMT and adduct peak can be observed from both CID and SID spectra. The mass of the adduct peaks matches with the TPMT-[AdoVin-AMBA] adduct (Figure 3.14B and C insets), indicating the major
TPMT-adduct peak observed in the mass spectrum of the *ex vivo* sample is the TPMT-[AdoVin-AMBA] adduct complex.

Figure 3.14. MS and MS/MS spectra of *ex vivo* TPMT-[AdoVin-AMBA] adduct complex. (A) Mass spectrum of *ex vivo* TPMT-[AdoVin-AMBA] adduct complex with 2 μM TPMT concentration in 20 mM ammonium acetate (pH 8.0). The 10+ charge (*) TPMT-[AdoVin-AMBA] adduct complex was dissociated by (B) CID and (C) SID with a collision energy of 500eV. The free AdoVin-AMBA adduct (highlighted and zoomed in in the insets). Adapted from Ref 234 with permission from John Wiley and Sons.234

The *ex vivo* sample was further diluted and sprayed by nESI. The mass spectra are shown in Figure 3.15. With a TPMT concentration of 0.4 μM, the TPMT-[AdoVin-AMBA] adduct complex can be observed in mass spectrum (Figure 3.15), while the complex peak was not resolved when the concentration decreased to 0.2 μM.
Figure 3.15. Mass spectra of \textit{ex vivo} TPMT-[AdoVin-AMBA] adduct complex with different concentrations. The mass spectra were obtained by spraying \textit{ex vivo} TPMT-[AdoVin-AMBA] adduct complex with (A) 2 µM, (B) 0.4 µM and (C) 0.2 µM TPMT concentration in 20 mM ammonium acetate (pH 8.0).

3.3.4. Conclusions

Both \textit{in vitro} and \textit{ex vivo} TPMT-adduct complex were studied by native mass spectrometry. The TPMT-bisubstrate adduct complex can be observed in the mass spectra of both \textit{in vitro} and \textit{ex vivo} samples, while the complex peak was not resolved when the TPMT concentration was lower than 0.4 µM.

Tandem mass spectrometry was used to identify the adduct bound to TPMT in the complex. The free AdoHcy was generated from the CID of TPMT-AdoHcy complex (K_d
~ 1 µM in aqueous solution). For the much stronger substrates binding to TPMT, AdoVin-TNB and Ado-AMBA were observed from both the CID and SID spectra of TPMT-adduct sample prepared in vitro and ex vivo. The fragmentation patterns observed in CID and SID are similar. The combination of stable isotope labeling and covalent fragments generated from high energy CID or SID helps in peak assignment of the fragment ions, confirming the identification of the structure of the adducts. Therefore, both CID and SID can be used to study the enzyme-substrate complex (K_d ≤ 1 µM in aqueous solution) and identify the structure of the substrate, providing a native MS method to use in broad screening for enzyme-subtract pairs.
Chapter 4. Comparison of solution phase and gas phase charge reduction via ion-ion reaction in Q-TOF instrument

4.1. Introduction

Gas phase ion-ion reaction, which has been developed in the past decades, has broad range of applications, such as charge reduction, charge reversion and gas phase cross-linking. In gas phase ion-ion reaction, both electron transfer reaction (ETR) and proton transfer reaction (PTR) have been shown to be able to generate charge reduced species, while c and z ions can be generated from the electron transfer reaction of protonated peptides. With the study of various reagent ions, the petitioning of ETR and PTR was reported to be related to the electron affinities associated with the anionic reagents. However, most of the ion-ion reaction studies were limited to peptides and small proteins. Among the few papers published on the study of protein complexes with gas phase ion-ion reactions, the charge-reduced species generated from proton transfer reaction has been shown to help distinguish protein complexes in the protein mixture.

In native mass spectrometry study, protein complexes with lower charge state preserve more native-like conformations by minimizing the effect of Coulombic repulsion on native structures. By adding additives with high gas-phase basicity into the protein solution, the charge reduction of protein complexes can be achieved. Charge reducing
reagents, such as triethylammonium acetate (TEAA), are commonly used to generate charge reduced protein complex ions.\(^{188,189}\) “Solution additive charge reduction” is used in this chapter to represent the charge reduction conducted by adding charge reducing reagent into the sample solution. Gas phase ion-ion reactions can also result in charge reduction of proteins and protein complexes in the gas phase.\(^{237,243}\) “Gas phase charge reduction” is used in this chapter to represent the charge reduction induced by the gas phase ion-ion reaction. Ubiquitin ions with different charge states have been shown to be refolded differently after gas phase activation.\(^{244}\) However, conformational differences between charge-reduced protein complex ions generated from gas phase ion-ion reactions of native protein complexes and solution additive charge reduction remains unclear. With the aid of ion mobility, protein complex ions with different charge states can be separated based on their difference in drift time, which provides conformational information on each protein complex ions, such as collision cross section.\(^{18,159}\) In addition, the previous study has shown that protein complex ions with the same drift time may have different conformations, and SID has been shown to be able to further monitor the conformational change of protein complexes.\(^{137}\) Therefore, in the present study, the charge reduction of protein complexes was performed in solution phase and in gas phase separately, and conformations of each of ions were studied by ion mobility cell and further dissociated by SID after the separation in the ion mobility. Conformational differences of the protein complex ions generated from the gas phase and solution additive charge reduction were investigated by studying the drift time distributions from ion mobility and the SID fragmentation patterns.
4.2. Method

4.2.1. Mass spectrometry

Experiments were performed by using a modified Waters Synapt G2S HDMS mass spectrometer (Waters Corp., Wilmslow, U.K.) coupled with a glow discharge ionization source to enable the ion-ion reaction. The 32 k quadrupole RF generator was used. The SID device was installed between the IM cell and the truncated transfer traveling wave ion guide (TWIG) as previously described. The schematic of the modified instrument is shown in Figure 4.1.

![Figure 4.1](image)

Figure 4.1. A schematic of a modified Waters Synapt G2S with a SID device installed between the ion mobility cell and the truncated Transfer TWIG. Adapted from Ref 186 with permission American Chemical Society, Copyright (2016).

The reagent anions generated from a glow discharge ionization source react with the protein cations generated from the nESI source in the Trap TWIG and the reaction time can be a few tens of milliseconds. The gas phase charge reduction experiment was
conducted by allowing the protein cations generated from nESI react with reagents anions in the Trap TWIG, and the solution additive charge reduction experiment was conducted by directly spraying the protein complexes mixed with charge reducing reagent (such as triethylammonium acetate) in solution.

4.2.2. Sample preparation

Perfluoro-1,3-dimethylcyclohexane (PDCH) was used as a reagent to generate anions for ion-ion reaction experiments. The reagent was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Avidin and streptavidin (Thermo Scientific Pierce Biotechnology, Rockford, IL, U.S.A.), recombinant human C-reactive protein (CRP, CalBiochem, San Diego, CA, U.S.A.), concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO, U.S.A.) and β-lactoglobulin from bovine milk (Sigma-Aldrich, St. Louis, MO, U.S.A.) were used as protein standards to generate cations for gas phase ion-ion reactions. All standard protein samples were buffer exchanged into 100 mM ammonium acetate (AA, Sigma-Aldrich, St. Louis, MO, U.S.A.) with 6-kDa cut-off size exclusion chromatography spin columns (Bio-Rad, Hercules, CA, U.S.A.) at a final concentration of 10 µM protein complex if not specifically noted. The solution phase charge reduced proteins were prepared by mixing the sample solution with 100 mM triethylammonium acetate (TEAA, Sigma-Aldrich, St. Louis, MO, U.S.A.) in a ratio of 4:1 (v:v). The solution phase supercharging proteins were prepared by mixing the sample solution with m-nitrobenzyl alcohol (mNBA, Sigma-Aldrich, St. Louis, MO, U.S.A.).190,191
4.2.3. Ion-ion reaction

Before starting the ion-ion reaction, the reagent was loaded in the reagent chamber (50 µL – 150 µL for PDCH, or 10 mg – 100 mg if solid reagent is used) and the instrument parameters were tuned in glow discharge (GD) mode, ETD mode and TOF mode to make sure that the signal of reagent ions reaches 10^7. The parameters for GD mode vary for each experiment, the main parameters tuned in the experiments were as follows: glow discharge current was 30 µA – 60 µA, source temperature was 80 °C, sampling cone voltage was 0 V- 100 V, source offset was 0 V – 20 V, the trap gas flow rate was 50 mL/min, LM resolution was 4.0 and HM resolution was 20.0. IMS DC bias was 10 V when SID was installed between ion mobility cell and Transfer TWIG, otherwise it was set as 3 V. The SID tuning parameters for the transmission of reagent ions in Glow Discharge mode are shown in Table A.3. Voltages on electrodes in Trap TWIG and IM cell, makeup gas flow and cone gas flow vary for signal optimization. All the gas phase charge reduction (ion-ion reaction) and solution additive charge reduction experiments were conducted in ESI mode, ETD mode and ion mobility (IM) mode after optimizing condition to maximize the signal for reagent ions.

The gas phase ion-ion reaction process in ETD mode is as described previously. In ETD mode, helium was used in both Trap TWIG and helium cell, nitrogen was used in IM cell, and argon was used in transfer TMIG. The data was acquired in sensitivity mode. The protein complex samples were sprayed via nanoelectrospray ionization. And ETD experiment was conducted in ETD mode and Ion Mobility mode.
The experimental parameters for gas phase ion-ion reaction were optimized for the production of high-intensity charge-reduced ions with relatively gentle conditions so that the activation of protein complex ions can be minimized. The conformation of ions generated from charge reduction was studied in this chapter. Thus the parameters were not tuned for generating charge-reduced ions with lowest charge state, instead, the parameters were tuned for better signal of charge-reduced ions with similar charge state to those generated in the solution additive charge reduction. For example, by increasing the trap DC bias, more charge-reduced ions with lower charge state can be generated, however, more CID activation can be introduced, thus 35 V was used to minimize the activation in the trap region and maintain ion transmission; decreasing the trap gas flow rate can also push the charge state of ions generated from ion-ion reaction to lower, while the signal of charge-reduced ions was too lower for further tandem mass spectrometry analysis, thus it the trap gas flow rate was set to 20 mL/min for the best transmission of charge-reduced ions.

The optimized parameters are listed as follows: sampling cone and source offset were 20 V, source temperature was 80 °C, trap DC bias was set to be 35 V and IMS DC bias was 10 V, LH resolution was 4.0 and HM resolution was 20.0 for quadrupole selection. The gas flow rate from Trap TWIG, helium cell, IM cell and Transfer TWIG were 20 mL/min, 120 mL/min, 40 mL/min and 2 mL/min respectively. The wave height and wave velocity for Trap TWIG were 0.2 V and 300 m/s. The wave velocity was set to 300 m/s and wave height was set to 20.0 V in the IM cell. And the wave height and wave velocity for Trap TWIG were 2.0 V and 65 m/s. The gas phase charge reduction experiment was conducted by isolating the protein complex ions with a specific charge state and allowing
them to react with the specific kind of reagent anions from glow discharge. The reagent refill time was set to be 0.1 s and the refill interval was 1.0 s. The solution phase gas reduction experiment was conducted by spraying the solution phase charge reduced protein samples under same conditions except that the glow discharge current was set to be 0 µA.

The charge reduced ions were then subject to SID for further structural characterization. SID tuning parameters for transmission and SID dissociation of protein complex ions in Ion Mobility mode are shown in Table A.3. The cone activation experiment was performed by activating the protein complex ion with various sampling cone voltage.

4.3. Comparison of different reagents in gas phase charge reduction

PDCH, 1,4-dicyanobenzene and 4-nitrotoluene are reagents for conducting gas phase charge reduction.\textsuperscript{237,240,242} 1,4-dicyanobenzene and 4-nitrotoluene can be used to conduct electron transfer reaction, while PDCH can be used to conduct PTR.\textsuperscript{237,240} The structures of the reagents are shown in Figure 4.2.

![Figure 4.2. The structure of reagents of the ion-ion reaction.](image)
In the present study, PDCH was used as a reagent to generate anions for gas phase charge reduction of protein complex ions via PTR so that only the charge reduced protein complex ions can be observed without the contamination from covalent ETD fragments.

Different kinds of radicals can be generated from PDCH, such as [PDCH-F]$^-$ ($m/z = 381$) and PDCH$^-$ ($m/z = 400$). Each of them can be generated under different experimental conditions. Spectra of PDCH radicals observed under different experimental conditions are shown in Figure 4.3. [PDCH-F]$^-$ can be observed at sampling cone voltage 100 V and PDCH$^-$ can be generated at sampling cone voltage 20 V. Each of the radical ions was isolated by the quadrupole (Figure 4.3B and D) and further reacted with protein complex ions in the Trap TWIG.

![Figure 4.3. Mass spectra of PDCH radicals generated under different conditions. (A) Mass spectrum of PDCH radical at sampling cone voltage 100 V and (B) mass spectrum of isolated [PDCH-F]$^-$ radical. (C) Mass spectrum of PDCH radical at sampling cone voltage 20 V and (D) mass spectrum of isolated PDCH$^-$ radical.](image)
The charge reduction behavior of different radicals generated from PDCH was tested with streptavidin. 10 µM streptavidin tetramer dissolved in 100 mM ammonium acetate was spray using nESI source, and 15+ streptavidin tetramer peak was selected to conduct the gas phase ion-ion reactions. The parameters for gas phase ion-ion reactions were optimized to produce high-intensity charge-reduced ions with relatively gentle conditions to minimize the gas phase activation of protein complex ions. Therefore, the charge state of ions generated from the gas phase ion-ion reaction in this chapter is not as low as the previously published results.\textsuperscript{237,242} The result from the gas phase ion-ion reactions of 15+ streptavidin tetramer with different reagent ions are shown in Figure 4.4.

\begin{center}
\includegraphics[width=\textwidth]{figure4.4.png}
\end{center}

Figure 4.4. Mass spectra of gas phase ion-ion reaction of 15+ streptavidin tetramer reacting with [PDCH-F]\textsuperscript{−} and [PDCH]\textsuperscript{−}. The intensity of m/z 5000 – 10000 region is amplified by ten.
Based on the results shown in Figure 4.4, under the same condition, 15+ streptavidin tetramer can be charge reduced to 7+ streptavidin tetramer with both [PDCH-F]$^-$ and PDCH$^-$. There is no significant difference in charge state distributions. To further confirm the conformation of the charge-reduced ions, the 11+ streptavidin tetramer ions generated via ion-ion reactions were separated by ion mobility and further dissociated by SID. The drift time distributions and SID fragmentation patterns of the 11+ streptavidin tetramer ions generated from the reaction with different PDCH radicals were compared, and the results are shown in Figure 4.5.

![Figure 4.5](image_url)

**Figure 4.5.** Drift time distributions and 50 V SID spectra of 11+ streptavidin tetramer generated from gas phase ion-ion reaction of 15+ streptavidin tetramer reacting with [PDCH-F]$^-$ and PDCH$^-$. The drift time distributions of 11+ streptavidin react generated from the reacting with (A) [PDCH-F]$^-$ and (C) PDCH$^-$ are similar. SID with an acceleration voltage of 50 V was used to dissociate ions generated from the reaction with (B) [PDCH-F]$^-$ and (D) PDCH$^-$. 

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Based on the results shown in Figure 4.5, both the drift time distributions and the SID fragmentation patterns are similar for the 11+ streptavidin ions generated from the reaction with [PDCH-F]− and PDCH−, indicating the similar structure of ions generated from reaction with different radicals. Therefore, [PDCH-F]− and PDCH− have no significant difference in gas phase ion-ion reaction behaviors when reacting with the 15+ streptavidin tetramer ions. PDCH− was used for further ion-ion reaction experiments because gentle source conditions, which are the same like those used for maintaining native-like structure of protein complexes, can be used to generate PDCH−.

4.4. CCS and drift time distribution comparison of ions generated from gas phase and solution additive charge reduction

The charge state distribution of ions generated from the gas phase charge reduction of the 15+ streptavidin tetramer ions was further compared with that from the solution additive charge reduction (Figure 4.6). As shown in Figure 4.6A, with gas phase charge reduction, the charge state of the 15+ streptavidin tetramer (black) can be reduced to as low as 6+. The 6+ ions can be observed from ion mobilogram, but the intensity is too low to be seen in the mass spectrum in Figure 4.6A. In solution additive charge reduction shown in Figure 4.6B, the predominant charge states of streptavidin tetramers are 10+ and 11+ (blue), and the charge state of streptavidin tetramers can be obtained is as low as 9+. With the decrease of capillary voltage, the charge state distribution of solution phase charge reduced sample can be shifted to higher so that streptavidin tetramer ions with charge state 10+ to 17+ can be detected.
Figure 4.6. Gas phase and solution additive charge reduction of streptavidin. (A) Gas phase ion-ion reaction of 15+ streptavidin tetramer (black) generates charge reduction streptavidin tetramer ions (red). (B) Mass spectrum of 10 µM streptavidin tetramer in 100 mM ammonium acetate (black) and 8 µM streptavidin tetramer in 80 mM ammonium acetate/20 mM TEAA (blue).

The conformations of ions generated from the gas phase and solution additive charge reduction were further compared by calculation their CCS values (Figure 4.7). The CCS calculation was introduced previously in Chapter 2.2.1. The theoretical CCS was calculated from the crystal structure (PDB ID: 1SWB) with scale PA method as described previously in Chapter 2.2.4. The CCS distributions of streptavidin tetramer ions with the same charge states are close to each other. When compared with the theoretical CCS, the CCS distributions of ions with charge state 11+ - 15+ are close to the theoretical CCS. The CCS of ions with lower charge state were observed to be slightly higher than the theoretical CCS. Note that the streptavidin tetramer ions with charge state lower than 10+ are out of
the calibration range covered by the standard CCS calibrants published, other techniques, such as linear drift cell, are needed for CCS calculation of ions with low charge states. Therefore, streptavidin tetramer ions with charge state 11+ to 15+, which is within the range covered by the CCS calibrants, will be used for CCS comparison. As shown in Figure 4.7, CCS values of streptavidin tetramer ions at the same charge states generated from gas phase and solution additive charge reduction are similar with the CCS of ions from solution additive charge reductions observed to be slightly lower. The drift time distribution comparison for more protein systems will be shown and discussed later in Figure 4.9.

Figure 4.7. CCS distributions of streptavidin tetramer ions generated from gas phase charge reduction and solution additive charge reduction. The CCS of streptavidin tetramer ions generated from gas phase charge reduction (red) and solution additive charge reduction (black) are compared with the theoretical CCS (solid line).
More protein complex systems were studied and concanavalin A tetramer is shown as an example to compare the gas phase charge reduction and solution additive charge reduction (Figure 4.8).

![Figure 4.8](image)

Figure 4.8. Gas phase and solution additive charge reduction of concanavalin A. (A) Gas phase ion-ion reaction of 21+ concanavalin A tetramer (black) generates charge reduction concanavalin A tetramer ions (red). (B) Mass spectrum of 20 µM concanavalin A tetramer in 100 mM ammonium acetate (black) and 16 µM concanavalin A tetramer in 80 mM ammonium acetate + 20 mM TEAA (blue).

Similar to the charge reduction of streptavidin shown in Figure 4.6, in the charge reduction of concanavalin A (Figure 4.8), the main charge states of concanavalin A tetramers generated from solution additive charge reduction (13+, 14+ and 15+) can also be generated by the gas phase charge reduction from the 21+ concanavalin A tetramer. The predominant peaks in the mass spectra of different protein complexes in 100 mM ammonium acetate were selected and charge reduced by gas phase ion-ion reaction.
Figure 4.9. Drift time distributions of precursor ions and charge-reduced ions generated from the gas phase and solution additive charge reduction. The protein complexes ions, (A) 13+ β-lactoglobulin, (B) 15+ streptavidin tetramer, (C) 17+ avidin tetramer, (D) 21+ concanavalin A tetramer and (E) 24+ CRP pentamer, were selected by the quadrupole (black) and charge reduced via gas phase ion-ion reaction (red), and compared with the ions with same charge state generated from solution additive charge reduction (blue).
The drift time distributions of protein complex ions with the same charge state generated from the gas phase and solution additive charge reduction are compared, and the results are shown in Figure 4.9. The gas phase charge reductions was conducted by using ions at the predomint charge state from 100 mM ammonium acetate as precursor ions for gas phase ion-ion reactions. The data were acquired with 300 m/s wave velocity and 20.0 wave height in the ion mobility cell. The drift time obtained from gas phase charge reduction (red line in Figure 4.9) is always slightly higher than that obtained from solution additive charge reduction (blue line in Figure 4.9), and the differences are within 3% (1.9% for the 8+ β-lactoglobulin dimer, 2.4% for the 11+ streptavidin tetramer, 2.2% for the 11+ avidin tetramer, 1.6% for the 14+ concanavalin A tetramer, and 2.4% for the 18+ CRP pentamer), which is a difference of one bin among the 200 bins in ion mobility data acquisition. Also, no significant difference was observed when comparing the drift time distributions of precursor ions generated with and without gas phase ion-ion reaction, demonstrating that different drift time distributions observed were not from different experimental conditions (with and without glow discharge current applied). To further validate the drift time difference observed in the results, the streptavidin sample was further tested with different wave velocities (300 m/s, 350 m/s, and 400 m/s) in the ion mobility cell. The drift time distributions of charge-reduced ions (11+ and 10+ streptavidin tetramer ions) generated from gas phase charge reduction were consistently slightly higher than those obtained from solution additive charge reduction under different wave conditions studied. Therefore, it is reasonable to conclude that the protein complex ions with the same charge state generated from solution additive charge reduction have slightly more compact
conformation than those generated from gas phase ion-ion reaction with precursor ions at higher charge states. It might be because of the retention of the slight less compact conformation of highly charged precursor ions in the gas phase ion-ion reactions than the conformation of solution phase charge-reduced ions. The minor conformational difference was further tested by SID.

4.5. SID fragmentation pattern of ions generated from gas phase and solution additive charge reduction

The charge reduced ions generated from gas phase ion-ion reactions were separated by ion mobility and then dissociated by SID. The process is shown in Figure 4.10 with streptavidin as an example.

Figure 4.10. Gas phase charge reduced streptavidin ions separated by ion mobility and dissociated by SID. (A) Ion mobilogram of charge-reduced ions generated from the gas phase charge reduction of the 15+ streptavidin tetramer. The pulse of each of the species was divided by dashed white lines. (B) Each of the pulse separated by ion mobility were dissociated by SID with an acceleration voltage of 50 V. The SID spectrum of each of streptavidin tetramer with different charge states can be exacted by summing the signal in each pulse.
Ions with different charge states (ranging from 15+ to 6+) generated from gas phase charge reduction of the 15+ streptavidin tetramer can be separated with different drift time distribution (y-axis in Figure 4.10A) in the ion mobility cell. Each of the pulses containing streptavidin tetramer ions with a specific charge state was then dissociated by SID, and all the SID fragments from the same precursor were recorded in the same drift time (horizontal slices in Figure 4.10B). The SID spectra of all the charge-reduced ions generated from gas phase ion-ion reaction can be acquired within one data acquisition. As expected, under the same SID acceleration voltage, more fragments can be observed from precursor ions with relatively higher charge state and fewer fragments can be observed when the precursor charge state gets lower due to different amount of energy deposition, for example, with an SID acceleration voltage of 50 V applied, the 15+ streptavidin tetramer was totally dissociated into monomers and dimers, while the 6+ streptavidin tetramer remains intact.

Since the peaks from 11+ and 10+ streptavidin tetramer are the predominant peaks observed from the solution additive charge reduction of streptavidin (Figure 4.6B), the SID dissociation patterns of 11+ and 10+ streptavidin tetramer generated from the gas phase and solution additive charge reduction are compared, and the corresponding spectra are shown in Figure 4.11. The SID spectra of 11+ and 10+ tetramer at an acceleration voltage of 50 V were extracted from the corresponding pulses shown in Figure 4.10B. The spectra in Figure 4.11 show that similar SID fragmentation patterns can be observed from streptavidin tetramer ions with the same charge state generated from the gas phase and solution additive charge reduction.
Figure 4.11. SID spectra of 11+ and 10+ streptavidin tetramer generated from the gas phase and solution additive charge reduction at an SID acceleration voltage of 50 V. SID spectra of the 11+ streptavidin tetramer generated from (A) gas phase (from 15+ streptavidin tetramer) and (B) solution additive charge reduction. SID spectra of the 10+ streptavidin tetramer generated from (C) gas phase (from 15+ streptavidin tetramer) and (D) solution additive charge reduction.
The SID fragmentation pattern of CRP pentamers generated from the gas phase and solution additive charge reduction in sensitivity mode are shown in Figure 4.12. The 18+ CRP pentamer generated in gas phase charge reduction was produced from the 24+ CRP pentamer.

![SID spectra of 18+ CRP pentamer generated from the gas phase and solution additive charge reduction at an SID acceleration voltage of 70 V. SID spectra of the 18+ CRP pentamer generated from (A) gas phase (from 24+ CRP pentamer) and (B) solution additive charge reduction.](image)

The SID spectra in Figure 4.12 show similar SID dissociation patterns of 18+ CRP pentamers generated from the gas phase and solution additive charge reduction, except for
more charge reduced pentamers and slightly less monomers observed in the SID of solution phase charge reduced CRP pentamer.

In summary, SID fragmentation patterns are similar for the protein complex ions (18+ CRP pentamer, 11+ and 10+ streptavidin tetramer) generated from the gas phase and solution additive charge reduction. Those ions generated in the gas phase and solution additive charge reduction were shown to have minor conformational difference based on differences observed in drift time (Figure 4.9). However, the no significant differences were observed in SID fragmentation patterns. The similar SID fragmentation patterns observed might be because the structural difference between the precursor ion and the charge reduced ion studied in the gas phase charge reduction, such as 15+ and 11+ streptavidin tetramer (as the CCS distribution shown in Figure 4.7), is too subtle to be distinguish by SID. Assuming the precursor conformation can be preserved during gas phase charge reduction, similar SID fragmentation patterns can be observed when the ions with similar conformations and same charge state are studied. To verify the assumption, the precursors with more significant conformational differences were used to conduct gas phase ion-ion reactions. The charge reduced ions were studied by SID, and the SID fragmentation patterns were compared with each other.

4.6. SID fragmentation pattern of ions generated in gas phase from different precursor conformations

The precursor ions with different conformations were generated in two ways: cone activation and supercharging. As described previously in Chapter 2.3 and Chapter 2.6, cone activation can lead to a conformational change of protein complexes which provides
different fragmentation patterns (Figure 2.13), and the addition of supercharging reagent has been shown to be able to generate unfolded protein complex ions (Figure 2.3).\textsuperscript{137,190,191}

The SID spectra of charge reduced streptavidin tetramer ions generated from the 15+ streptavidin tetramer activated via different cone voltages in sensitivity mode are shown in Figure 4.13.

![Figure 4.13](image.png)

Figure 4.13. Drift time distributions and SID spectra of 11+ streptavidin tetramer ions generated the gas phase charge reduction from the 15+ precursor under different cone voltages. The mobilograms of gas phase charge reduction of the 15+ streptavidin tetramer activated under cone voltage (A) 80 V and (B) 20 V. Under 20 V cone voltage, The drift time distributions of the 11+ streptavidin tetramer were extracted with (C) 20 V and (D) 80 V cone voltage, and dissociated by SID with an acceleration voltage of 50 V with (E) 20 V and (F) 80 V cone voltage.

As shown in Figure 4.13B, the 15+ streptavidin tetramer has relatively elongated drift time distribution, indicating that it was already partially unfolded under the activated cone voltage. In Figure 4.13D, the low intensity tails remaining in drift time distributions of the charge-reduced ions generated from gas phase ion-ion reaction show that the
partially unfolded structure of the precursor ion can be preserved in the gas phase charge reduction. When comparing the 11+ streptavidin tetramer generated from different precursors (Figure 4.13C and D), the major drift time distributions were similar to each other with the 11+ tetramer generated from 80 V cone activated precursor remains partially unfolded. The further SID fragmentations of the 11+ streptavidin tetramer generated from precursors activation under different cone voltages in Figure 4.13E and F show that more streptavidin tetramer precursors and monomers can be observed under the same SID activation energy, which agrees with the previous observation (Figure 2.13) from the cone activation of the 11+ streptavidin tetramer. Thus, the charge reduced streptavidin ions preserve the unfolded structure of the precursor ions induced from cone activation in the gas phase charge reduction.

The protein complexes generated from supercharging and normal charge protein complex precursors were charge reduced via gas phase ion-ion reactions, SID fragmentation patterns of charge-reduced ions were compared with each other. Two protein complex systems, streptavidin and CRP, were tested. The previous study has shown that streptavidin ions generated from the buffer with m-NBA added presents more unfolded structure than those generated from 100 mM ammonium acetate (Figure 2.3). In order to generate highly charged streptavidin tetramer, streptavidin with 5% m-NBA added was sprayed, and 21+ was observed and isolated for gas phase ion-ion reaction. The 15+ streptavidin tetramer generated from streptavidin in 100 mM ammonium acetate was also studied under the same condition. SID spectra of the 11+ streptavidin tetramer generated
The 11+ streptavidin tetramer can be generated from the gas phase charge reduction of 15+ and 21+ streptavidin tetramers. As shown in Figure 4.14A and B, the 21+ streptavidin tetramer has a broad drift time distribution when compared with the 15+ streptavidin tetramer, indicating more unfolded structure. In addition, with the aid of the dashed white line labeled in the same position in Figure 4.14A and B, the drift time
distribution of charge-reduced ions generated from the 15+ and 21+ streptavidin tetramers can be directly compared, the charge-reduced ions from the 21+ streptavidin tetramer have broader and higher drift time distributions. The SID spectra from 11+ streptavidin tetramers were further compared. Lower intensities of highly charged (6+ and 5+) monomers observed in Figure 4.14D than those observed in Figure 4.14C. As the addition of supercharging reagent produces streptavidin dimers in the solution phase (Figure 2.2), the Coulombic repulsion may give rise to the conformation which weaken the dimer-dimer interaction in the 21+ streptavidin tetramer, contributing to the broad drift time distribution. Thus, the weak dimer-dimer interaction in the 11+ streptavidin tetramer from the gas phase charge reduction of the 21+ precursor lead to the higher portion of dimer production in SID when the charge state gets lower, which support the assumption that the precursor conformation can be preserved in the gas phase ion-ion reactions.

CRP pentamers generated from gas phase charge reduction of precursors with different charge states were also studied. The supercharged CRP pentamer was generated with 2% m-NBA added into the 10 µM CRP pentamer in 100 mM ammonium acetate. The predominant charge state, 28+, was isolated by the quadrupole. However, the isolation window of 28+ CRP pentamer was not narrow enough with a 32 k quadrupole RF generator installed, thus an isolation of multiple peaks with 28+ as the predominant charge state was used for the gas phase ion-ion reactions. The gas phase ion-ion reactions of the 24+ CRP pentamer isolated from the mass spectrum of 24+ pentamer from 10 µM CRP pentamer in 100 mM ammonium acetate was also studied for comparison. The charge reduced ions were then dissociated by SID with an SID acceleration voltage of 70 V. The SID spectra
of 18+ CRP pentamers generated from the gas phase ion-ion reaction from the 24+ and 28+ CRP pentamers are shown in Figure 4.15.

The 18+ CRP pentamer can be generated from the charge reduction of the 24+ and 28+ CRP pentamers (Figure 4.15A and B). No obvious unfolding can be observed from the 28+ CRP pentamer and drift time distributions of charge-reduced ions generated from different precursors (24+ and 28+) are similar to each other, indicating similar conformations of the 24+ and 28+ CRP pentamers. From the SID spectra shown in Figure 4.15C and D, the SID fragmentation patterns are similar except for the relatively higher
intensity of 5+ CRP monomer in the SID of the 18+ CRP pentamer generated from the 28+ precursor. The observation that higher intensity of 5+ CRP monomer and lower intensity of 4+ CRP monomer/ 8+ CRP dimer from the SID of charge-reduced ions with higher precursor charge state (28+), demonstrates that the CRP pentamer precursor ions with higher charge state has more charges distributed on each of the monomers, thus more charges preserved on monomers after gas phase ion-ion reaction even when the SID precursor has the same charge states. It supports the assumption that the conformation of precursor ions can be partially preserved in the gas phase ion-ion reaction.

4.7. Conclusions

The structures of charge-reduced ions generated from the gas phase and solution additive charge reduction were compared in this chapter with the aid of ion mobility and SID. With PDCH⁻ used as a reagent ion to conduct gas phase charge reduction and TEAA added into the sample solution to induce solution additive charge reduction, drift time distributions and SID fragmentation patterns of the protein complex ions generated from those two different charge reduction methods lead to the conclusion that the precursor conformation can be preserved in the gas phase ion-ion reactions. The statement is investigated in four aspects:

1) Drift time distributions of protein complex ions (8+ β-lactoglobulin, 11+ streptavidin tetramer, 11+ avidin tetramer, 14+ concanavalin A tetramer and 18+ CRP pentamer) generated from gas phase charge reduction are slightly higher than those generated from solution additive charge reduction. It might be because of the retention of
the slightly less compact conformation of highly charged precursor ions in the gas phase ion-ion reactions than the conformation of solution phase charge-reduced ions.

2) The SID spectra of the 10+ and 11+ streptavidin tetramers from the 15+ precursor and the 18+ CRP pentamer from the 24+ precursor present similar SID fragmentation patterns to the SID spectra of the ions at the same charge state generated solution additive charge reduction. The minor differences observed in drift time distributions may not be enough to be distinguished from SID dissociation. The structural difference between the precursor ion and the charge reduced ion studied in the gas phase charge reduction is too subtle to be distinguish by SID.

3) The more unfolded drift time distribution of 11+ streptavidin tetramer generated from gas phase charge reduction of the cone activated 15+ streptavidin tetramer shows the preservation of unfolded precursor structure after experiencing gas phase ion-ion reactions. Additionally, the similar SID fragmentation pattern of the 11+ streptavidin tetramer ions generated from cone activated 15+ streptavidin tetramer to that of cone activation 11+ streptavidin tetramer generated from solution additive charge reduction further confirms the preservation of the unfolded precursor conformation.

4) The different SID fragmentation patterns of gas phase charge-reduced ions generated from precursors with different charge states support the statement that the preservation of precursor conformation in gas phase ion-ion reactions. For streptavidin, higher portion of dimers generated from the SID of 11+ tetramer from gas phase charge reduction of the unfolded 21+ precursor than that from 15+ precursor, indicates the preservation of the precursor conformation with a weaker dimer-dimer interaction. For
CRP, the higher intensity of 5+ monomer and lower intensity of 4+ monomer /8+ dimer observed in the SID of 18+ charge-reduced pentamer ions from the 28+ precursor than that from 24+ precursor agree with the fact that the CRP pentamer precursor ions with higher charge state has more charges distributed on each of the monomers, supporting the preservation of the precursor conformation in gas phase ion-ion reaction.
Chapter 5. Monitoring conformation change of protein ions by trapping in a Q-TOF instrument

Some content in this chapter is derived from the paper Sophie R. Harvey, Jing Yan, Jeffery M. Brown, Emmy Hoyes, and Vicki H. Wysocki, Extended Gas-Phase Trapping Followed by Surface-Induced Dissociation of Noncovalent Protein Complexes, *Analytical Chemistry* 2016, 88 (2), 1218-1221.

5.1. Introduction

Native mass spectrometry has become an important technique in structural biology studying protein assemblies, which are essential in cellular biological processes.248 With the utilization of various activation methods to activate protein complex ions in the gas phase, the quaternary structure can be studied.103 In structural studies of protein complexes, quadrupole time-of-flight (Q-TOF) instruments have been shown to be capable of detecting protein complexes with a molecular weight in MDa range.249,250 With fast ion transmission, the Q-TOF instrument has been shown to be able to fragment ions with collision induced dissociation (CID) and surface induced dissociation (SID).104,105,181 However, when it comes to electron transfer dissociation (ETD) and ultraviolet photodissociation (UVPD), which require extended time frame to conduct the gas phase reaction,107,246 the fast
transmission in the Q-TOF instrument can be a limitation. As each gas phase activation method has advantages and disadvantages,\textsuperscript{103} the extension of time for protein ions to be trapped in the gas phase in the Q-TOF instrument helps in coupling different activation techniques for better understanding of the structure of proteins.

The successful trapping of mononucleotide and cytochrome c for UVPD study\textsuperscript{251} has shown the possibility of the trapping ions in the gas phase in a Q-TOF instrument. In this chapter, proteins and protein complexes were managed to be trapped in the Trap TWIG in a modified Waters Synapt G2S quadrupole time-of-flight mass spectrometer with an SID device installed between the truncated Trap TWIG and the ion mobility cell (Figure 2.1).\textsuperscript{131} To further investigate the conformational change of protein complexes during trapping, the trapped protein complex ions were further studied by SID fragmentation.\textsuperscript{186}

5.2. Method

5.2.1. Sample preparation

Cytochrome c from horse heart and β-lactoglobulin from bovine milk were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Streptavidin was purchased from Thermo Scientific Pierce Biotechnology (Rockford, IL, U.S.A.). The protein samples (cytochrome c, β-lactoglobulin and streptavidin) were buffer exchanged into 100 mM ammonium acetate \(\geq 99\%\) (Sigma-Aldrich, St. Louis, MO, U.S.A.) with 6-kDa cut-off size exclusion chromatography spin columns (Bio-Rad, Hercules, CA, U.S.A.). The charge reduced streptavidin was prepared by mixing the streptavidin sample in 100 mM ammonium acetate with 100 mM triethylammonium acetate (TEAA) in a ratio of 4:1 (v/v).
In the experiment, protein ions with specific charge states were studied. The 7+ cytochrome c ions were generated by spraying 10 µM cytochrome c. The 13+ β-lactoglobulin dimer ions were generated by spraying 20 µM β-lactoglobulin dimer. The 15+ and 11+ streptavidin tetramers ions were generated by spraying 10 µM streptavidin tetramer in 100 mM ammonium acetate and 8 µM streptavidin tetramer in 80 mM ammonium acetate + 20 mM TEAA, respectively.

5.2.2. Mass spectrometry

All the MS and MS/MS experiments were conducted a modified Synapt G2S quadrupole time-of-flight mass spectrometer (Waters Corp., Wilmslow, U.K.) with an SID device installed between the truncated Trap TWIG and the ion mobility cell (Figure 2.1). Nanoelectrospray ionization (nESI) source was used to spray protein samples using 0.8 – 1.1 x 90 mm glass capillaries (Kimble, Vineland, NJ, U.S.A.) pulled by a Sutter Instruments P-97 micropipette puller (Novato, CA, U.S.A.). The experiments were conducted in TOF mode with He and N₂ gas flow rate turned off, thus no ion mobility separation was performed. The capillary voltage for spraying protein samples was set to 1.5 kV and was controlled by the trapping script when the script started to run. The sampling cone voltage was set to 20 V. The trap entrance and trap DC bias are key parameters for the trapping experiment, and they were kept constant during the trapping process. The pressure in the Trap TWIG was 4.97 x 10⁻³ mbar (gas flow rate 1.0 mL/min) or 8.72 x 10⁻³ mbar (gas flow rate 2.0 mL/min). All the precursor ions were selected by quadrupole and trapped in the Trap TWIG. The tuning parameters varied when different
species were trapped. And the parameters optimized for maximal extraction of different precursor ions are shown in Table 5.1.

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<td>Decrease by 0.3 V for 60 times</td>
<td>Decrease by 0.3 V for 60 times</td>
<td>Decrease by 0.3 V for 60 times</td>
</tr>
</tbody>
</table>

Table 5.1. Tuning parameter for trapping of different species.
The SID experiments were conducted in TOF mode with the instrument setup as shown previously.\textsuperscript{131,136} The tuning parameters for the SID device for the SID experiments are shown in Table A.1 in Appendix A. All the spectra were acquired with 1s per scan.

5.2.3. Workflow of the trapping process

The trapping experiment was conducted with WREnS (Waters Research enabled software). The potentials on electrodes were controlled and changed sequentially by running a script in WREnS. The original trapping script was created by Jeff Brown from Waters Corp. (Wilmslow, U.K.) and the parameters were optimized for each of the protein systems studied. There are four steps in the trapping process (Figure 5.1). The traveling wave in the Trap TWIG was disabled during the trapping process.
In each of the steps, the capillary voltage, voltage on the exit lens of the Trap TWIG and the length for each of the steps were controlled by the script. The first step (Beam) was used to check the signal of the spray with the capillary voltage on and exit lens of the Trap TWIG set to 0 V relative to Trap DC bias so that all the ions can transmit through the Trap TWIG. In the second step (Fill), the voltage on the exit lens of the Trap TWIG was increased by 7 – 12 V with the capillary voltage on so that ions can be filled in the Trap TWIG by being stopped by the exit lens. The capillary voltage was turned off in the third step (Trap) so that the ions can be confined in the Trap TWIG. In the fourth step (Trap
extract), the exit lens of the Trap TWIG decreased at a certain rate with the capillary voltage kept off so that the ions trapped in the Trap TWIG can be ejected for further detection in the TOF analyzer. The typical tuning parameters tuned controlled in the trapping script are shown in Table 5.2.

<table>
<thead>
<tr>
<th>Step number</th>
<th>Step name</th>
<th>Capillary voltage/kV</th>
<th>Trap exit voltage/V</th>
<th>Time/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Beam</td>
<td>1.5</td>
<td>0</td>
<td>5 – 10</td>
</tr>
<tr>
<td>II</td>
<td>Fill</td>
<td>1.5</td>
<td>5 – 15</td>
<td>2 - 120</td>
</tr>
<tr>
<td>III</td>
<td>Trap</td>
<td>0</td>
<td>5 - 15</td>
<td>2 - 120</td>
</tr>
<tr>
<td>IV</td>
<td>Trap extract</td>
<td>0</td>
<td>Decrease by 0.3 V for 60 times</td>
<td>40 - 60</td>
</tr>
</tbody>
</table>

Table 5.2. Typical tuning parameters for the trapping process.

5.3. MS of protein ions trapped for different amount of time

The cytochrome c sample in 100 mM ammonium acetate was sprayed by nESI source and the 7+ cytochrome c peak was selected by quadrupole to conduct the trapping experiment.

The signal of 7+ cytochrome c in one cycle of the trapping process, which provides relatively low intensity per scan of extracted ions (Trap extract) that are comparable with the intensity per scan of ions in transmission (Beam), is shown in Figure 5.2 as an example. The normalized intensity of 7+ cytochrome c vs. time during the trapping process is shown
in Figure 5.2A. With optimized conditions applied, the intensity per scan of ions in Trap extract is much higher than that shown in Figure 5.2A.

For the signal of ions in one trapping cycle shown in Figure 5.2A: I) During the 5 s Beam step, stable precursor signal can be observed (Figure 5.2B). II) In the 10 s Fill step, stopping potential in the exit lens of the Trap TWIG was applied, signal loss was observed in Figure 5.2A and no signal of the precursor ion was detected in the TOF analyzer (Figure 5.2C), indicating that ions were accumulated and there was no ion leaking from the Trap TWIG with the source capillary voltage on. III) In 10 s Trap step, no signal of the precursor ion was detected in the TOF analyzer (Figure 5.2D), demonstrating the efficient trapping without ion leaking. IV) In Trap extract step, the signal can be detected in the TOF analyzer with capillary voltage off (Figure 5.2E), indicating the release of ions trapped in the Trap TWIG after the 10 s trapping.
Figure 5.2. The relative intensity and mass spectra of 7+ cytochrome c in each step during the trapping process. (A) The relative intensity of 7+ cytochrome c in one cycle of the trapping process. Each step was separated by the dashed line. The mass spectra of ions detected in (B) Beam, (C) Fill, (D) Trap and (E) Trap exact step of the trapping process.
The 7+ cytochrome c ions were trapped with the trapping script using the optimized parameters shown in Table 5.1 with different trapping time. The mass spectra obtained by combining 5 pulses acquired in Trap extract step are shown in Figure 5.3.

Figure 5.3. Mass spectra obtained after trapping 7+ cytochrome c ions with different trapping time. The spectra were combined from five extraction pulses.
As shown in Figure 5.3, 7+ cytochrome c ions can be successfully trapped and extracted. However, the 6+ charge state, due to charge stripping, can be observed with increased trapping time. The relative intensity of 6+ cytochrome c also increases with increased trapping time. The charge stripping peaks might be generated by collisions with neutrals during the trapping process. In order to better understand the observation, the trapping of protein complex systems was also performed. The spectra extracted by trapping complex ions with different trapping time are shown in Figure 5.4.

![Mass spectra obtained after trapping 13+ β-lactoglobulin dimer, 15+ streptavidin tetramer and 11+ streptavidin tetramer ions with different trapping time. The spectra were combined from five extraction pulses.](image)

Figure 5.4.
As shown in Figure 5.4, 13+ β-lactoglobulin dimer, 15+ streptavidin and 11+ streptavidin tetramer ions were trapped and extracted from the truncated Trap TWIG. The sources of β-lactoglobulin and streptavidin are heterogeneous, where β-lactoglobulin contains variants A and B and streptavidin has partial N-terminal methionine excision.\textsuperscript{132,235,252} Because of the heterogeneity, peak splitting can be observed in the spectra extracted from those two samples. With the increase of trapping time, the charge stripping from the precursor ions can be observed in different degrees for different precursors. The 13+ β-lactoglobulin dimer experienced the most significant charge stripping with low precursor peak remaining at a trapping time of 120 s, while the intensity of charge stripping peak observed from the 11+ streptavidin tetramer is low after a 120 s trapping. With the solvent-accessible surface area (SASA) obtained from the previous publication\textsuperscript{112} and PISA (Protein Interfaces, Surfaces and Assemblies)\textsuperscript{197} analysis, surface charge density ($z$/SASA) of each of the precursors were calculated in Table 5.3.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>PDB</th>
<th>z</th>
<th>SASA (Å$^2$)</th>
<th>$z$/SASA (x 10$^3$Å$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7+ cytochrome c monomer</td>
<td>1HRC</td>
<td>7</td>
<td>6264.3</td>
<td>1.11</td>
</tr>
<tr>
<td>13+ β-lactoglobulin dimer</td>
<td>2Q2M</td>
<td>13</td>
<td>16013</td>
<td>0.81</td>
</tr>
<tr>
<td>15+ streptavidin tetramer</td>
<td>1SWB</td>
<td>15</td>
<td>19820</td>
<td>0.76</td>
</tr>
<tr>
<td>11+ streptavidin tetramer</td>
<td>1SWB</td>
<td>11</td>
<td>19820</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Table 5.3. The charge state, SASA and surface charge density of cytochrome c, β-lactoglobulin and streptavidin ions studied.\textsuperscript{112,197}
Based on results shown in Table 5.3, surface charge density (z/SASA) increases in the following order, 11+ streptavidin tetramer, 15+ streptavidin tetramer, and 13+ β-lactoglobulin dimer, which is the same as the order for more significant charge stripping observed. However, the surface charge density of cytochrome c does not fall into the trend discussed above. We believe this is due to the fact that cytochrome c was partially unfolded at charge state 7+, leading to a much larger SASA than that was calculated from the folded crystal structure, thus the surface charge density of 7+ cytochrome c is much lower than 1.11, the value calculated from the corresponding crystal structure (Table 5.3). Therefore, for protein complex with higher surface charge density, more charge stripping can be observed with longer trapping time.

5.4. The effect of different parameters on trapping efficiency

For the trapping of different protein systems, different parameters were applied to maximize the intensity of ions extracted as shown in Table 5.1. The effect of different parameters on trapping efficiency was studied. The trapping efficiency was calculated by dividing the total intensity of ions extracted by the total intensity of ions filled. The effect of key parameters, such as the trap entrance voltage, the trap DC bias, trap gas flow rate and voltage gradient for ion extraction were all studied to maximize the trapping efficiency, and they are different for different precursor ions. For example, the optimized trap pressure for the trapping of 11+ and 15+ streptavidin tetramers was 8.72 x 10^{-3} mbar (trap gas flow rate 2 mL/min), while the optimized trap pressure for the trapping of 7+ cytochrome c and 13+ β-lactoglobulin dimer is lower, around 5 x 10^{-3} mbar (trap gas flow rate around 1 mL/min). The study of the effect of fill time and trapping time is shown as examples in
Figure 5.5 and Figure 5.6. In Figure 5.5, the effect of fill time was studied with constant trapping time applied for each of the species.

![Figure 5.5](image)

**Figure 5.5.** The effect of fill time on trapping efficiency. The trapping time was 10 s for 7+ cytochrome c and 20 s for all the other ions. The error bars were calculated with three repeats.

As shown in Figure 5.5, the data was acquired with the optimized conditions shown in Table 5.1 except for the fill time. For 15+ and 11+ streptavidin tetramers, and 7+ cytochrome c the trapping efficiency starts to decrease with trapping time higher than 5 s. For 15+ streptavidin tetramer, and 7+ cytochrome c, the trapping efficiency is maximized with 5s fill time. For the 11+ streptavidin tetramer, the trapping efficiency is stable with
fill time less than 5 s. For the 13+ β-lactoglobulin dimer, the trapping efficiency is relatively stable in all the fill times studied (2 s – 20 s). With the optimized fill time determined for trapping different species, the trapping efficiency with various trapping time was further studied. The results are shown in Figure 5.6.

![Figure 5.6](image.png)

Figure 5.6. The effect of trapping time on trapping efficiency. The fill time was 3 s for 7+ cytochrome c and 5 s for all the other ions. The error bars were calculated with three repeats.

As shown in Figure 5.6, within 60 s trapping, the trapping efficiency of all the species studied was stable. The trapping efficiency for 13+ β-lactoglobulin dimer and 7+ cytochrome c started to decrease when trapped for more than 1 min, while the trapping efficiency for 11+ and 15+ streptavidin tetramers were stable even after trapped for 2
minutes. Therefore, for different protein and protein complex ions studied in this chapter, there was no significant change in the trapping efficiency within 1-minute trapping.

5.5. SID of protein ions shows no significant conformational change with increase of trapping time

As shown in Figure 5.6, the ions can be trapped in the truncated Trap TWIG, especially for 11+ streptavidin tetramer ions, which can be efficiently trapped for 2 minutes. The potential conformational changes of the ions during trapping were further monitored by fragmenting trapped ions with SID. The charge stripping of precursor ions can be observed in Figure 5.4. The precursor ions with different charge states can generate different SID fragmentation patterns, which interfere with the comparison of protein conformation based on the difference of SID fragmentation patterns. Among all the ions studied in this chapter, least charge stripping was observed in the trapping of the 11+ streptavidin tetramer (Figure 5.4), making it the best model for the conformational study with SID. In addition, as discussed previously in Chapter 2.3, charge reduced streptavidin can better preserve compact conformation, thus 11+ streptavidin tetramer generated from the charge reduced streptavidin sample was used to monitor the conformation change during trapping process via SID. The 11+ streptavidin tetramer ions generated from the sample prepared with charge reducing reagent TEAA were isolated by the quadrupole and trapped in the truncated Trap TWIG. The trapped ions were fragmented in the SID device between the truncated Trap TWIG and the ion mobility cell. The mass spectra of streptavidin tetramer ions extracted after trapping with different lengths of trapping time and their corresponding SID results are shown in Figure 5.7.
As shown in Figure 5.7, minor charge stripping peak (10+ streptavidin tetramer) can be observed with 120 s trapping (left column in Figure 5.7). With an SID collision energy of 550 eV, the 11+ streptavidin tetramer ions dissociate into monomers, dimers, and trimers with some remaining precursor ions. The SID fragmentation patterns generated from ions trapped for different amount of trapping time are similar to each other (right column in Figure 5.7), showing no significant conformational change during the trapping process. The slight difference in relative intensities of dimer and precursor ions observed
in SID spectra were shown to be within error bars obtained from three repeats,\textsuperscript{186} thus the SID fragmentation patterns of the 11+ streptavidin tetramer trapped for a different amount of time are similar.

5.6. Conclusions

Different protein and protein complexes have been successfully trapped in the truncated Trap TWIG in a modified Waters Synapt G2S Mass Spectrometer. The optimized parameters are different for trapping different ions with different molecular weight and different charge states. For the ions studied in this chapter (7+ cytochrome c, 13+ β-lactoglobulin dimer, 11+ and 15+ streptavidin tetramers), no significant change in trapping efficiency was observed. Increased charge stripping can, however, be observed for ions with higher surface charge density. The similar SID fragmentation patterns of the 11+ streptavidin tetramer trapped for different amount of time demonstrate no significant conformational change during trapping. More protein systems have been trapped and studied by Dr. Sophie Harvey and the similar result has been observed that no significant conformational change can be observed during the during time studied.\textsuperscript{186}
Chapter 6. Application of SID on FT-ICR platform


6.1. Introduction

Mass spectrometry has been shown to be a useful tool in the structural analysis, providing stoichiometry and quaternary structure of protein complexes.\(^8,17,25,27\) Previous studies have shown that protein complex ions generated by electrospray ionization (ESI) can preserve their native states in the gas phase.\(^27,254\) Tandem mass spectrometry with various activation methods, can further provide structural information on protein complexes.\(^99,105,246,255,256\) For example, collision induced dissociation (CID) of protein complexes ejects an unfolded monomer subunit, providing stoichiometric information.\(^104\) It has been shown that electron transfer dissociation (ETD) and electron capture dissociation (ECD) provide backbone cleavage, allowing surface mapping of protein
Ultraviolet photodissociation (UVPD) provides secondary and quaternary structural information on protein complexes by producing covalent and noncovalent fragments. As discussed in Chapter 2 and previous studies, surface induced dissociation (SID) has been shown to provide insight into the quaternary structure of protein complexes by producing fragments indicative of the relative strength of protein subunit interfaces. SID has successfully applied in quadrupole time-of-flight (Q-TOF) instruments and much of the work with SID of non-covalent protein complexes were performed on Q-TOF platforms. With the aid of ion mobility providing additional separation in Q-TOF instruments, the fragments with the same m/z can be assigned based on the drift time difference. However, when systems with a minor mass difference are studied, the mass resolution of Q-TOF instruments can limit the information that can be obtained from the SID of protein complexes. For example, as mentioned in Chapter 3.1, the wild-type λ exonuclease trimer needs to be tagged with His-tag in order to be distinguished with its single point K131A mutant, which is 57 Da lower than that of untagged wild-type λ exonuclease in each subunit. The resolution of TOF detector fails in distinguishing the wild type and its mutant when His-tag was not added. However, the addition of His-tag sometimes affects the structure of protein complexes. In this case, mass spectrometers with higher resolution are needed to distinguish the minor mass difference of protein complexes in order to avoid the conformational change from the addition of His-tag.

Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR MS) provide ultrahigh resolution, ultra-high mass accuracy m/z measurements. The analysis of
protein complexes in FT-ICR mass spectrometers has been improved with the ParaCell mass analyzer, which increases the collection of long transients with dynamic harmonization of the electric potential of ion motion at any radius, providing more effective in ion motion harmonization at higher cyclotron radii.\textsuperscript{97-99} Protein complexes, with molecular weight up to 186 kDa, has been shown to be isotopically resolved in FT-ICR instruments.\textsuperscript{99,258} Additionally, CID of large protein complexes in the commercial quadrupole CID collision cell leads to loss of signal at the high collision energy required for the dissociation of large protein complex ions.\textsuperscript{259} Therefore, an efficient activation method is needed for the dissociation of large protein complexes in FT-ICR instruments. SID has been applied to FT-ICR instruments with a surface installed in the ICR cell and used to study the peptide fragmentation in previous studies.\textsuperscript{123,260,261} However, the SID in FT-ICR was not used for large protein complex studies. In this chapter, a SID device was designed, installed and tested with several protein complex systems in a hybrid FT-ICR instrument (external to the ICR cell). Some content in this chapter is derived from the paper from J. Yan \textit{et al.}.\textsuperscript{132}

6.2. Method

6.2.1. Design of SID device in FT-ICR

The SID device was designed to replace the original CID collision cell, and the design was optimized by SIMION 8.0 simulations. A model of the SID device is shown in Figure 6.1. The script used for simulation was modified from a script from a previous dissertation.\textsuperscript{262} The protein complex ions were simulated to start from the aperture of the
entrance lens (Electrode 1 in Figure 6.1) with initial kinetic energies ranging from 50 eV to 100 eV, and then accelerate to collide with the surface (Electrode 6 in Figure 6.1) with kinetic energies of approximately 500 eV. Assuming the dissociation of the precursor ions happens between the surface and the exit lens of the SID device (Electrode 6 and Electrode 10 in Figure 6.1), generation of both precursor and fragment ions after the surface collision was considered in the simulation. Therefore, the mass and the charge state parameters of a portion of precursor ions were changed to values which represent the potential fragments when the precursor ions reach the surface. 10% - 20% kinetic energy retention (0.32 - 0.45 of velocity loss factor in x, y and z-axis in the script) in the surface activation was considered.
Figure 6.1. The design of the SID device in an FT-ICR instrument. A) The front view, B) the 3D model, C) the 3D section view and D) the photo of the SID device. The electrodes labelled in A) are as follows: 1, 2 – entrance lens for the SID region; 3, 4, 5, 7, 8 – deflection lenses; 6 – surface; 9, 10 – exit lens for the SID region; 11 – entrance lens for the trap region; 12 – rectilinear quadrupole; 13 – asymptotic electrodes; 14 – exit lens of the trap region. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).132

As shown in Figure 6.1A, there are two parts in the SID device, a 2.75 cm long SID region and a 2.79 cm long trap region. The detailed dimension information on each of the electrodes in the SID device is shown in Appendix B. In the SID region, DC potentials were applied on the ten electrodes (Electrode 1 -10 in Figure 6.1A) and controlled by
external DC power supply. The trap region consists of entrance lens, exit lens, four asymptotic electrodes and rectilinear quadrupole (Electrode 11, 14, 13 and 12 in Figure 6.1). DC voltages on the entrance and exit lens were controlled by the corresponding DC pulsing power supply for the entrance and exit lens of the original collision cell. The DC voltage applied on asymptotic electrodes is controlled by the repurposed internal extra DC pulsing power supply in the FT-ICR instrument that was previously used for trapping and pulsing ions into the ICR cell. The RF and DC power supply for the hexapole in the original collision cell was used as power supply for the rectilinear quadrupole of the SID device. Ions transmitted through the SID region into the trap region are trapped and pulsed/extracted into the ICR cell. Therefore, there are two sets of voltages for the DC applied on the trap region for trapping and pulsing/extracting functions, respectively, in each scan of m/z measurement. The typical DC voltages applied on each of the electrodes in the SID device (Figure 1.1A) for MS and MS/MS experiment are shown in Table 6.1. There are two sets of voltages used for the trap entrance and trap exit (Electrode 11 and Electrode 14) for trapping and pulsing/extracting functions, respectively.
<table>
<thead>
<tr>
<th>Electrode</th>
<th>Transmission mode (V)</th>
<th>SID 35 V (V)</th>
<th>SID 35 V + ΔV (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SID region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>0.0</td>
<td>ΔV</td>
</tr>
<tr>
<td>2</td>
<td>-3.0</td>
<td>0.0</td>
<td>ΔV</td>
</tr>
<tr>
<td>3</td>
<td>-2.0</td>
<td>-32.0</td>
<td>-32.0</td>
</tr>
<tr>
<td>4</td>
<td>-1.0</td>
<td>0.0</td>
<td>ΔV</td>
</tr>
<tr>
<td>5</td>
<td>-4.0</td>
<td>-25.0</td>
<td>-25.0</td>
</tr>
<tr>
<td>6</td>
<td>-3.0</td>
<td>-35.0</td>
<td>-35.0</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>-70.0</td>
<td>-70.0</td>
</tr>
<tr>
<td>8</td>
<td>-7.5</td>
<td>-30.0</td>
<td>-30.0</td>
</tr>
<tr>
<td>9</td>
<td>-5.0</td>
<td>-80.0</td>
<td>-80.0</td>
</tr>
<tr>
<td>10</td>
<td>-7.0</td>
<td>-100.0</td>
<td>-100.0</td>
</tr>
<tr>
<td>Trap region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 (Entrance lens trap)</td>
<td>3.5</td>
<td>-30</td>
<td>-30</td>
</tr>
<tr>
<td>11 (Entrance lens extract)</td>
<td>15</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>12 (Collision voltage)</td>
<td>-4</td>
<td>-40</td>
<td>-40</td>
</tr>
<tr>
<td>13 (Asymptotic electrodes)</td>
<td>-10</td>
<td>-10</td>
<td>-10</td>
</tr>
<tr>
<td>14 (Exit lens trap)</td>
<td>5</td>
<td>-28</td>
<td>-28</td>
</tr>
<tr>
<td>14 (Exit lens extract)</td>
<td>-10</td>
<td>-15</td>
<td>-15</td>
</tr>
</tbody>
</table>

Table 6.1. DC voltages applied on each of the electrodes in the SID experiment. For SID 35 V + ΔV experiment, all the voltages from Funnel 2 to Post-filter DC bias were increased by ΔV. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).132
Notice that the DC voltages applied on each of the electrodes vary after each SID installation. All of the DC parameters are retuned based on the parameters listed in Table 6.1 after each SID installation because of the minor repositioning of the SID device.

For voltages on the asymptotic electrodes, -10 V in both trapping and pulsing/extracting when 2.0 V applied on the “DC Extract Bias” were used in MS and MS/MS experiments. The combination of 30 V for trap and 55 V for extract on the asymptotic electrodes when 0.0 V was applied for the “DC Extract Bias” have been shown to better trap the ions in the region near the exit electrode. However, there was no significant difference in the total intensity of ions extracted. The RF applied on the rectilinear quadrupole (Electrode 12 in Figure 6.1A) was set to 1.4 MHz, corresponding to a typical readback of 1576.2 ± 0.7 Hz, and the peak to peak amplitude of 2000 V.

6.2.2. Mass spectrometry experiment parameters for protein complexes

All MS and MS/MS experiments were performed on a Bruker SolariX XR 15 T FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany) with the customized SID device (Figure 6.1D) installed replacing the original CID collision cell. A home-made nanoelectrospray ionization (nESI) source (Figure 6.2) was used to conduct all the MS and MS/MS experiments. The home-made nanoelectrospray ionization source consists of a positioning station and a capillary holder with a grounded platinum wire as shown in Figure 6.2. The protein solution was loaded into a 0.8 – 1.1 x 90 mm glass capillary (Kimble, Vineland, NJ, U.S.A.) pulled by P-97 micropipette puller (Sutter Instruments, Novato, CA, U.S.A.) and a capillary voltage of -1.25 ± 0.15 kV was applied on the heating glass capillary in the source region of the instrument to spray protein ions into the gas phase.
Except for the DC voltages applied to the SID device, all the instrument settings were controlled by Bruker FTICR control software. In “API Source” settings, the capillary voltage was set to $-1.25 \pm 0.15 \text{kV}$, the “Drying Gas Flow Rate” was set to 1.5 mL/min and the “Drying Gas Temperature” was set to 200°C for nanoelectrospray ionization. In “Collision cell” settings, the “Collision Voltage” was set as shown in Table 6.1. The “DC Extract Bias” was set to 1.5 V. The gas flow was set to 80% for protein complex analysis. The UHV pressure readback for the experiment was around $2.2 \times 10^{-9} \text{mbar}$. Depending on the protein system tested, the “Time of Flight” varied from 1.2 ms to 1.8 ms. The RF frequency was set to be the lower limit and the RF amplitude was set to the upper limit of the instrument in the Bruker ftms Control software for the analysis of large protein complexes ($m/z > 3000$). The RF frequency was set to higher and RF amplitude was set to
lower when systems with lower m/z were analyzed. The schematics of DC potentials applied on the electrodes in the instrument for transmission and SID modes are shown in Figure 6.3

Figure 6.3. Schematics of a modified Bruker SolariX XR 15 T FT-ICR and its corresponding DC potentials applied for transmission mode (middle row) and SID mode (bottom row) in FT-ICR. The cutaway views of the SID device are highlighted in the shaded box on the left. In SID mode, the DC potential on the surface is −35 V (red dot), providing an acceleration voltage of 35 V or 35 V + ΔV (as shown by the blue solid line and the blue dashed line. Product ions are accelerated through the SID exit lens and trapped and thermalized in the rectilinear quadrupole. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).132

For ion transmission, the DC voltages applied on the SID device were set to values shown in transmission mode in Table 6.1, and the corresponding potential diagram is shown in the middle row in Figure 6.3. The ions were directly transmitted through the SID
region without colliding with any electrodes in the SID device and trapped in the Trap region before pulsed into the ICR cell for mass detection. The SID device has been tested with small molecules, peptides, and protein complexes and it has been shown to be able to efficiently transmit those ions with the same DC but different RF tuning parameters. For the SID experiment, the DC voltages applied on the SID device were set to values shown in SID mode in Table 6.1, and the corresponding potential diagram is shown in the bottom row in Figure 6.3. The voltage on the first entrance electrode of the SID device (Electrode 1 in Figure 6.1) is similar to that applied on the quadrupole. The voltage difference between the first entrance electrode and the surface (Electrode 1 and Electrode 6 in Figure 6.1) of the SID device was used to represent the SID acceleration voltage.

This is the first-generation SID device in an FT-ICR mass spectrometer installed external to the ICR cell, which was successfully tested with several protein systems. The trap region of the SID device, in which the ions are trapped and thermalized, is much shorter than the original collision cell. It has been observed that a 0.5 s accumulation time with the SID device can be used to generate a comparable signal to that obtained in the original collision cell with a 0.05 s accumulation time. The transient time, which is related to the resolution of the spectrum, is affected by the lower bound of the m/z range, but not the upper bound. Therefore, different upper bound settings for spectrum acquisition do not affect the resolution of mass spectra. In the MS and MS/MS experiments performed with protein complexes, all the peaks detected were at the m/z lower than 14,000, thus the m/z range was set to 506 – 14,000. Various numbers of scans and different transient time were used to acquire the spectra of different protein complex systems so that the isotopic
distribution of all peaks can be observed. Due to the loss of signal from quadrupole selection, MS/MS spectra were acquired with a larger number of scans than that used to acquire mass spectra.

6.2.3. Sample preparation

Streptavidin (Thermo Scientific Pierce Biotechnology, Rockford, IL, U.S.A.), cholera toxin B (Sigma-Aldrich, St. Louis, MO, U.S.A.), recombinant human C-reactive protein (CalBiochem, San Diego, CA, U.S.A.), toyocamycin nitrile hydratase (from Dr. Vahe Bandarian’s lab, University of Utah),265 serum amyloid P (CalBiochem, San Diego, CA, U.S.A.), glutamate dehydrogenase from bovine liver (Sigma-Aldrich, St. Louis, MO, U.S.A.), carbonic anhydrase (Sigma-Aldrich, St. Louis, MO, U.S.A.), and ubiquitin from bovine erythrocytes (Sigma-Aldrich, St. Louis, MO, U.S.A.) were studied to test the SID device. Ubiquitin, carbonic anhydrase, streptavidin, serum amyloid P (SAP) and glutamate dehydrogenase from bovine liver (GDH) were used to test the transmission of the SID device. The carbonic anhydrase, streptavidin, SAP and GDH samples for transmission tests were buffer exchanged into 100 mM ammonium acetate ≥ 99% (Sigma-Aldrich, St. Louis, MO, U.S.A.) with 6-kDa cut-off size exclusion chromatography spin columns (Bio-Rad, Hercules, CA, U.S.A.). Ubiquitin was dissolved in water at a concentration of 2 µM for mass spectrometry analysis. The protein samples for SID fragmentation test (streptavidin, cholera toxin B, C-reactive protein, and toyocamycin nitrile hydratase) were buffer exchanged into 100 mM ethylenediammonium diacetate (EDDA, Sigma-Aldrich, St. Louis, MO, U.S.A.) with 6-kDa cut-off size exclusion chromatography spin columns. Concentrations of buffer exchanged protein samples were measured with a Nanodrop
2000c spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.). The samples for transmission test were diluted to the final concentration of 5 - 10 µM. The samples for SID fragmentation test were diluted to a protein complex concentration of 5 µM (streptavidin), 4 µM (cholera toxin B) and 5 µM (toyocamycin nitrile hydratase) for mass spectrometry analysis. C-reactive protein (CRP) samples in different buffers were prepared by buffer exchanging the protein sample into 100 mM ammonium acetate. The CRP samples charge reduced by EDDA and triethylammonium acetate (TEAA, Sigma-Aldrich, St. Louis, MO, U.S.A.) were prepared by mixing CRP sample, which was buffer exchanged into 100 mM ammonium acetate, with 100 mM TEAA in 4:1 (v/v) ratio or 100 mM EDDA in 1:1 (v/v) ratio. The final concentration of CRP complex was 3 µM. The streptavidin–biotin complex was obtained by mixing streptavidin sample with 500 µM biotin (Sigma-Aldrich) stock solution. The final concentration ratio of streptavidin monomer: biotin is 1: 1.5.

6.2.4. m/z calibration

Perfluoroheptanoic acid (PFHA) purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) was used as an m/z calibrant for protein complex study. The stock solution was prepared by dissolving 1 µL of PFHA into 500 µL isopropanol with an addition of 300 µL 16.7 mM NaHCO₃ solution. Since the melting point of PFHA is 54.3 °C, which is higher than room temperature, PFHA was heated to melt before diluted. The stock solution was stored in -20 °C freezer. The m/z calibrant was prepared by 10-fold dilution of the stock solution into 1:1 isopropanol: acetonitrile solution. The m/z from 794 to 7742 was calibrated for the SID experiment of streptavidin, cholera toxin B (CTB), C-reactive protein (CRP) and toyocamycin nitrile hydratase (TNH). In “Ion Transfer” panel,
“Collision Voltage”, “DC Extract Bias” and “Time of Flight” were set to -1.5 V, 0.5 V and 2.0 ms, respectively. Calibration of m/z higher than 7742 was covered by increasing the “Time of Flight” parameter when higher m/z calibration is needed in the experiment.

6.3. Transmission test with different protein systems

With the SID device installed, the transmission of proteins and protein complexes has been tested. Ubiquitin was tested with an ESI source. All the other proteins and protein complexes were tested with a nESI source. The tuning parameters for protein complex transmission were shown in Chapter 6.2.2. The tuning parameters for transmitting different species are similar, except for the “Time of Flight” of the transfer optics between the collision cell and the ICR cell, the RF frequency and amplitude applied on the other transfer multipoles, and the DC voltage applied on the quadrupole in the trap region of the SID device. For species with lower m/z (m/z < 3000), the “Time of Flight” was set to lower than 1.0 ms (typically 0.6 – 1.0 ms). For all the transfer multipoles, the RF frequency increased and the RF amplitude was set to lower for transmitting lower m/z species. For the DC settings, the “Collision Voltage” was increased to -1.5 V, the “DC Extract Bias” was decreased to 0.5 V.

The mass spectra of proteins (ubiquitin and carbonic anhydrase) are shown in Figure 6.4. As shown in Figure 6.4, ubiquitin (8.6 kDa) and carbonic anhydrase (29 kDa) can be efficiently transmitted through the SID device. Efficient transmission can also be obtained with small peptides and molecules were also tested (data no shown).
Figure 6.4. MS of ubiquitin and carbonic anhydrase. (A) Mass spectrum of 2 μM ubiquitin in water by averaging 20 scans with a transient of 2.3 s. (B) Mass spectrum of 10 μM carbonic anhydrase in 100 mM ammonium acetate by averaging 20 scans with a transient of 4.6 s.

The protein complexes with higher m/z were further tested. The results are shown in Figure 6.5. In Figure 6.5, protein complexes with various molecular weight (streptavidin tetramer – 53 kDa, streptavidin octamer – 106 kDa, SAP pentamer – 125 kDa, SAP decamer – 250 kDa, and GDH hexamer - 334 kDa) can be transmitted through the SID device. Based on the baseline noise level, the signal of species with higher m/z or higher molecular weight (for example, GDH shown in Figure 6.5C) is lower than that of species with relatively lower m/z or lower molecular weight (for example, streptavidin and SAP shown in Figure 6.5A and B), indicating that the trapping efficiency might be lower for high m/z species.
Figure 6.5. Mass spectra of streptavidin, SAP and GDH in 100 mM ammonium acetate. (A) Mass spectrum of streptavidin by averaging 41 scans with a transient of 4.6 s. (B) Mass spectrum of SAP by averaging 53 scans with a transient of 9.2 s. (C) Mass spectrum of GDH by averaging 100 scans with a transient of 4.6 s.

6.4. SID of protein complexes

6.4.1. Streptavidin

For the homotetramer, streptavidin, the interface interaction and SID dissociation behavior in Q-TOF instrument have been discussed previously in Chapter 2.4. Unlike the resultant ejection of an unfolded monomer in CID, SID has shown to be able to produce dimers from a streptavidin tetramer, indicating the relatively weak interaction between dimers (Figure 6.6A).\textsuperscript{136} Here, streptavidin charge reduced by EDDA was used to test the SID device in the FTICR instrument. A 4.6 s transient was used to study streptavidin, and as a result, all the peaks in both MS and MS/MS can be isotopically resolved.
Figure 6.6. The MS and SID spectra of 5 μM streptavidin tetramer in 100 mM EDDA acquired with a transient of 4.6 s. (A) The relative interaction between subunits in a streptavidin tetramer (PDB ID: 1SWB) from PISA (Protein Interfaces, Surfaces and Assemblies) analysis. (B) Mass spectrum of streptavidin with 51 scans. (C) SID of 12+ streptavidin tetramer under different acceleration voltages by averaging 50 scans and all the peaks are assigned with mass errors of less than 2 ppm. The region highlighted in blue is zoomed in and shown in (D). The theoretical isotopic distributions of the 12+ tetramer, 6+ dimer and 3+ monomer are shown in purple, red and blue, respectively. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017). 132

In the mass spectrum of 5 μM streptavidin tetramer in 100 mM EDDA (Figure 6.6B), the predominant peak is from the 12+ streptavidin tetramer with a resolution of approximately 110,000. The 12+ charge state ion of streptavidin tetramer was then isolated by the quadrupole with an isolation window of m/z 50. The isolated ions were then fragmented in the SID region of the SID device, trapped in the Trap region and
pulsed/extracted into the ICR cell from the trap region (Figure 6.1A). The SID spectra of streptavidin acquired under different acceleration voltages (35 V, 45 V and 55 V) are shown in Figure 6.6C with the corresponding tuning parameters of the SID device shown in Table 6.1. At relatively low SID acceleration voltage (35 V), the major products of 12+ streptavidin tetramer are 5+, 6+ and 7+ streptavidin dimers, matching the prediction of cleavage of the relatively weak interaction between dimers (Figure 6.6A). Symmetric dissociation of tetramer into dimers with around half of the charge state of the precursor is the major pathway for SID dissociation of 12+ streptavidin tetramer, which is similar to the observation on of SID of 11+ streptavidin tetramer on Q-TOF platform. The observation of 4+ monomer and its complementary 8+ trimer indicates the existence of a minor dissociation pathway.

The peak at approximately $m/z$ 4425 is the superposition of 12+ tetramer, 6+ dimer and 3+ monomer with the same nominal mass but different isotopic distribution. The resolution is approximately 150,000. In order to better analyze the relative intensities of different species, the $m/z$ region was zoomed in and shown in Figure 6.6D. The experimental data was plotted in grey. The theoretical isotopic distribution of different species generated in Bruker Compass DataAnalysis 4.2 software to compare to the experimental data. The sequence of streptavidin from the PDB file (PDB ID: 1SWB) was used to generate the formula of streptavidin monomer, $C_{585}H_{883}N_{163}O_{192}$. A peak width of 0.02 Th was used to generate the individual theoretical isotopic pattern of different species. The theoretical distributions of each species were then scaled and added up to reach the best match with the experimental data. The process was simplified by starting a scaling
factor to unique peaks which just contribute to one species, in this case, 12+ tetramer. After the scaling factor for the 12+ tetramer had been assigned, only peaks that contribute to the 12+ tetramer and the 6+ dimer were used to assign the scaling factor of the 6+ dimer. Eventually, the scaling factor of the 3+ monomer can be assigned. The isotopic distributions of each of the species with their individual scaling factor considered are shown in Figure 6.6D (12+ tetramer in purple, 6+ dimer in red, and 3+ monomer in blue). Therefore, the change of relative intensity under different SID acceleration voltages can be observed.

With the increase of SID acceleration voltage, the relative intensities of 6+ and 7+ dimers decrease, while the relative intensities of 3+ and 4+ monomers increase (Figure 6.6C), indicating the presence of a secondary fragmentation from dimers to monomers with higher energy involved in a surface collision. However, the absolute intensity of the predominant peak at m/z 4425 (mainly 6+ dimer and 3+ monomer) is higher than that of the precursor peak acquired with the same number of scans. It might be because of the relatively lower trapping efficiency of the 12+ tetramer than that of the smaller species, such as the 3+ monomer and the 6+ dimer. As a result, calculating the efficiency of SID fragmentation is challenging.

6.4.2. Streptavidin-biotin

The streptavidin-biotin complex was further analyzed with the same parameters used for the above streptavidin results (Chapter 6.4.1). The MS and SID spectra of streptavidin-biotin tetramer are shown in Figure 6.7.
Similar to the previous streptavidin results (Figure 6.6), in Figure 6.7, the major peaks in the mass spectrum is from the 12+ streptavidin-biotin tetramer. With an SID acceleration voltage of 35 V, the 5+, 6+, 7+ dimers are the major products from the 12+ streptavidin-biotin tetramer. With a transient of 4.6 s used. The zoom in of 12+ streptavidin-biotin tetramer can be seen in Figure 6.8A. Because the streptavidin sample is a mixture of streptavidin subunits with and without N-terminal methionine (Met), the 12+ streptavidin-biotin peak is not a single isotopic distribution. As shown in the zoom in spectrum in Figure 6.8A, 12+ streptavidin-biotin tetramer with different numbers of subunits with Met as well as various degrees of sodium adduction can be well distinguished. The resolution of 12+ streptavidin-biotin tetramer peaks is approximately 120,000.
In Figure 6.8B, the ultrahigh resolution of FT-ICR makes it possible for the number of biotin binding as well as the sodium adduct to be assigned based on match experimental isotopic distributions with theoretical isotopic distributions. The mass errors for the assigned peaks are less than 2 ppm. Comparisons of experimental and theoretical isotopic distributions of are shown as examples in Figure 6.9.
Figure 6.9. Zoom in of the 12+ streptavidin – biotin tetramer and 6+ streptavidin – biotin dimer peaks, and their corresponding theoretical distributions. (A) Zoom in of the 12+ streptavidin – biotin tetramer peak ((Q + 4b)\textsuperscript{12+} peak in Figure 6.7A is compared with (B) the simulated isotopic distribution. (C) Zoom in of the 6+ streptavidin – biotin dimer ((D + 2b)\textsuperscript{6+} in Figure 6.7B is compared with (D) the simulated isotopic distribution. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).\textsuperscript{132}

The formulas of two streptavidin subunits and 2 biotins (C\textsubscript{1190}H\textsubscript{1798}N\textsubscript{330}O\textsubscript{390}S\textsubscript{2}) and four streptavidin subunits and 4 biotins (C\textsubscript{2380}H\textsubscript{3596}N\textsubscript{660}O\textsubscript{780}S\textsubscript{4}) were used to generate the theoretical isotopic distribution in Figure 6.9B and D. The mass errors are less than 2 ppm. The resolution of peaks in Figure 6.9A and C is approximately 150,000.

When comparing the relative intensity of nonspecific adducts on the streptavidin-biotin tetramer precursor with the streptavidin dimer product in Figure 6.8, there are much fewer nonspecific adducts on the product than observed on the precursor, indicating the loss of nonspecific adducts when ions were activated in the SID device. The fact that most of streptavidin dimers lost their biotin ligands as shown in Figure 6.8B demonstrates that
there might be secondary activation in the collision cell after the fragment ions were generated from the SID process, as previous biotin-binding experiments performed on Q-TOF platforms yielded more products with retained ligands.\textsuperscript{136}

6.4.3. Cholera toxin B (CTB)

Cholera toxin B (CTB) is a homopentamer with a ring structure, which is different from the structure of streptavidin as a dimer of dimers discussed previously. The structure of CTB is shown in the inset of Figure 6.10A. As shown in the mass spectrum (Figure 6.10A), the predominant peak is from the 13+ CTB pentamer. The resolution of 13+ CTB pentamer peak is approximately 130,000. The 13+ CTB pentamer ions were selected by the quadrupole with a selection window of \textit{m/z} 100 and dissociated with SID with an acceleration voltage of 35 V. Based on the similar interactions between subunits in a ring-structured CTB pentamer, the production of monomer + tetramer, and dimer + trimer from the pentamer has similar possibility. In the SID spectrum shown in Figure 6.10B, monomers, dimers, trimers and tetramers can be observed, which matches with the prediction from the cyclic pentamer structure. The SID fragmentation pattern is also similar to the observation acquired from a Q-SID-TOF platform.\textsuperscript{267}

The overlapping peak shown in Figure 6.10B was zoomed in in Figure 6.10C. The theoretical isotopic distribution of 2+ monomer, 4+ dimer, 6+ trimer and 8+ tetramer were used to fit the experimental data with the method as described previously in Chapter 6.4.1. The formula of CTB monomer (C\textsubscript{51}H\textsubscript{81}N\textsubscript{142}O\textsubscript{156}S\textsubscript{5}) was obtained from the PDB 1FGB with the disulfide bond considered.
Figure 6.10. The MS and SID spectra of 4 μM CTB pentamer in 100 mM EDDA. (A) MS of CTB acquired with a 4.6 s transient by averaging 30 scans. Inset: connectivity between subunits in a CTB pentamer (PDB ID: 1FGB) from PISA (Protein Interfaces, Surfaces and Assemblies) analysis. The 13+ CTB pentamer peak was isolated by quadrupole with an isolation window of m/z 100 for SID fragmentation. (B) SID of 13+ CTB pentamer with a 35 V SID acceleration voltage. The spectrum was acquired with a 9.2 s transient by averaging 181 scans. The region highlighted in blue was zoomed in and shown in (C). The experimental data is plotted in black. The simulated isotopic distribution is plotted in grey with each species shown in different colors (8+ tetramer in purple, 6+ trimer in green, 4+ dimer in red and 2+ monomer in blue). The region around m/z 5803.7 was further zoomed in in (D). Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).132

Based on the simulated result, the relative intensities of different species are shown in Figure 6.10C. With a transient of 9.2 s, the resolution of peaks in the zoomed in region is greater than 200,000. The peaks for different species are labeled in Figure 6.10D, and
the mass errors are within 2.2 ppm. Therefore, SID of CTB pentamer in FT-ICR platform provides fragments with high resolution and high mass accuracy, matching the prediction from the known crystal structure.

6.4.4. Toyocamycin nitrile hydratase (TNH)

Different from the structures streptavidin and CTB, toyocamycin nitrile hydratase (TNH) is a protein complex without known crystal structure. Based on the result from previous studies,\textsuperscript{133,268} TNH is a heterohexamer with three different subunits $\alpha$, $\beta$ and $\gamma$. The structural model of TNH built based multiple mass spectrometry techniques has shown that TNH is a dimer of trimers, and in each of the trimers, there is one copy of $\alpha$, $\beta$ and $\gamma$ subunits.\textsuperscript{133,268} TNH was used to test the SID device in the FT-ICR instrument, and the results are shown in Figure 6.11. The 15$^{+}$ TNH hexamer peak is the predominant peak in the mass spectrum shown in Figure 6.11A, and the resolution of 15$^{+}$ TNH hexamer peaks is approximately 200,000.

Because the $m/z$ of 15$^{+}$ TNH hexamer is close to the upper $m/z$ selection limit of the quadrupole, which is 6000, an isolation window of $m/z$ 500 was used, leaving three charge states (14$,^+$, 15$,^+$ and 16$,^+$) isolated after the quadrupole isolation (highlighted in Figure 6.11A).
Figure 6.11. The MS and SID spectra of 5 µM TNH hexamer in 100 mM EDDA, and the experimental & theoretical isotopic distribution of 6+ α subunit generated in SID spectrum. (A) Mass spectrum of TNH acquired with a 9.2 s transient by averaging 50 scans. The hexamers in the highlight region were selected by quadrupole and (B) dissociated by SID with an acceleration voltage of 45 V. The SID spectrum was acquired with a transient of 9.2 s by averaging 255 scans. The 6+ α subunit generated in SID spectrum was zoomed in (C), and the corresponding theoretical isotopic distributions are shown in (D). Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).
From the SID spectrum shown in Figure 6.11B, with an SID acceleration voltage of 45 V, the TNH hexamer ions were mainly dissociated into $\alpha\beta\gamma$ trimers with around half of the precursor charge state. The fragmentation pattern is similar to the SID results obtained by spraying the same sample on the Q-TOF platform described in Chapter 2.2.1 (Figure 6.12).

Figure 6.12. The SID spectrum of 15+ TNH hexamer in 100 mM EDDA at an SID acceleration voltage of 50 V acquired in a quadrupole ion mobility time-of-flight (Q-IM-TOF) instrument (Synapt G2S, Waters Corp., Wilmslow, U.K.) with a customized SID device installed between the trap traveling wave ion guide and the ion mobility cell.\textsuperscript{131,136} Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).\textsuperscript{132}

The symmetric dissociation pattern matches with previous observation on SID fragmentation of noncovalent protein complexes, indicating the preservation of native-like structure of protein complexes.\textsuperscript{105,203} The production of $\alpha\beta\gamma$ trimers as well as $\alpha\beta$ dimers as
minor fragments matches with the previous statement that TNH is a dimer of trimers with the relatively strong interaction between $\alpha$ and $\beta$ subunits in each of $\alpha\beta\gamma$ trimers.\textsuperscript{38}

In the SID spectrum of TNH hexamer (Figure 6.11B), two forms of $\alpha$ subunit can be observed in all the $\alpha$ containing fragments, namely, $\alpha$ subunit with and without one cysteine-sulfenate oxygen, two cysteine-sulfinate oxygens and one cobalt.\textsuperscript{265} For example, in the zoom in region in the SID spectrum of TNH two isotopic distributions of 6+ $\alpha$ subunit can be observed (Figure 6.11C). The sequence of $\alpha$ subunit was obtained from previous publication.\textsuperscript{38} With a disulfide bond considered, $C_{931}H_{1501}N_{271}O_{277}S_5$ was used as the formula of $\alpha$ subunit without three oxygens and one cobalt. With a disulfide bond reduced (+ 2H), and the addition of three oxygens (+ 3O) and one cobalt (+ Co – 2H), $C_{931}H_{1501}N_{271}O_{280}S_5Co$ was used as the formula of $\alpha$ subunit with three oxygens and one cobalt. Therefore, $C_{931}H_{1501}N_{271}O_{277}S_5$ and $C_{931}H_{1501}N_{271}O_{280}S_5Co$ were used to generate theoretical isotopic distributions of the $\alpha$ subunit without (purple in Figure 6.11D, Figure 6.13B and D) and with (red in Figure 6.11D, Figure 6.13B and D) three oxygens and one cobalt by using Bruker Compass DataAnalysis 4.2 software. A similar pattern can also be observed in $\alpha\beta$ dimers ($C_{1378}H_{2182}N_{392}O_{408}S_9$ and $C_{1378}H_{2182}N_{392}O_{411}S_9Co$) and $\alpha\beta\gamma$ trimers ($C_{1889}H_{2979}N_{549}O_{548}S_{11}$ and $C_{1889}H_{2979}N_{549}O_{551}S_{11}Co$) produced in SID fragmentation as shown in Figure 6.13. The mass errors are within 1.5 ppm.

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Figure 6.13. Zoom in region of 7+ αβ dimer and 8+ αβγ trimer in SID spectrum of the TNH hexamer, and their corresponding theoretical isotopic distributions. (A) Zoomed in region of 7+ αβ dimer in SID spectrum of the TNH hexamer and (B) the corresponding simulated isotopic distributions. (C) Zoomed in region of 8+ charge state of the αβγ trimer in SID spectrum of the TNH hexamer and (D) the corresponding simulated isotopic distributions. Reprinted from Ref 132 with permission from American Chemical Society, Copyright (2017).
6.4.5. C-reactive protein (CRP)

C-reactive protein (CRP) is a homopentamer with a ring structure. The structure is similar to that of CTB, while its molecular weight is around twice of that of CTB. In this section, CRP ions with different charge states were tested with SID in FT-ICR. The CRP ions with different charge states were generated by spraying CRP samples dissolved in different buffers (100 mM ammonium acetate (AA), 50 mM AA + 50 mM EDDA, and 80 mM AA + 20 mM TEAA) and their mass spectra are shown in Figure 6.14.

![Mass spectra of CRP pentamer with different charge state distributions acquired with a transient of 9.2 s. (A) Mass spectrum of 3 µM CRP pentamer in 100 mM ammonium acetate (AA) by averaging 42 scans. (B) Mass spectrum of 3 µM CRP pentamer in 50 mM AA and 50 mM EDDA by averaging 52 scans. (C) Mass spectrum of 3 µM CRP pentamer in 80 mM AA and 20 mM TEAA by averaging 42 scans.](image)
The mass spectra in Figure 6.14 show that different charge state distributions can be generated by spraying CRP samples dissolved in different buffers via a nESI source. Under the buffer conditions listed (100 mM AA, 50 mM AA + 50 mM EDDA, 80 mM AA + 20 mM TEAA), the predominant charge states are 23+, 20+ and 17+, respectively.

The CRP pentamer ions were then fragmented by SID. Because the predominant charge state of CRP in 80 mM AA + 20 mM TEAA is above the upper m/z selection limit (\(m/z 6000\)) of the quadrupole and the major charge state of CRP in 50 mM AA + 50 mM EDDA is close the the upper m/z selection limit of the quadrupole, it is difficult to select a single charge state from those two samples. Therefore, there was no quadrupole selection applied in the SID fragmentation of CRP pentamers and CRP pentamers with all different charge states were transmitted to the SID device and dissociated at an acceleration voltage of 35 V. The “Time of Flight” of the transfer optics between the collision cell and the ICR cell was set to 1.8 ms so that the relative intensity of the same fragment in SID spectra can be compared for those three samples.

The SID spectra are shown in Figure 6.15. As an SID acceleration voltage of 35 V, the major products that can be observed were CRP monomers. The SID fragmentation pattern is different from previous observation on the Q-TOF platform where CRP monomers, dimers, trimers and tetramers can be generated from charge reduced (18+) CRP pentamer.\(^{114}\)
Figure 6.15. SID spectra of CRP pentamers with different charge state distributions at an acceleration voltage of 35 V with a transient of 4.6 s. (A) SID spectrum of 3 µM CRP pentamer in 100 mM ammonium acetate (AA) by averaging 59 scans. (B) SID spectrum of 3 µM CRP pentamer in 50 mM AA and 50 mM EDDA by averaging 71 scans. (C) SID spectrum of 3 µM CRP pentamer in 80 mM AA and 20 mM TEAA by averaging 52 scans.

There are two possible explanations for the difference in the SID of CRP: 1) The trapping efficiency for high mass species might be low in the trap region of the SID device as discussed in Chapter 6.4.1. However, CTB tetramers and TNH trimers, which are with similar molecular weight, have been shown to be able to be trapped and detected as shown in Chapter 6.4.3 and Chapter 6.4.4. Therefore, the low trapping efficiency for high mass species is not a likely explanation. 2) The difference of the time the activated ions experienced before detection on FT-ICR and Q-TOF may lead to different SID fragmentation pattern. In Q-TOF platform, with ion mobility mode on, the ions activated
by the surface around 10 µs before thermalized in the He cell between the SID device and the ion mobility cell, while in FT-ICR, the long accumulation time (0.5 s) as well as the collision gas (Ar) may lead to secondary fragmentation of SID products before being thermalized.

The charge state distributions of CRP monomers in Figure 6.15 show that charge state distributions of CRP monomer products shift in the same direction as their precursors. By calculating the average charge state of the CRP pentamers (using the relative abundance of each peak) to roughly show the difference of the charge state distributions for each buffer system, the average charge states of the samples in 100 mM AA, 50 mM AA/50 mM EDDA and 80 mM AA/20 mM TEAA are 23.1, 20.4, and 17.2, respectively. Therefore, assuming all the charges are symmetrically distributed on each of the CRP pentamer, the theoretical charge state on each of the monomers are 4.62, 4.08 and 3.44. When calculating the average charge state of the SID products in Figure 6.15, the average charge states for the CRP monomers from the samples in 100 mM AA, 50 mM AA/50 mM EDDA and 80 mM AA/20 mM TEAA are 4.88, 4.17 and 3.95. While the average charge state of CRP monomers from the SID results matches with the theoretical monomer charge state for the samples in 100 mM AA and 50 mM AA/50 mM EDDA (4.62 is close to 4.88 and 4.08 is close to 4.17), the experimental and predicted average charge states of the CRP sample in 80 mM AA/20 mM TEAA do not, the average charge state of monomers generated by SID is much higher than that calculated from the average charge state of the precursors in this sample. It might be because the trapping efficiency of the 3+ monomer is relatively lower than that of 5+ and 4+ monomers. Therefore, outside of the buffer condition of 80 mM AA/20 mM TEAA,
the charge state of the CRP monomer generated from CRP pentamers matches with the expected charge state of monomers in the CRP pentamer, indicating direct fragmentation of CRP pentamers during the SID process without unfolding.

Figure 6.16. Zoom in of 4+ CRP monomer generated by SID at an acceleration voltage of 35 V and its corresponding theoretical isotopic distributions. (A) Zoom in of 4+ monomer generated by SID of 3 µM CRP pentamer in 100 mM ammonium acetate at an acceleration of 35 V, which was shown in Figure 6.15A. (B) Theoretical isotopic distribution of 4+ CRP monomer.

With a 4.6 s transient, CRP monomers generated by SID fragmentation can be isotopically resolved. The experimental isotopic distribution of CRP monomers was compared with the theoretical isotopic distributions. The sequence of CRP was obtained from the PDB file (PDB ID: 1GNH). Considering the disulfide bond as well as the (pyroglutamic acid) pyrrolidone carboxylic acid in the N-terminal,269 the formula of the
CRP monomer is $\text{C}_{1058}\text{H}_{1594}\text{N}_{260}\text{O}_{309}\text{S}_4$. The experimental and theoretical isotopic distributions of 4+ CRP monomers are shown in Figure 6.16 as an example. The experimental isotopic distribution of 4+ CRP monomer matches well with the corresponding theoretical isotopic distribution. The mass errors are less than 1 ppm. In Figure 6.16A, a low-intensity isotopic distribution can be observed between the peaks in the main monomer isotopic distribution, indicating the existence of low intensity 8+ CRP dimers in SID fragmentation of CRP pentamers in 100 mM ammonium acetate.

In summary, by comparing the SID behavior of CRP pentamers with different charge state distributions, monomers have been shown to be major SID products with average charge states that scale with the original charges on pentamer precursors, indicating that pentamer precursors remain compact structure during SID activation. The activation of fragment ions in the collision cell before being thermalized might be the reason that leads to this different result from previous observation on Q-TOF platform. The observation.\textsuperscript{114}

6.5. Effect of collision gas on the behavior of the SID device

All SID spectra shown previously were acquired with Ar gas collision gas in the trap region of the SID device (or modified collision cell). Nitrogen was used as an alternative to mitigate secondary activation. The effect of collision gas on the transmission of protein complexes was tested.
Figure 6.17. Mass spectra of streptavidin and CRP with Ar and N$_2$ filled in the modified collision cell. The 5 µM streptavidin tetramer in 100 mM EDDA was sprayed (A) Ar and (B) N$_2$ filled in the modified collision cell by averaging 40 scans and 31 scans with a 4.6 s transient. 3 µM CRP pentamer in 50 mM ammonium acetate + 50 mM EDDA was sprayed (C) Ar and (D) N$_2$ filled in the modified collision cell by averaging 36 scans and 31 scans with a 9.2 s transient.
Streptavidin and CRP were used to test the transmission of protein complex ions in the SID device with argon and nitrogen filled in the modified collision cell. The mass spectra are shown in Figure 6.17. The tuning parameters matched those of when the spectra of the same samples were acquired with different collision gas (argon and nitrogen) used. The “Collision Gas Flow Rate” in “Ion Transfer” panel were set to 80% for the acquisition of all the spectra shown in Figure 6.17. The mass spectra of streptavidin with using either argon or nitrogen filled in the modified collision cell are similar, with the major peaks correspond to the streptavidin tetramer \((m/z 4,000 – 5,000\) in Figure 6.17A and B). Minor streptavidin octamer can be observed with nitrogen used as the collision gas. In the mass spectra of CRP, the difference is more significant. While the CRP pentamers are the predominant ions \((m/z 5,000 – 6,500\) in Figure 6.17C and D), the peaks corresponding to CRP decamers \((m/z 7,500 – 10,000\) in Figure 6.17C and D), are more significant in the mass spectrum acquired with nitrogen filled in the modified collision cell than what is observed with argon used as the collision gas. Therefore, nitrogen used as collision gas provides better trapping efficiency in the transmission of high molecular weight species. This is exemplified by the presence of streptavidin octamer and CRP decamer.

The streptavidin and streptavidin-biotin systems were used to study the effect of collision gas on SID fragmentation behavior. The effect of nitrogen and argon as collision gases in the trap region of the SID device was examined with streptavidin and streptavidin-biotin as well. The mass spectra of the streptavidin tetramer and the streptavidin-biotin tetramer in 100 mM EDDA with Ar collision gas have been shown in Figure 6.6B and
Figure 6.7A, respectively. The peak corresponding to the 12+ streptavidin tetramer and the 12+ streptavidin-biotin tetramer, which are the predominant peaks, were isolated by quadrupole with an isolation window of $m/z$ 100. The 12+ streptavidin tetramer and 12+ streptavidin-biotin tetramer ions were fragmented via surface collision, and the fragments were trapped in the collision cell with Ar or nitrogen filled. The SID spectra of streptavidin tetramer at an SID acceleration voltage of 35 V are shown in Figure 6.18. All of the tuning parameters were set to be the same when either Ar or nitrogen was filled in the modified collision cell. The “Time of Flight” of the transfer optics between the SID device and the ICR cell was set to 1.3 ms. The “Collision Gas Flow Rate” was set to 80% when argon or nitrogen was connected to the modified collision cell.

Figure 6.18. The SID spectra of the 12+ streptavidin tetramer at an SID acceleration voltage of 35 V with Ar and $N_2$ filled in the modified collision cell. The SID spectrum of 5 µM streptavidin tetramer in 100 mM EDDA with (A) Ar and (B) $N_2$ filled in the modified collision cell. Both of the spectra were acquired with a transient of 4.6 s by averaging 200 scans.
As shown in Figure 6.18, the SID fragmentation patterns shown in the two spectra are similar to the same products collected in the modified collision cell and detected in the ICR cell. The relative intensities of ions in high m/z region (11+ tetramer and 5+ dimer) are relatively higher with N\textsubscript{2} filled in the modified collision cell than that obtained with Ar filled. The effect of collision gas on SID fragmentation behavior was further studied with the protein-ligand system. The 12+ streptavidin-biotin tetramer ions were isolated by quadrupole and fragmented with the same tuning parameters applied, and SID spectra are shown in Figure 6.19.

![Image](image_url)

**Figure 6.19.** The SID spectra of the 12+ streptavidin-biotin tetramer at an SID acceleration voltage of 35 V with Ar and N\textsubscript{2} filled in the modified collision cell. The SID spectrum of 5 \muM streptavidin-biotin tetramer in 100 mM EDDA with (A) Ar and (B) N\textsubscript{2} filled in the modified collision cell. The spectra were acquired with a transient of 4.6 s by averaging 200 scans and 100 scans, respectively.
As shown in Figure 6.19, streptavidin dimers with around half of the precursor charge state are the major product at the SID acceleration voltage of 35 V. Same SID products were trapped and detected when Ar or N$_2$ was filled in the modified collision cell. Similar to the observation from Figure 6.18, in Figure 6.19, the relative intensities of species with higher m/z (11+ tetramer, 6+ and 5+ dimers) are higher when nitrogen was filled in the modified collision cell than those obtained with argon filled.

![Figure 6.20. Zoom in spectra of the 6+ streptavidin dimer generated by SID of the 12+ streptavidin-biotin tetramer at an SID acceleration voltage of 35 V with Ar and N$_2$ filled in the modified collision cell. Zoom in spectra of Figure 6.19 with (A) Ar and (B) N$_2$ filled in the modified collision cell. “b” represents biotin and “Met” represents the N-terminal methionine in the streptavidin monomer.](image)

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The retention of biotin on SID products was also studied by zooming in the spectra in Figure 6.19. The zoomed in spectra of 6+ dimers are shown in Figure 6.20 as an example. Similar species can be observed with Ar or N₂ filled in the modified collision cell similar to what is observed in Figure 6.19. When comparing the relative intensity of streptavidin dimer with two biotins ((D+2b)⁶⁺ peak in Figure 6.20), the relative intensity is 25.6% with argon filled in the modified collision cell, and the relative intensity is 26.0% with nitrogen filled. Therefore, with argon or nitrogen filled in the modified collision cell, the ratios of the amount of 6+ streptavidin dimers with two biotins retaining to the amount of all the 6+ streptavidin dimers are similar. The nitrogen and argon have similar effects on the products generated by SID dissociation.

In summary, with the transmission test with streptavidin and CRP systems (Figure 6.17), and the SID fragmentation test with streptavidin and streptavidin-biotin systems (Figure 6.18 and Figure 6.19), nitrogen has been shown to be able to provide better signal of species with high m/z in the systems studied when same parameters were applied to the acquisition of the spectra of the same systems. The SID fragmentation patterns of streptavidin and streptavidin-biotin were similar were not affected by switching between nitrogen and argon collision gas.

6.6. Conclusions

An SID device has been designed and successfully implemented to a hybrid 15 T Bruker SolariX XR FT-ICR instrument (external to the ICR cell) in place of the original quadrupole collision cell. The SID device has been shown to be capable of transmitting ions, including small molecules, peptides, proteins and protein complexes. With the
transmission test using protein complexes, loss of signal can be observed when large protein complexes, especially with molecular weight higher than 300 kDa was studied.

Utilizing the ultrahigh resolution inherent to the ICR, SID fragments of protein complexes studied can be isotopically resolved, making it possible for accurate mass assignment. The mass errors are within 2.2 ppm. The SID fragmentation patterns of several protein complex systems (the homotetramer streptavidin, the homopentamer CTB and the heterohexamer TNH) were similar to the previous results from Q-TOF platform, matching with the prediction from their known structures. However, SID of CRP within the ICR platform largely yields monomer products, while previous Q-TOF experiments produced a range of CRP oligomers. This may be due to secondary activation of ions in the modified collision cell. The charge state of monomers generated by SID matches with predictions based off of precursor charge states. Therefore, the SID of protein complexes in FT-ICR instrument can provide SID fragments with ultrahigh resolution and mass accuracy, which reflect native-like structures of the precursor ions.

The SID of the streptavidin-biotin system has shown biotin retention with streptavidin dimers, indicating the preservation of native-like structure during SID fragmentation. However, the low intensity of streptavidin-biotin dimer may due to the activation after the surface collision. Fewer nonspecific adducts binding in the SID spectrum than those observed in the precursor mass spectrum indicates the loss of nonspecific adducts during the activation in the SID device.

The SID fragmentation patterns of streptavidin and streptavidin-biotin were similar were not affected by switching between nitrogen and argon collision gas. However, the
high $m/z$ trapping efficiency is greater while using nitrogen rather than argon as the collision gas.
Chapter 7. Conclusions and future directions

This dissertation focuses on characterizing the structure of protein complexes by native mass spectrometry coupled to a surface induced dissociation (SID) device, helping to solve structural biology questions. The application of SID, a tandem mass spectrometry activation method, to study the structure of protein complexes is promising because of its ability to overcome some limitations of conventional collision induced dissociation (CID).\textsuperscript{114,134,203} To better understand the correlations between SID (collision energy and fragmentation pattern) and protein complex structure, several protein complex systems with known structure were studied with SID (Chapter 2).

In the work described in Chapter 2, streptavidin and neutravidin, globular homotetramers with similar molecular weights and structures, were studied with ion mobility and SID in a Q-TOF platform. The tetramers studied have monomer structures that show no intertwining/domain swapping. SID provides protein structural information in several aspects: 1) The major SID dissociation pathway was shown to be tetramer $\rightarrow$ dimer $\rightarrow$ monomer for both streptavidin and neutravidin, which matches with the prediction from their interface analysis that suggests that the interface with the smallest interface area (dimer-dimer interface) will be cleaved first. Therefore, the SID products of those homotetramers studied match well with the prediction from known structures. 2)
Combined with the study of transthyretin, the SID collision energy required for cleaving the interface with the smallest interface area (dimer-dimer interface) of those three homotetramers was found to be correlated with the corresponding interface area. 3) The different SID fragmentation patterns of precursors with different conformations, induced by collisional activation in the ion source, show that SID is capable of monitoring conformational change of globular protein complexes in the gas phase.

However, the effects of subunit interactions at the interface (e.g., salt bridges, hydrogen bonds and hydrophobic interactions) on the SID collision energy remain unclear. Also, the flexibility of protein subunit in protein complexes on the SID collision energy is not well known. Therefore, more various protein complex systems with known structures, such as protein oligomers with different assemblies, DNA-protein complexes, RNA-protein complexes and membrane proteins, need to be studied by SID in the future to better understand the effects of the subunit interactions and the flexibility of protein subunits on the SID collision energy, in order to better unitize SID as a tool to characterize the structure of protein complexes.

With the correlations between SID and protein complexes with known structures studied, the further application of SID to uncharacterized protein systems to solve biological problems is introduced in Chapter 3. Three systems, including a protein oligomer (lambda exonuclease), a DNA-protein complex (nucleosome) and an enzyme-substrate complex (methyltransferase substrates), were studied with SID in Q-TOF platforms. The data of Chapter 3.1 confirms the stoichiometries of oligomers in the mixture of lambda exonuclease wild-type and K131A mutant by MS and SID, demonstrating that
subunit exchange happened during the incubation of the wild type and the mutant. Chapter 3.2 presents the SID of two nucleosomes with different stabilities, wild type and the H2A.Z variant.\textsuperscript{213,214} Two nucleosomes with different stabilities, wild type and the H2A.Z variant nucleosomes, were studied by SID. The collision energy required for SID fragmentation of wild-type nucleosome is slightly higher than that of the H2A.Z variant, which matches with the fact that the H2A.Z has a less stable structure.\textsuperscript{213,214} However, the correlations between the stability and the SID collision energy still need to be further confirmed by studying the SID fragmentation patterns of precursor ions with a specific charge state selected. The energy required for the dissociation of different nucleosome samples can be calculated by multiplying the charge state of the precursor ions and the SID acceleration voltage used to dissociate the precursor ions. In Chapter 3.3, the interactions between a methyltransferase and its substrates (K\textsubscript{d} \leq 1 \mu M in aqueous solution) can be preserved in native mass spectrometry. The substrate can be identified by both CID and SID for both \textit{in vitro} and \textit{ex vivo} samples, providing a native MS method to use in broad screening for enzyme-substrate pairs.

Chapter 4 and Chapter 5 describe experiments in which SID was applied to better understand the influence of experimental conditions on structures of globular protein complexes in the gas phase. The conformational changes of protein complexes generated from gas phase & solution additive charge reduction (Chapter 4) and gas phase trapping (Chapter 5) were monitored by SID in a Q-TOF platform.

The conformational changes of globular protein complex ions generated from solution additive charge reduction (addition of charge reduction reagent in sample solution)
and gas phase charge reductions (gas phase ion-ion reactions) were monitored by both ion mobility and SID (Chapter 4). Globular protein complexes with monomer structures that remain folded upon SID, streptavidin\textsuperscript{136} and C-reactive protein,\textsuperscript{189} were selected because they have been well studied by SID previously. Both drift time distributions and SID fragmentation patterns of protein complexes were used to study the conformational change of protein complexes. For both streptavidin tetramer and C-reactive protein pentamer, no significant difference was observed between gas phase charge reduced ions generated from compact precursors and ions generated from solution additive charge reduction, indicating similar conformation of the precursor ions and the charge reduced ions. When the conformations of the precursor ions were disrupted by supercharging or cone activation, differences in both drift time distributions and SID fragmentation patterns can be observed between gas phase charge reduced ions generated from precursors with and without conformational disruption, indicating that the unfolded structure of the supercharged or cone activated protein complex ions can be preserved after gas phase ion-ion reactions. Therefore, the precursor conformation of the streptavidin tetramer and C-reactive protein pentamer studied are preserved in the gas phase ion-ion reaction. More protein complex systems, especially those with different conformations under different charge states, need to be studied for further confirmation of this conclusion.

The conformational changes of cytochrome c monomer, β-lactoglobulin dimer and streptavidin tetramer trapped in the gas phase were monitored by using SID in the work described in Chapter 5. The protein and protein complex ions were successfully trapped in a Q-TOF instrument. There was no significant conformational change observed during the
5 s – 120 s of trapping of streptavidin. The study of more systems (pyruvate kinase, concanavalin A and C-reactive protein) has been published.186 For the future work, ion mobility will need to be utilized to better understand the conformational changes of protein complexes during the gas phase trapping by monitoring the change of CCS.

To explore the application of SID on an ultrahigh resolution platform, SID was designed and installed in FT-ICR (Chapter 6). SID of different protein complexes (streptavidin tetramer, streptavidin-biotin tetramer, cholera toxin B pentamer and toyocamycin nitrile hydratase hexamer) gave similar fragmentation patterns on FT-ICR platform in comparison to those obtained previously from Q-TOF platforms. In addition to the stoichiometry and connectivity information that can be obtained from SID on Q-TOF platforms, the modifications (e.g., oxidation or attachment of cofactors) on each of the protein complex subunits can also be clearly assigned with the ultrahigh resolution of FT-ICR.270

For the future work, the conformation of SID products from FT-ICR will be further investigated by combining SID with various techniques, including gas phase hydrogen-deuterium exchange (HDX)271 and electron induced dissociation (EID)144 in the ICR cell to explore the conformations of protein complex ions generated by SID. Trapped ion mobility spectrometry (TIMS) in the source region will provide another option to study the conformation of protein complexes in the FT-ICR platform.272

TIMS has been commercialized in a Bruker micrOTOF-Q Q-TOF instrument. The TIMS design is shown in Figure 7.1.273 It can be used to measure the CCS of proteins,272 providing gas phase topology information of proteins.
TIMS can also be coupled to the FT-ICR by placing it to replace Ion Funnel 2 in the source region. In recent experiments, the author of this dissertation was able to show (through work at Bruker, Billerica, MA) that TIMS does allow noncovalent protein complexes (streptavidin and C-reactive protein) to remain intact in the device. With the incorporation of TIMS, protein complexes different conformations can be separated, isolated and then dissociated by SID.
In gas phase HDX experiments, ND$_3$ gas is pulsed into the ICR cell and reacts for a given amount of time with the ions trapped in the ICR cell; the $m/z$ of the ions are measured after the pressure in the ICR cell is pumped down. The gas line for SORI-CID was connected to ND$_3$, rather than Ar, so that ND$_3$ can be pulsed into the ICR cell through the reservoir for SORI-CID gas. The pressure of the reservoir, the pulse length for entry of the ND$_3$ gas and the pump down time can be tuned for the gas phase HDX experiment. The pulse length is limited by the reservoir pressure because the pressure in the ICR cell should be lower than 10$^{-6}$ mbar. With the current setup, if the reservoir pressure set to 0.1 mbar, the pulse length can be as long as 60 s. The intact protein complex ions, CID products, and SID products are then studied by gas phase HDX in the ICR cell. The relative mass shift observed in this gas phase HDX has been shown to match well with the drift time distribution acquired from ion mobility cell of Q-TOF instruments. Application of this gas phase HDX approach in the ICR will be applied in future for protein structural study.

Intact protein complex ions, CID products, and SID products can also be studied by EID in the ICR cell. The EID experiment can be conducted with no extra modification to the current 15 T Bruker SolariX XR FT-ICR instrument. With the ECD bias set to 30 V, 30 eV electrons activate the protein ions in EID. For the study of intact protein complexes, more covalent fragmentation and fewer charge reduction products can be generated in EID than those generated from ECD with the ECD bias set to 1.5 V. However, the low charge states of SID products limit the covalent fragmentation of SID products in EID.

In summary, SID on different platforms can not only provide connectivity, stoichiometry and ligand/adduct binding information on various protein complex systems,
but it can also monitor the conformational changes of globular protein complexes with monomer structure that shows no intertwining/domain swapping in the gas phase. The work in this dissertation illustrated that SID can be used to both answer real-life structural biology questions and to help to gain insight into the structural study of protein complexes.
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Appendix A. SID tuning parameters on Q-TOF instruments

Figure A.1. SID model in Waters Synapt G2 and G2S
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<td>IMS bias</td>
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Table A.1. SID tuning parameters for different samples on a modified Waters Synapt G2S instrument with SID incorporated between the Trap TWIG and the ion mobility cell.
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Table A.2. SID tuning parameters for nucleosome samples on a modified Waters Synapt G2 instrument with SID incorporated between the Trap TWIG and the ion mobility cell.
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Table A.3. SID tuning parameters for different samples on a modified Waters Synapt G2S instrument with SID incorporated between the ion mobility cell and the Transfer TWIG.
Appendix B. Design and dimension of each electrode of SID in FT-ICR

Figure B.1. Photo of the SID device installed in a hybrid 15 T Bruker SolariX XR FT-ICR instrument.
Figure B.2. The relative position of all the electrodes in the SID device (Figure 6.1A).
Figure B.3. Drawing of the entrance lens of the SID device (Electrode 1 and 2 in Figure 6.1A).

Figure B.4. Drawing on the front top (left) and back top (right) deflectors of the SID device (Electrode 3 and 7 in Figure 6.1A).
Figure B.5. Drawing of the front bottom deflector of the SID device (Electrode 4 in Figure 6.1A).

Figure B.6. Drawing of the middle bottom deflector of the SID device (Electrode 5 in Figure 6.1A).
Figure B.7. Drawing of the surface holder of the SID device (Electrode 6 in Figure 6.1A).

Figure B.8. Drawing of the back bottom of the SID device (Electrode 8 in Figure 6.1A).
Figure B.9. Drawing of the exit lens in the SID region of the SID device (Electrode 9 and 10 in Figure 6.1A).

Figure B.10. Drawing of the entrance lens of the trap region in the SID device (Electrode 11 in Figure 6.1A).
Figure B.11. Drawing of the rectilinear quadrupole in the trap region in the SID device (Electrode 12 in Figure 6.1A).

Figure B.12. Drawing of asymptotic electrodes in the trap region in the SID device (Electrode 13 in Figure 6.1A).
Figure B.13. Drawing of the exit lens of the trap region in the SID device (Electrode 14 in Figure 6.1A).