COLLISION CROSS SECTIONS OF PROTEIN IONS AND
NONCOVALENT COMPLEXES OF PROTEINS STUDIED BY
ELECTROSPRAY IONIZATION-MASS SPECTROMETRY

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
Department of Chemistry

We accept this thesis as confirming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
November 1999
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Date 03/03/2000

DE-6 (2/88)
Abstract

The advent of gentle ionization sources has made it possible to study the higher-order structure of proteins, and noncovalent interactions between proteins and small molecules or other proteins with mass spectrometry (MS). Electrospray ionization (ESI), which allows formation of intact gas phase protein ions, is one such technique. This work focuses on fundamental aspects of the structure and stability of gas phase proteins formed by ESI.

Collision cross sections, a measure of ion "size", provide insight into the conformations of protein ions. The first part of this thesis concerns the experimental and theoretical descriptions of collision cross sections of gas phase protein ions. The energy losses of protein ions (myoglobin and cytochrome c), produced by ESI, in collisions with Ne, Ar, and Kr were measured with a triple quadrupole mass spectrometer. The results were interpreted with a drag coefficient model, in which the thermal motion of the target gas, the scattering angle distribution, and inelastic collisions have been effectively included by introducing a drag coefficient to a previously proposed simple hard sphere model. Comparisons of cross sections obtained with different gases and comparisons to literature cross sections measured by the ion mobility method, suggest that a "diffuse" scattering model, suitable for collisions with a rough surface, gives the best description of collisions between protein ions and neutrals. The drag coefficient model can also applied to interpret mobility experiments. The drag coefficient model suggests the projection areas obtained from the ion mobility should be reduced by a factor of about 0.74. Cross section results obtained from the energy loss method and the ion mobility method agree within 3% when both are interpreted with the diffuse scattering model. This model also shows that, for a given charge state, collision cross sections with Ne, Ar, and Kr are similar but have small differences in the order Ne < Ar < Kr. No
evidence is found for substantial contributions to the cross section from ion-induced dipole interactions. This work has successfully unified two methods of cross section measurements, energy loss and ion mobility.

The second part of this work develops and evaluates a new collision model which can be used to calculate relative energies transferred to protein ions in tandem mass spectrometry. This collision model considers the collision cross sections and the energy losses of ions in the activation process in the collision cell of a triple quadrupole tandem mass spectrometer system. The model can reduce the ca. 250% change in dissociation voltages over a range of pressures from 0.50 to 1.50 millitorr to a better than 10% spread in calculated internal energy required to cause dissociation.

Noncovalent complexes of proteins binding a small molecule or another protein were studied using the approaches developed in this work. In the case of myoglobin, highly charged holomyoglobin ions were observed by ESI-MS with a novel continuous-flow mixing setup. Collision cross section measurements show that the protein has unfolded appreciably in high charge states. However, measurements of the energies needed to dissociate heme show that the heme binding energy decreases only slightly in these more highly charged ions. Thus, much of the heme pocket appears to remain in this protein as it unfolds in the gas phase. Further, noncovalent interactions of bovine liver cytochrome b$_5$ and a series of yeast iso-1-cytochrome c mutants (wild type, trimethyl-Lys72Ala, Lys73Ala, Lys79Ala and Lys87Ala) were studied by collision cross section measurements and tandem mass spectrometry. The results show that similar energies are required to dissociate gas phase complexes of these mutants with cytochrome b$_5$. This illustrates that these mutations do not cause substantial perturbation for the formation, structure and stability of cytochrome c-cytochrome b$_5$ noncovalent complexes in the gas phase.
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<thead>
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<th>Symbol or Abbreviation</th>
<th>Physical meaning or Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>$\alpha = (m_1^2 + m_2^2)/(m_1 + m_2)^2$, where $m_1$ and $m_2$ are masses of the collision partners</td>
</tr>
<tr>
<td>$\alpha'$</td>
<td>$\alpha' = 1/\alpha$</td>
</tr>
<tr>
<td>$a_u, q_u$</td>
<td>Mathieu parameters</td>
</tr>
<tr>
<td>$\beta_u$</td>
<td>Complex function of $a_u$ and $q_u$</td>
</tr>
<tr>
<td>$u$</td>
<td>General spatial coordinate</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Atomic polarizability</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Collision cross section</td>
</tr>
<tr>
<td>$A$</td>
<td>Projection area</td>
</tr>
<tr>
<td>$n$</td>
<td>Gas number density</td>
</tr>
<tr>
<td>$n_0$</td>
<td>Gas number density at STP</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Energy transfer efficiency</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Reaction time</td>
</tr>
<tr>
<td>$r_f$</td>
<td>Total flow rate</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Inner diameter of capillary or column</td>
</tr>
<tr>
<td>$r$</td>
<td>Inner radius of mixing capillary</td>
</tr>
<tr>
<td>$s$</td>
<td>Speed ratio (ratio of ion speed to thermal speed of target gas)</td>
</tr>
<tr>
<td>$v$</td>
<td>Ion speed</td>
</tr>
</tbody>
</table>
\( \theta, \phi, \gamma \)  
Three angles that define the collision geometry

\( \chi(\theta, \phi, \gamma, b) \)  
Scattering angle

\( b_{\text{min}} \)  
Minimum impact parameter

\( \Omega \)  
Collision integral

\( K \)  
Mobility

\( K_0 \)  
Reduced mobility

\( E_{\text{lab}} \)  
Laboratory energy

\( E_{\text{cm}} \)  
Center-of-mass energy

\( E_{\text{int}} \)  
Internal energy

\( \theta_{\text{cm}} \)  
Scattering angle in center-of-mass coordinate

\( N_f \)  
Number of degrees of freedom

\( N \)  
Number of collisions

\( r.s.d. \)  
Relative standard deviation

\( m.w. \)  
Molecular weight

ESI-MS  
Electrospray ionization-mass spectrometry

MS/MS  
Tandem mass spectrometry

Mb  
Myoglobin

aMb  
Apomyoglobin

hMb  
Holomyoglobin

Cyt c  
Cytochrome c

J72A-Cyt c  
Trimethyl lysine at position 72 of cyt c is replaced by alanine

or trimethyl-Lys72Ala

K73A-Cyt c or Lys73Ala  
Lysine at position 73 of cyt c is replaced by alanine

K79A-Cyt c or Lys79Ala  
Lysine at position 79 of cyt c is replaced by alanine
K87A-Cyt c or Lys87Ala

Lysine at position 87 of cyt c is replaced by alanine

Cyt \( b_5 \)

Cytochrome \( b_5 \)

Cyt \( c-\text{cyt} \) \( b_5 \) or \( c-b_5 \)

Cytochrome \( c-\text{cytochrome} \) \( b_5 \) complex

Apo-cyt \( b_5 \) or \( ab_5 \)

Apo-cytochrome \( b_5 \)

BPTI

Bovine pancreatic trypsin inhibitor

Q0

Quadrupole ion guide

Q1

First quadrupole

Q2

Second quadrupole

Q3

Third quadrupole

\( \lambda \)

Mean free path

\( D \)

Diameter

\( m_1 \)

Ion mass

\( m_2 \)

Collision gas mass

\( K_n \)

Knudsen number

\( T \)

Temperature

\( \mu \)

Reduced mass

\( k_B \)

Boltzmann constant

\( z \)

Number of charges

\( q \)

\( q = ze \) where \( z \) is the number of charges and \( e \) is the electron charge

\( a \)

Sum of radii of collision partners

\( l \)

Length of a collision cell or drift tube

\( L \)

Length of mixing capillary

\( v_d \)

Drift speed
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$t_d$</td>
<td>Drift time</td>
</tr>
<tr>
<td>$t_{det}$</td>
<td>Detection time</td>
</tr>
<tr>
<td>$GB$</td>
<td>Gas phase basicity</td>
</tr>
<tr>
<td>H/D exchange</td>
<td>Hydrogen/deuterium exchange</td>
</tr>
<tr>
<td>$C_D$</td>
<td>Drag coefficient</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Electric field</td>
</tr>
<tr>
<td>$F_D$</td>
<td>Drag force</td>
</tr>
<tr>
<td>$\sigma_L$</td>
<td>Langevin cross section</td>
</tr>
<tr>
<td>CEM</td>
<td>Channel electron multiplier</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform-ion cyclotron resonance</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>LIT</td>
<td>Linear ion trap</td>
</tr>
<tr>
<td>IT</td>
<td>Ion trap</td>
</tr>
<tr>
<td>CAD</td>
<td>Collisionally activated dissociation</td>
</tr>
<tr>
<td>CID</td>
<td>Collisionally induced dissociation</td>
</tr>
<tr>
<td>FP</td>
<td>Faraday plate</td>
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Acknowledgments

I would like to express my heartfelt thanks to my supervisor, Dr. Don Douglas, for his consistent and patient guidance, his support of this work, and his advice and comments on this thesis. From Don, I have learned a lot. I also thank Don for generously sending me to several conferences to present my results. I am indebted to Dr. Bruce Collings for providing me considerable assistance with his expertise in instrumentation. I also would like to express my gratitude to Dr. Grant Mauk and his group members, Fred Rosell and Marcia Mauk, for their generous offering me a series of commercially unavailable protein samples. My special thanks also go out to Dr. Lars Konermann. In addition, I want to extend my thanks to all other former and present members in Don's group, Jennifer, Zhaohui, Wei, BC, Fan, Ravi, Samir and Victor et al. I also appreciate my friend Letian Wang for his kind assistance.

For the financial support, research funding was provided through Don's NSERC-SCIEX Industrial Chair. I also greatly acknowledge NSERC, Faculty of Graduate Studies of UBC, Department of Chemistry of UBC for awarding me several fellowships or scholarships.

Finally, I remain grateful to my parents, brothers and sisters, and my wife Jing Fang for their continuous support and encouragement. Also my little daughter Michelle has brought me lots of fun during the last year of my study. This thesis is dedicated to them.
Chapter 1  Introduction

The advent of electrospray ionization-mass spectrometry (ESI-MS) has provided a new tool for studying higher-order structures of gas phase proteins and other biomolecules. In the past decade, ESI-MS techniques have been extensively developed. ESI has been coupled to various mass analyzers to fulfill different analytical purposes, and ESI-MS has also been combined with separation techniques such as liquid chromatography (LC) and capillary electrophoresis (CE). Developing new approaches for probing higher-order structures of gas-phase biomolecules with ESI-MS is the theme of this thesis. In this thesis, particular emphasis is placed on cross section measurements, tandem mass spectrometry and their application to protein noncovalent complexes. As the introduction of the thesis, this chapter first attempts to provide an overview of ESI and corresponding combined techniques, then address method developments and some applications which are directly relevant to this work. Section 1.1 briefly reviews the history of ESI, the features of ESI, the coupling of ESI to various mass analyzers and separation techniques. Section 1.2 reviews the approaches under development for probing higher-order structure of gas-phase biomolecules and section 1.3 summarizes major applications of ESI-MS to proteins and noncovalent protein complexes. The goals of this research and an outline of this work are described in section 1.4 and 1.5, respectively.
1.1 Electrospray ionization-mass spectrometry

1.1.1 General

The origin of mass spectroscopy and mass spectrometry can be dated back a century [1]. J. J. Thomson’s book on "Rays of Positive Electricity and Their Application to Chemical Analysis" [2], published in 1913, symbolized the inception of modern mass spectrometry. The development of mass spectrometry mainly has focussed on two areas: mass analyzers and ion sources. To date, magnetic-sector mass analyzers [3], double-focusing mass spectrometers [4], time-of-flight (TOF) mass analyzers [5], quadrupole mass analyzers [6], quadrupole ion-trap (QIT) mass analyzers [7] and Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometers [8] have all had extensive development and have been applied to various analytical purposes. In 1989 the Nobel Prize in Physics was awarded jointly to Paul for his work on radio frequency (RF) ion traps and to two other physicists Dehmelt and Ramsey for their work on atomic precision spectroscopy [9]. With the advance of vacuum technology and electronics, the resolving power and sensitivity of various mass analyzers have been drastically improved. One of the most impressive features of modern mass spectrometry is its ultra-high sensitivity. At present, a picomole to low femtomole or even attomole of sample is often enough to obtain information on molecular weight and sequence of biomolecules. In contrast to this, a micromole-level of sample is usually required for NMR, 2-D gel electrophoresis and X-ray crystallography.

Ionization techniques for mass spectrometry have also had extensive development. Previous ionization methods include electron-impact sources [10], vacuum-spark sources [11], thermal-ionization sources [12], gas-discharge sources [13], photoionization sources
[14], field-ionization sources [15] and positive-ion-bombardment sources [16]. The early development of ion sources has been reviewed by Elliott [17] and recently summarized by Judson [18]. In the past two decades, soft ionization techniques have been developed. This has led to the extensive application of mass spectrometry to biomolecules such as proteins, peptides and nucleic acids [19-22]. In 1969, Bekey first described intact molecular ions of high molecular weight compounds formed by field desorption mass spectrometry, reviewed in [22]. In the early 1980s, fast atom bombardment mass spectrometry (FAB-MS) was introduced by Barber et al. [23, 24] to measure molecular weights for polypeptides and small proteins. In 1988, Fenn and co-workers first reported ESI-MS spectra of intact multiply protonated molecular ions of proteins [25]. Almost at the same time, another soft ionization method - matrix-assisted laser desorption ionization (MALDI) was discovered by Karas and Hillenkamp [26]. In the last decade, ESI-MS has developed at a tremendous pace. Along with MALDI-MS, it has permitted new possibilities for mass spectrometric studies of various types of high-molecular-weight compounds such as proteins, nucleotides, and synthetic polymers. Most other analytical techniques cannot provide the same level of detailed information regarding molecular weight and structure from extremely small samples.

1.1.2 Development of ESI-MS

Dole and his colleagues [27, 28] first described electrospray ionization as a source of gas-phase ions in 1968. Their work suggested that ESI could be used to ionize macromolecules [27-30]. Dole et al. [31] also discussed the possibility of using mass spectrometry to study macromolecular ions. Mass analysis of electrosprayed macromolecular ions obtained by a retarding potential method produced data that were interpreted as resulting
from singly and multiply charged ions. Eventually it was concluded by Dole et al. that accurate molecular weights of polymers could not be determined by electrospray ionization [32]. Over a decade later, Fenn and co-workers successfully interfaced ESI to a quadrupole mass spectrometer [33, 34]. Almost at the same time, Aleksandrov et al. reported a separate experiment on the interfacing of ESI to a magnetic sector mass spectrometer [35, 36]. In the 1970s, Thomson and Iribarne studied the mechanism of ESI processes and proposed an "ion evaporation" model to describe the formation of gas-phase ions from ESI [37, 38]. In 1987, Henion's group [39] described an ion spray interface for combined liquid chromatography/atmospheric pressure ionization-quadrupole mass spectrometry. They used nitrogen to assist nebulization at increasing liquid flow rates. These works formed the foundation of ESI-MS and demonstrated the utility of ESI for the analysis of macromolecules and as a potential interface for the combination of liquid chromatography with mass spectrometry.

Electrospray ionization did not receive extensive attention until Fenn and co-workers reported that high-mass proteins were observed by ESI as multiply charged ions and could thus be mass analyzed using existing quadrupole mass spectrometers [40-42]. In ESI, a solution containing ions is passed through a capillary held at a high potential. Charged drops leave the capillary and ions of the solution species are formed in the gas phase. Two mechanisms have been proposed for electrospray ionization process. The first is ion evaporation, which results in analyte ion desorption from a droplet due to the high-field strength generated by charged droplets [37, 38, 43-46]. The second is based on Rayleigh fission to produce tiny charged droplets followed by solvent evaporation to result in a gas-phase ion [44, 46-48]. Improvements for the nebulization and desolvation processes have been made by using pneumatically-assisted nebulization, additional drying gas and heat [39, 49-51]. Currently the most popular ESI sources in commercial mass spectrometers include
pneumatically-assisted electrospray sources [39, 49-50] and thermally-assisted spray sources [51]. The history of ESI has been reviewed by Hamdan and Curcuruto [52]. Recently, the development of low solution flow rate ESI sources such as microspray has drastically lowered the overall sample size of ESI-MS detection to the femtomolar regime [53-55]. The most impressive advance with ESI sources in recent years is the nanospray technique, developed first by Wilm and Mann et al. [56, 57]. The key analytical features for nano-ESI ion source include an extremely small sample size, the freedom to choose a wide range of buffer compositions and pH, and a strong tolerance to salt contamination [56].

1.1.3 Features of ESI sources

Several key features of ESI sources have made them a popular ionization technique for mass spectrometry. These are summarized as follows.

The first unique feature is the "softness" of the ESI process. This permits the preservation in the gas phase of ions of intact large biomolecules including proteins and noncovalent complexes between molecules which exist in solution. This attribute makes it possible to probe the higher-order structure of biomolecules by mass spectrometry.

A second feature is the ability of the ESI source to produce multiply charged ions. The formation of highly charged ions results in a relatively low mass-to-charge (m/z) ratio for macromolecules. This allows mass analysis for large biopolymers on virtually all types of mass spectrometers.

A third distinguishing feature of ESI is that samples are introduced in solution. This results in a natural compatibility of ESI with many types of separation techniques, particularly
those most suitable for separations of mixtures of large molecules, such as liquid chromatography (LC) and capillary electrophoresis (CE).

Finally, ESI sources, especially the recently developed micro- and nano- ESI sources, have achieved extremely low detection limits: a picomole for the conventional ESI source and low femtomole, or even attomole for micro- and nano- spray sources. This makes ESI-MS a critical tool for the analysis of many real biological samples.

1.1.4 Coupling of ESI to various mass spectrometers

ESI has been coupled to various mass analyzers. ESI-MS was first demonstrated and commercialized with quadrupole mass spectrometers [41, 42, 58]. Because of the relatively low cost, modest resolution and mass-to-charge range, and ease of operation, ESI with quadrupole mass spectrometers has been the most popular combination. Because other mass analyzers have some special advantages over quadrupole mass spectrometers, ESI has also been interfaced to different mass analyzers.

Several groups [36, 59-66] have reported the combination of ESI with magnetic sector mass analyzers. The most attractive features of ESI-magnetic sector systems include higher resolution, better mass accuracy and higher collision energy for tandem mass spectrometry. The coupling of ESI to a time-of-flight (TOF) mass analyzer using an orthogonal injection design was first proposed by Dodonov et al. in 1987 [67]. The interfacing of ESI to TOF mass analyzers with the sample injection directly along the spectrometer axis leads to a tremendous decrease in sensitivity relative to an orthogonal design. Subsequently, several other ESI-TOF systems and their applications have been reported with high sensitivity and moderate
resolution (typically 1000-5000) [68-72]. The unlimited mass-to-charge range of TOF mass analyzers is important for macrobiomolecules with relatively low charge states. Chernushevich et al. [73] reported the detection of protein multimers with masses greater than 1 million Daltons, with multiply charged ions extending beyond a mass-to-charge ratio \((m/z)\) of 10,000. Recently, Lubman and co-workers [74-76] have developed an alternative approach for interfacing ESI to TOF spectrometers. An on-axis configuration of an ESI source, an ion trap, and a TOF mass analyzer was used. This hybrid system made a great improvement in the duty cycle of the TOF. More recently, a new hybrid linear ion trap (LIT) time-of-flight mass spectrometer (LIT/TOF-MS) with MS/MS capabilities has been reported [77].

Because of several unique features of ion trap mass spectrometry (IT-MS) such as high efficiency for MS/MS, the capability for multiple stages of tandem mass spectrometry i.e. \(MS^n\) \((n > 2)\), and the capability for measurements of kinetics of ion-molecule or ion-ion reactions, the interfacing of ESI with IT-MS has formed an extremely useful analytical tool [78-86]. ESI combined with a quadrupole ion trap mass spectrometer (ESI-QIT-MS) was first reported by McLuckey and co-workers [78] in 1990. Two years later, Henion's group coupled an ESI source to the first bench-top QIT spectrometer for use in LC and CE studies [79]. Recently, Schwartz and Bier et al. [80, 81] at Finnigan Corporation developed an ES-QIT mass spectrometer (LCQ\textsuperscript{TM}). McLuckey et al. [83, 84] and Schwartz et al. [85] have published several review papers on high pressure ion sources interfaced to ion trap mass spectrometers or especially on ESI-IT-MS. More recently, Bier and Schwartz [86 and references therein] have given a comprehensive review on the principles of QIT, ESI-QIT-MS instrumentation, the capabilities and operation modes of ESI-QIT, and the applications of ESI-QIT in analyses of peptides, proteins and oligonucleotides.
ESI was first coupled to an FT-ICR system by McLafferty and co-workers in 1989 [87]. They used a radiofrequency (RF) quadrupole lens for focusing in a 2.8 Tesla ESI-FT-ICR instrument to achieve routine high-resolution (~5000) mass spectra [88]. Later McLafferty's group demonstrated high-resolution (20,000-70,000), high-mass-accuracy FT-ICR mass spectra of proteins [89] and ion dissociation mass spectra [90] with a new 6.2 Tesla ESI-FT-ICR instrument described in detail in reference [91]. In 1993, a 7 Tesla ESI-FT-ICR system was constructed by Smith and co-workers [92]. They employed two RF-only quadrupoles, including a second 1-m length set of rods, as ion optics to guide the ions through the fringing field. This instrument has achieved a mass resolution of 700,000 for the +4 charge state of insulin with \textit{m/z} 1434. Recently, Smith's group has constructed an 11.5 Tesla ESI-FT-ICR instrument with very high resolution [93] and this instrument has been and is being used to study proteomics [94]. Marshall's group has also made distinguished contributions to the advances of FT-ICR-MS [95]. In recent years, instruments with magnet fields of 9.4 Tesla [96], 20 Tesla [97], and 25 Tesla [98] have been developed by Marshall and co-workers. These high magnet field instruments have achieved resolution up to \(8 \times 10^6\) [96b].

1.1.5 Combinations of separation techniques and ESI-MS

The introduction of samples in solution makes it very convenient to interface ESI with solution-based separation techniques such as liquid chromatography (LC) and capillary electrophoresis (CE). As early as 1985, LC was first coupled to an ESI-quadrupole mass spectrometer by Fenn and co-workers [34]. Henion's group [39, 48] employed pneumatically-assisted nebulization to make ESI compatible with flows from a conventional LC column (φ
4.6 mm) with a flow rate of 1-2 mL/min. Most commercial ESI sources are compatible with a 1.0 mm (ϕ) microbore column (the flow rate is 40-50 µL/min. [48]). ESI-TOF-MS has been coupled recently to LC [99, 100]. The TOF-MS is capable of recording full spectra on a millisecond scale. LC-MS has been extensively applied to the analyses of many biomedical, biological and environmental samples. Recently Voyksner and Lee [101] have reported an off-axis nebulizer which improves the sensitivity and precision for LC-ESI-ITMS. To date, a number of review papers [102-106] on LC/MS and its applications have been published.

ESI is also an ideal source to couple to capillary electrophoresis (CE). The first work on CE-MS was reported by Smith et al. in 1987 [107]. In this initial work, a metal-coated capillary tip made contact with a metal sheath capillary to which the ESI voltage was applied. In this way, the sheath capillary acted as both the CE cathode and the ESI source. CE has been interfaced to ESI on quadrupole [108, 109] and magnetic sector mass spectrometers [110]. Smith et al. developed the first successful CE-ESI-FT-ICR interface and acquired the first on-line high-resolution mass spectra of CE samples [111-114]. In the first work [111], a mixture of six proteins was separated and detected by on-line CE-ESI-FT-ICR with sufficiently high resolving power to observe the individual isotopes in charge states for proteins as large as carbonic anhydrase (m.w. 28,802 Da). In later work, an MS/MS experiment was performed in which sprayed CE solute bands were subjected to sustained off-resonance irradiation to produce dissociation spectra for euquine apomyoglobin with a resolving power of 50,000 [113]. More recent work has applied CE-ESI-FT-ICR to directly analyze cellular proteins [114]. In addition, CE has also been coupled recently to ESI-TOF-MS [115, 116].
1.2 Experimental approaches for studying biomolecules

1.2.1 Ion mobility measurements

In ion mobility measurements, ions generated by ESI or other ion sources are injected into a drift tube through a small aperture. The drift tube is usually filled with a few torr of buffer gas (typically helium) and has a uniform electric field produced by applying electric potentials to a series of electrodes. The electric field accelerates ions, while collisions with the buffer gas decelerate them, leading to a constant drift speed. The mobility, \( K \), is defined as the ratio of the drift speed \( (v_d) \) to the electric field \( (\mathcal{E}) \), i.e. \( K = \frac{v_d}{\mathcal{E}} \). The mobility contains information about the interaction between the ions and the buffer gas. For a large polyatomic ion, the mobility depends on the average cross section. An ion with a larger cross section undergoes more collisions with the buffer gas and travels more slowly than an ion with a smaller cross section. Thus ion mobility measurements can be used to separate ions with different sizes and geometries. Ion mobility spectrometry was first developed in 1970 by Cohen and Karasek [117]. About two decades later, mobility-based methods were first applied to the determination of conformations of gas phase ions by Bowers group [118]. These methods have been successfully used to study small rigid systems such as carbon clusters [119] and metal carbon clusters [120], some more flexible "floppy" molecules like polyethylene glycol [121], crown ethers [122], polypeptides [123] and oligoglycines [124]. More recently, Jarrold, Clemmer and co-workers have performed a series of mobility measurements for protein ions produced by ESI [125-131]. These proteins include cytochrome c [125-128], bovine pancreatic trypsin inhibitor (BPTI) [126, 127], apomyoglobin [128, 129], lysozyme [130], and ubiquitin [131]. In these investigations, numerous
conformers for protein ions were resolved, and unfolding and refolding of these gas phase proteins were examined using collisional heating and proton stripping reactions. Jarrold et al. [126b] have demonstrated that mobility measurements of gas phase ions are capable of distinguishing between the native state of cytochrome c in solution and the methanol-denatured form in solution. In addition, mobility measurements have also been performed for oligonucleotides [132] and oligosaccharides [133]. In the development of instruments, Jarrold and co-workers [134] recently have reported a new apparatus which used a longer (63 cm) drift tube, operated with a higher drift voltage (up to 14,000 V) and a higher helium buffer gas pressure of ~500 torr. With this new apparatus, the resolving power for ion mobility measurements is over an order of magnitude higher than was achieved using conventional ion drift tube techniques. More recently, Clemmer's group has described an interface for coupling an ion trap with ESI-ion mobility experiments [135] and also a new instrument that coupled ESI to an injected-ion drift tube/TOF-MS [136]. With the ion trap/ESI-ion mobility approach [135], the trap allowed the ion beam to be accumulated between pulses of the mobility experiments. This method has improved signal-to-noise (S/N) ratios by factors of ~10-30 and achieved experimental duty cycles of nearly 100%. With the ESI-ion mobility/TOFMS analysis, individual components in a mixture of ions were separated by mobility difference in the drift tube and subsequently dispersed by m/z ratios in a TOF instrument, resulting in a three-dimensional spectrum that contains collision cross section, m/z, and ion abundance information [136]. Several review articles have summarized the recent advances in ion mobility methods and their applications to complex ions such as atomic clusters and various large biomolecules [137, 138], as well as the unfolding/refolding and hydration of gas phase proteins [139].
1.2.2 Collision cross section measurements

A collision cross section is a measure of ion "size", which can give direct evidence for changes of higher-order structures for protein and other ions. Measurements of collision cross sections can also provide benefits for better understanding of ion transport and collisionally activated dissociation (CAD) of ions in tandem mass spectrometry. ESI allows the transfer of intact native or denatured protein ions from solution into the gas phase. This makes it possible to study the conformational transition in the process of folding/unfolding for proteins. Collision cross sections provide insight into these conformational changes. In 1990, Smith and Barinaga [140] estimated a dissociation cross section of about 1000 Å² for cytochrome c ions. Later, Douglas and French [141] studied the effect of collisional focusing in an RF quadrupole and concluded that collision cross sections must be approximately 1000 Å² or greater for such ions.

Collision cross section measurements for protein ions were initiated by Covey and Douglas in 1993 [142]. In this work, they described an energy loss method for the determination of collision cross sections for gas phase protein ions. This approach was based on the axial kinetic energy loss of an ion as it passes through a collision cell containing a neutral target gas. Collision cross sections were obtained from the experimental energy loss data interpreted using a simple hard sphere model [142]. This model is expressed as [142]

\[ \frac{E}{E_0} = \alpha^{\text{ml}} \]  

(1-1)

where \( E_0 \) is the initial kinetic energy of the ion entering the collision cell, \( E \) is the energy of the ion leaving the collision cell, \( \sigma \) is the collision cross section, \( n \) is the target gas number.
density, $l$ is the collision cell length, and $\alpha = \frac{m_1^2 + m_2^2}{(m_1 + m_2)^2}$, where $m_1$ is the ion mass and $m_2$ is the target gas mass. Based upon this method, collision cross sections for a series of proteins such as motilin, ubiquitin, cytochrome $c$, myoglobin, and bovine serum albumin were measured. A similar but separate observation of energy losses for protein ions was reported by Cooks’ group [143]. Collings and Douglas [144] have probed the conformation of gas phase holo- and apo- myoglobin ions by cross section measurements done in this way. All these studies found that cross sections generally increased with the number of charges on the ion.

An alternative approach for obtaining cross section data is ion mobility measurements [145]. Mobility measurements have been described in section 1.2.1. Ion mobility determines the drift time of an ion under a uniform electric field. The drift speed of the ion depends on the strength of the electric field of the drift tube, the pressure and nature of buffer gas, and the cross section of the ion. The drift time of an ion at a given length drift tube can be experimentally determined. According to ion mobility theory [146], the drift time ($t_d$) can be converted into a collision integral ($\Omega$) by

$$
\Omega = \frac{\sqrt{18\pi}}{16} \left[ \frac{1}{m_1} + \frac{1}{m_2} \right]^{1/2} \frac{ze}{\sqrt{k_B T n}} \frac{1}{l^2} t_d V
$$

(1-2)

where $m_1$ is the mass of the ion, $m_2$ is the mass of the buffer gas, $ze$ is the charge, $n$ is the buffer gas number density, $k_B$ is the Boltzmann constant, $T$ is the temperature, $l$ is the drift tube length, and $V$ is the voltage applied across the drift tube. For hard-sphere collisions, the collision integral $\Omega = \pi a^2$, where $a$ is the sum of the radii of the spheres, and so $\Omega$ equals the
projection area $A$. Jarrold et al. [147] have developed an exact hard-spheres scattering model for calculating the gas phase mobilities of polyatomic ions. Ion mobility measurements have been used to determine the cross sections for polypeptides by Bowers and co-workers [124] and proteins by Jarrold, Clemmer and co-workers [137]. Comparison of the collision integrals calculated using the exact hard-spheres scattering model with those estimated using the projection area from mobility shows that a large deviation, over 20%, occurs for some geometries with grossly concave surfaces [147]. Cross sections derived from ion mobilities have been extensively applied to study the conformers of protein ions, the unfolding, refolding and hydration of proteins in the gas phase [137, 139]. Due to the lack of accurate cross section data for protein ions, direct evaluation on the accuracy of measured cross sections can not be made. The mobility measurements are an old technique which was originally based on hard sphere scattering theory [146]. This method was used to describe the drift behavior of simple molecules or ions in buffer gases with great success. Proteins and other biomolecules have more complicated structures and conformations than simple molecules or ions. Using the simple hard sphere model to describe the drift behavior of these macromolecules in the buffer gas may be inaccurate.

Comparison of cross sections of gas phase protein ions measured by the energy loss method based on a simple hard sphere model [142] and by ion mobility [126a, 129] shows substantial differences. Aside from the limitations of present mobility methods, the simple hard sphere model used to interpret energy loss experiments is oversimplified. An improved method of describing both mobility measurements and energy loss experiments is required. The first major part of this thesis (Chapter 3) discusses this issue in detail. By introducing a drag coefficient based on diffuse scattering, collisions between protein ions and neutrals can be better described [148]. In this model, cross sections from both energy loss and ion mobility
measurements agree within 3% [148]. The cross section measurements based on this new model have recently been applied to probe the conformations of ions of BPTI and its mutants [149], and the structure of highly charged holo-myoglobin ions [150].

1.2.3 Ion/molecule chemistry

Ion/molecule reactions are an important approach for studying gas-phase intermolecular interactions. These have been used to derive three-dimensional structure information and intrinsic reactivity of proteins, peptides and other biomolecules in the gas phase by monitoring the relative reactivity or basicity of different charge states [151-157]. The gas-phase basicity of a compound A ($GB_A$) is defined as the negative of the standard free energy change for the protonation reaction i.e.

$$ A + H^+ \rightarrow AH^+ $$

$$ GB_A = -\Delta G_A^0 $$

Since the gas-phase basicity is related to intrinsic bond strain and molecular conformation, it is of interest to determine the gas-phase basicity of proteins and other biopolymers. When an unknown compound or group A is equilibrated with a reference base BH⁺, the standard free-energy change ($\Delta G_{net}^0$) for the net proton-transfer reaction

$$ A + BH^+ \rightarrow AH^+ + B $$

can be experimentally determined from the relative abundances of $AH^+$ and $BH^+$. Thus, the gas-phase basicity of an unknown compound A can be calculated from $\Delta G_{net}^0$ and the known basicity of the reference base B ($GB_B$)
Major techniques used in studying ion/molecule chemistry include proton transfer kinetics [153-156], H/D exchange kinetics [151, 152, 157] and more recently developed neutral/molecule or ion reaction kinetics [158-160]. Gas phase H/D exchange has produced clear evidence for distinguishing protein conformers. McLafferty's group [152, 157, 161] has found six different conformations of cytochrome c in the gas phase based on H/D exchange measurements. Two different ion structures for the charge state +12 of ubiquitin have been recognized by Cassady and Carr [156] using H/D exchange. Due to the Coulomb repulsion between charges in multiply charged ions, highly protonated protein ions have more open conformations and thus they usually have greater proton transfer rates. However, an exception was observed for equine cytochrome c by McLafferty and co-workers [161]. The measured basicity (relative to a reference base) of each individual charge state can be obtained in a gas-phase "titration" experiment [155, 162]. Williams and co-workers have studied the small peptide gramicidin S [163], cytochrome c [164], and lysozyme [165] by measuring proton transfer rates. Differences in gas-phase protein conformations can be inferred from differences in rates. For example, distinct gas-phase conformations of intact and reduced lysozyme show dramatically different proton transfer rates [163]. McLuckey et al. [164] have compared the proton transfer rates for hMb and aMb ions of the same charge state reacting with anions derived from perfluoro-1, 3-dimethylcyclohexane and found that these proton transfer rates are indistinguishable. This observation supports the expectation that ion-ion proton transfer rates are insensitive to ion structure and are determined primarily by the attractive Coulombic field [166]. Recently McLuckey and co-workers [158-160] have

\[
\Delta G_{net}^0 = -RT \ln K_{eq} = -RT \ln \left[ \frac{[B][AH^+]}{[A][BH^+]} \right] \tag{1-6}
\]

\[
GB_A = GB_B - \Delta G_{net}^0 \tag{1-7}
\]
reported the measurement of the kinetics of hydroiodic acid (HI) attachment to polypeptides [158, 159] and proteins [160] as a chemical probe of gaseous protein ion structure. The attachment of HI to a series of neutral basic residues such as arginines, histidines, lysines and the N-terminus has been observed [158]. This specific ion/molecule chemistry can be used to determine the number of basic sites in a polypeptide by adding the total charges to the maximum number of HI molecules, HI only attaches to neutral basic sites. An attached HI molecule is equivalent to a proton in reacting with a basic group, but doesn't change the charge [159]. Significant difference in the kinetics of HI attachment to native and reduced BPTI ions shows that this ion/molecule chemistry is an effective means for monitoring the difference in the three-dimensional structure resulting from the presence or absence of three disulfide linkages in the protein [160].

### 1.2.4 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is one of the most common experimental techniques for identifying unknown compounds, interpreting chemical structures and protein sequencing [167]. The book "Metastable Ions" marked the beginning of the modern era of tandem mass spectrometry [168]. Other previous key contributions to this field included the development of collision-activated dissociation [169, 170] and the introduction of the triple quadrupole mass spectrometer [171]. In 1988, Busch et al. published the book "Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry", in which the early history, the instrumentation, the fundamentals and analytical applications of MS/MS were comprehensively reviewed [172]. In terms of the mechanism for MS/MS, mass spectrometers can be divided into two types: tandem-in-space
and tandem-in-time [172]. The tandem-in-space instrument is made up of instruments in which two mass analyzers are assembled in tandem such as two mass-analyzing quadrupoles [171], two magnetic-sector analyzer instruments, and a quadrupole time-of-flight (Q-TOF) system [173]. The tandem-in-time instrument consists of mass analyzers capable of storing ions such as a quadrupole ion trap [174], ICR mass spectrometers [175], and the recently developed linear ion trap-TOF instrument [77]. At present, the triple quadrupole mass spectrometer [171] is the most widely used for all types of MS/MS experiments. Three main scan modes including the precursor ion scan, the fragment ion scan, and the neutral loss scan have been used for different applications [176]. Collisionally-activated dissociation (CAD) or collisionally-induced dissociation (CID) is the most frequently used dissociation technique in tandem mass spectrometry [177]. Hayes and Gross [178], Bruins [179], and Shukla and Futrell [180] have described the fundamental aspects of the CAD processes in detail. Recently, ESI-MS/MS has been used to identify proteins [181] and protein adducts [182], to characterize modified human hemoglobin [183] and to study of the dissociation of noncovalent protein-substrate complexes [49, 73, 184-186].

1.3 Applications of ESI-MS to the study of proteins

1.3.1 Protein conformation and folding/unfolding

The higher-order structure of a protein plays a key role in its biological function. Intact protein molecular ions can be generated by ESI [41]. This has raised an interesting question: how does the gas-phase structure of a protein correlate with its solution structure? If
conditions can be found under which a gas-phase protein ion preserves its solution conformation, then ESI-MS will become a powerful tool in solving biochemical and biomedical problems. In the past decade, a number of new experimental approaches have been developed for studying protein ion conformation in the gas phase. These newly developed techniques, as reviewed in section 1.2, mainly include ion mobility measurements [121, 137], collision cross section measurement [142], H/D exchange [151, 152], the gas-phase basicity [164, 165] and the relative rates of ion/molecule or ion/ion reactions [155, 187, 188].

The conformation of a protein in solution is determined by several factors such as temperature, pH, and solvent composition. Usually, high temperature, high acidity i.e. low pH, and high content of an organic solvent lead to the denaturation of a protein and a conformation transition from the native to an unfolded state for the protein occurs. As a result, conformation-related chemical/physical properties have a substantial change. Some of these changes have been monitored by ESI-MS. The simplest observation is a shift of charge state distribution [44, 189-196]. An unfolded protein has larger surface area [44] and more basic amino acid residues are exposed to the solvent [189-192], thus higher charge states are observed. Gas-phase H/D exchange and ion/molecule reactions have been used to study the conformation change between disulfide-intact and disulfide-reduced proteins [151, 152, 157, 197, 198]. These results showed that the H/D exchange in disulfide-intact proteins in the gas phase initially was faster than the corresponding disulfide-reduced proteins, but disulfide-reduced proteins finally exchange to a larger extent.

Cross section measurements based on ion energy loss [142] or ion mobility [137] have also been applied to study the conformation of gas-phase protein ions [126a, 130, 131, 144, 149]. Both methods have shown that cross sections for protein ions increase with increasing
charge. Larger cross sections for higher charge states show that higher charge states favor more unfolded conformations. These experiments have also demonstrated that cross sections for a given charge state increase with the interface voltage i.e. orifice-skimmer voltage difference on an ESI-triple quadrupole mass spectrometer (energy loss measurements) [144] or ion injection energy (ion mobility) [126a]. Most previous studies show that protein ions formed from denaturing conditions have larger cross sections (compared to the same charge state protein ions formed from the native state) which suggested that the solution structure of protein ions could be preserved, at least somewhat, in the gas phase.

Recently, ESI-MS has been used for monitoring solution protein folding/unfolding reactions [199-204]. Konermann and Douglas [199] used ESI-MS to detect changes in the tertiary structure for the acid-induced unfolding of cytochrome c at different methanol concentrations. They introduced "time-resolved" ESI-MS for monitoring the kinetics of protein folding or unfolding and other (bio)chemical reactions in solution [200-203]. Folding or unfolding for a number of proteins such as cytochrome c [199, 200, 202, 204], myoglobin [201, 202]; lysozyme [204], ubiquitin [202, 204] and BPTI [202] etc has been studied by ESI-MS. In a more recent report, Konermann and Douglas [204] have compared the unfolding of proteins monitored by ESI-MS in both positive and negative ion modes. The results show that the acid-induced unfolding for cytochrome c as well as the acid- and the base-induced unfolding for ubiquitin only lead to marginal changes in the negative ion charge state distributions, whereas in the positive ion mode all cases studied show significant shifts to higher charge states.
A more recent application of ESI-MS is to the study of noncovalent interactions between biomolecules. The gentleness of the ESI technique allows intact protein noncovalent complexes to be detected by mass spectrometry. The pioneering work in this field includes the observation of a receptor-ligand complex and enzyme-substrates with ESI-MS by Henion et al. [205] and the observation of the heme-globin complex of native myoglobin by Katta and Chait [191] in 1991. Since then, a number of other types of noncovalent complexes have been observed by ESI-MS. These noncovalent complexes can be classified into several types: protein-metal ions [206-215], protein-small molecules [184, 191, 193, 194, 216-230], protein quaternary structure complexes [220, 221, 231-252], protein-cofactor [253], antibody-antigen [248, 254], inclusion complexes [255-257], and protein-nucleic acid complexes [239, 258-261]. A number of reviews on this subject have appeared [186, 228, 262-267]. Several typical examples are discussed here.

Protein-metal ion complexes are one group of the most common protein noncovalent complexes. Metal ions play an essential role to the catalytic function and structural stability of many metalloenzymes. A well-studied case is calmodulin-calcium binding [208-211, 214]. Loo et al. [208, 209] used ESI-MS to determine the stoichiometry of the calmodulin-calcium binding complex. Their studies found that bovine α-lactalbumin, rabbit parvalbumin, and bovine calmodulin bind specifically to one, two, and four calcium ions, respectively. Lafitte et al. [210] reported the metal ion binding sites in calmodulin and the effect of Mg and Tb on the calcium binding to calmodulin. Loo's group [209] used a calcium ion titration study to observe the changes of the relative abundance of the coexisting species containing various numbers of calcium ions. The results showed that with an increase of calcium concentration,
the abundance of calmodulin decreased and calmodulin-4-calcium increased, whereas other
calcium-binding species remained at relatively low levels. A more recent study with ESI-MS
by Veenstra et al. [211] has demonstrated that, per mole rat brain calbindin D28K binds 4
moles of calcium, i.e. two of the EF-hands (the EF-hand was defined as a helix-loop-helix
structural motif [266]) do not participate in binding calcium.

The myoglobin-heme complex is a model case of a protein-small molecule
noncovalent complex. This system has been extensively investigated by ESI-MS [191, 193,
194, 219, 222, 223, 226, 229]. Organic solvents and acidic solution conditions are known to
denature myoglobin, removing the heme from the pocket. Usually, ESI-mass spectra observed
from neutral aqueous solutions show only ions for the myoglobin-heme protein. The mass
spectrum of myoglobin taken from solutions of acidic pH contains a group of peaks for the
apo-protein and a singly charged heme peak. Loo et al. reported that the detection limit for
observing the holo-myoglobin noncovalent complex was 100 fmol by ESI-double focusing
mass spectrometer [223]. Horse heart (holo-) myoglobin in 10-mM ammonium acetate
solution (pH 6.7) shows ions for the 8+ and 9+ charge states of myoglobin-heme complex.
Collisionally activated dissociation (CAD) of the complex may occur when the ESI interface
voltage (orifice-skimmer voltage difference in the ESI-quadrupole mass spectrometer system)
increases, resulting in apo-myoglobin ions with 7+ to 9+ charge states and a singly charged
ion at \( m/z \) 616 for heme [223]. Similar CAD experiments for dissociating the myoglobin-
heme complex in the sampling interface have also been performed by Feng and Konishi [194,
219], Jaquinod et al. [225], and Hunter et al. [229]. Hunter et al. [229] have described
evidence that the hydrogen bonds known to bind the heme-protein complex in solution persist
in the gas-phase ion and contribute to the stability of the complex in the gas-phase. This study
concluded that the gas-phase ion retained much of the solution structure around the heme over
at least the millisecond time scale. More recently, Gross et al. have measured the activation energy of the dissociation reaction for pseudo-native holomyoglobin ions in the gas-phase by the blackbody infrared radiative dissociation (BIRD) technique [222]. In all of these experiments, holomyoglobin ions up to a maximum +14 were observed by ESI-MS. These ions retain a native or pseudo-native conformation in solution.

Protein multimers are typical protein-protein noncovalent complexes. ESI-MS provides direct, unambiguous molecular weight evidence to show the formation of protein-protein complexes. A very common example is hemoglobin [220, 221, 231, 232]. Hemoglobin is a tetrameric complex composed of two α-chains and two β-chains, each chain bound to one heme group. At physiological pH, the tetramer is in equilibrium with the heterodimer species [268]. From an aqueous solution of human hemoglobin, the αβ complexes were observed with mass spectrometry by Ganem and Henion [217]. Loo et al. [220] reported that at m/z greater than 2400, multiply charged ions for the 32.2 kDa αβ heterodimer complex were observed for human hemoglobin in 10 mM ammonium acetate (pH 6.7). No multiply charged α-dimer and β-dimer complexes were observed, with the conclusion that the observed complexes were those formed in solution and not due to random aggregation. Furthermore, ESI-MS experiments were run for turkey hemoglobin under identical solution and instrumental conditions. The results showed that turkey hemoglobin has higher relative abundance of the αβ heterodimer (relative to human hemoglobin), suggesting that the equilibrium between monomer and heterodimer in turkey hemoglobin is shifted towards the multimeric form. Smith's group [231] studied the noncovalent tetrameric active forms of avidin, concanavalin A (Con A), and adult human hemoglobin by ESI-MS. They observed that the ESI interface conditions strongly influenced the retention of these weak
noncovalent solution associations into the gas phase, as well as the average extent of charging for the subunits upon dissociation. In this case, the known solution pH dependence of the dimer-tetramer equilibrium of Con A was observed by ESI-MS, and the intact heterodimeric and tetrameric forms of adult human hemoglobin, \((\alpha\beta)\) and \((\alpha\beta)_2\), with the prosthetic heme groups have also been observed by ESI-MS. Meanwhile, ions corresponding to a trimer, not known to be formed under physiological conditions, were observed for each of the proteins as well. Differences in stability of the gas phase ions of these tetrameric proteins observed with ESI-MS were found to be qualitatively consistent with the known solution stabilities of these ions. They also compared the relative stability of several protein tetramers. The results showed that the hemoglobin tetramer is less stable in the gas phase than either the Con A or avidin tetramer, which is consistent with solution dissociation constants [231]. Aplin et al. [221] also reported the observation of the tetramers \((\alpha_2\beta_2)\) for hemoglobin at pH 8. Wang et al. [232] used ESI-MS with high mass range scans (up to 20,000 \(m/z\)) to study several tetramer proteins such as Concanavalin A (Con A), soybean agglutinin (SBA), and human hemoglobin. The ESI-mass spectra contain peaks representing tetrameric species and higher order complexes such as dimers, trimers of the tetrameric proteins. A number of other multimeric proteins including the streptavidin tetramer [244-247], the catfish roe lectin trimer [249], the 4-oxalocrotonate tautomerase hexamer [250], the citrate synthase-NADH hexamer [251], and the hemerythrin octamer [252] etc. have been observed by ESI-MS as well. Normally, multimeric forms of proteins have relatively low charge states, thus a higher mass-to-charge ratio range scan is required.

Another important group includes protein-nucleic acid complexes. This group is of particular interest because the expression of the genetic information found in nucleic acids is dependent upon the specificity of their interactions with proteins [265]. Proteins serve as the
regulators of the genetic information provided by nucleic acids. Recently, ESI-MS has extended its application to the observation of intact protein-DNA/RNA noncovalent complexes [239, 258-261]. Greig et al. [258] observed albumin-DNA complexes and measured the dissociation constants for the complexes by ESI-MS. Cheng et al. [239] have studied a single-stranded DNA binding protein, gene V (9.7 kDa), with ESI-MS. This protein exists as a dimer under physiological conditions and the protein dimer binds to DNA with a stoichiometry of 1:1. The observation of the protein dimer-DNA complex in the gas phase was found to be consistent with the solution phase results obtained from NMR and gel-shift experiments [239]. In addition, ESI-MS has been used to study protein-RNA complexes [259, 260]. Harms et al. [259] have reported mass spectra of the regA protein-RNA complexes. Sannes and Loo et al. [260] used ESI-MS to detect the complex formation between Tat protein (9.8 kDa) and TAR RNA (31-mer, 9.2 kDa). The Tat protein-TAR RNA complex highlights the importance of RNA structure in protein recognition of RNA. Tat protein contains an arginine-rich region which is essential for RNA binding. Compared to the protein, a 40-residue polypeptide containing this basic region has very similar binding characteristics to TAR RNA. Under competitive binding conditions, ESI-mass spectra show that the Tat peptide affinity for TAR RNA is greatly reduced for the 28-mer RNA, which is consistent with solution phase measurements [260b].

Whether the ESI-MS spectrum observed accurately reflects solution equilibrium is a question which must be addressed when studying any noncovalent biomolecular interaction using this technique. To date it has not been conclusively demonstrated that a gas phase complex is identical in its activity, binding affinity, and conformation to the same complex in solution. In a recent review article [267], Veenstra has discussed this issue. Most of the examples mentioned above have shown a positive correlation between a complex's stability in
the gas phase and solution phase. However in some cases, non-specific aggregates have been observed [269, 270]. The observations of unexpected oligomers are believed to be a result of both high solution concentrations and artifacts of the ESI processes [267]. Thus careful control experiments to prove that the biomolecular complex observed in the gas phase is specific and indicative of that observed in solution are often necessary when using ESI-MS.

1.4 Goals of this research

ESI-MS is a powerful tool for the study of gas-phase protein structures because it allows the formation of intact protein ions in the gas phase. The study of protein higher-order structure through measuring collision cross sections is a new subject [142]. Collision cross sections can provide insight into the structures of gas phase protein ions and also reflect the conformation transition when a gas-phase protein ion unfolds. Collision cross sections are required to model the transport of protein ions through low-density gases. As described in section 1.2.2, two methods i.e. ion energy loss measurements [142] and ion mobility measurements [137] have been used to determine collision cross sections for protein ions. For a given protein ion, cross sections obtained from the energy loss method interpreted with the simple hard sphere model and from the ion mobility method show significant differences which can not be simply explained by experimental errors. In previous energy loss measurements, collision cross sections were derived from the energy loss data interpreted with a simple hard sphere model. This simple model neglects the thermal motion of the target gas and assumes an average center-of-mass scattering angle of 90°. To describe the more realistic behavior of protein ions in a low-density gas, this model is oversimplified. In
addition, in previous energy loss measurements, only argon was used as the collision gas. The effect of the ion-induced dipole potential on cross section was not evaluated in detail. The dynamics of protein-ion neutral collisions are mostly unknown. How does the nature of the collision gas affect the collision cross sections of protein ions? How is the collision cross section related to the nature of the protein itself?

Collision gases with different masses have different thermal speeds. Different collision gases produce different average forces to prevent the transport of protein ions. This force is defined as the drag force \( F_D \) [271] given by

\[
F_D = m_1 \frac{dv_d}{dt} = -C_D \frac{Anm_1v_d^2}{2}
\]

where \( m_1 \) is the mass of the particle (a protein ion in this case), \( m_2 \) is the mass of the bath gas atom, \( n \) is the gas number density, \( A \) is the projection area i.e. cross section, \( v_d \) is the speed of the ion, and \( C_D \) is a drag coefficient. The drag coefficient \( (C_D) \) is determined by many factors. These factors include the gas density, viscosity, particle speed, the particle size and geometric shape, and the ratio of the particle speed to the thermal speed of the gas, i.e. the speed ratio. For the same ion but with different speed ratios, the \( C_D \) values may change by a factor of 2 or more. The energy loss experiments and ion mobility measurements described in this thesis are an example of this. Thus experimental tests are needed to determine the \( C_D \) value required to correct the simple model. More details are discussed in chapter 3. Introducing a drag coefficient to modify the simple hard sphere model should be expected to give a better interpretation of the energy loss experiments with different collision gases. This will contribute to improving our understanding of the dynamics of collisions between protein ions and target gas atoms.
One purpose of this work is to develop and evaluate the simple hard sphere model and the drag coefficient model which are used to interpret energy loss experiments. This is done by measuring collision cross sections of protein ions with the different target gases Ne, Ar, and Kr. The motion of a protein ion in different buffer gases has different drag forces. It is shown that the drag coefficient model can effectively describe protein ion-neutral collisions. Comparison of cross sections obtained from energy loss experiments with the drag coefficient model and from the literature data derived from ion mobility experiments provides some evidence supporting this new model. In addition, the effect of ion-induced dipole interactions is also evaluated. According to the Langevin theory [272], the collision cross section, $\sigma_L$, is given by

$$\sigma_L = \frac{2\pi e^2}{v_{rel}} \sqrt{\frac{\beta}{\mu}}$$

(1-9)

where $z$ is the number of charges on the ion, $e$ is the electron charge, $v_{rel}$ is the relative velocity of the collision partners, $\beta$ is the polarizability of the collision gas, and $\mu$ the reduced mass of the collision partners, i.e. $\mu = m_1 m_2 / (m_1 + m_2)$. The differences in the polarizabilities and masses of the gases lead to Langevin collision cross sections in the ratio 0.70 : 1.00 : 0.85 for Ne, Ar, and Kr, respectively, for a given charge state. However, comparison of collision cross sections obtained with different collision gases reveals that ion-induced dipole interactions make no contribution to the cross section. Detailed discussions are in Chapter 3.

In tandem mass spectrometry, fragmentation or dissociation of a selected protein ion results from the deposit of translational energy into ion internal energy in a series of collisions. In conventional MS/MS experiments, the relative abundances of fragment or precursor ions are measured by varying the initial kinetic energy of precursor ions in collision
with a given target gas at a fixed pressure. Previous MS/MS experiments were typically used to fragment small organic molecules. In these studies a few collisions or even a single collision in some cases, is enough to reach the threshold energy and lead to the fragmentation of molecules. Protein ions contain thousands of atoms and have a very large number of degrees of freedom and large cross sections. The CAD of protein ions requires many relatively low-energy collisions. Based on a more thorough understanding of the protein-target gas atom collision behavior, a second purpose of this work is to evaluate a collision model which can be used to calculate the relative energy transferred into internal energy of ions in MS/MS experiments under conditions where ions have many collisions. The internal energy calculated from this collision model can be used to compare the relative stability or binding energies of different protein noncovalent complexes. This model is evaluated by comparing the relative energies required for the dissociation of a given ion at different cell pressures and with different gases.

A third goal of this work is to apply the above newly developed approaches, i.e. cross section measurements and calculations of relative energies required for dissociation, to study noncovalent protein complexes. One interesting case investigates the strength of heme-binding in highly charged holomyoglobin ions. The other case involves cytochrome c-cytochrome b5 noncovalent complexes. Cross section measurements provide information on the structure or structural changes of protein ions. The internal energy required for the dissociation of noncovalent complexes gives evidence for the relative binding energies of different noncovalent complexes and different charge state ions.
1.5 Outline of the thesis

This work focuses on fundamental aspects of the structure and stability of gas phase proteins and noncovalent protein complexes studied with ESI-MS and/or ESI-tandem mass spectrometry.

Chapter 2 describes the experimental apparatus used in this study, the working principles of quadrupole mass analyzers and sample preparation.

Chapter 3 is on the experimental and theoretical descriptions of collision cross sections of gas phase protein ions. The energy losses of protein ions (apomyoglobin and cytochrome c) in collision with Ne, Ar, and Kr have been measured with a triple quadrupole mass spectrometer. The thermal motion of the target gas and inelastic collisions have been effectively modeled by introducing a drag coefficient model to correct the simple hard sphere model. Comparisons of cross sections obtained with different gases and comparisons to literature cross sections measured by ion mobility method suggest that a "diffuse" scattering model gives the best description of collisions between protein ions and neutrals. In addition, the contribution of different polarizabilities to cross sections of protein ions is also discussed.

Chapter 4 evaluates a new collision model for the description of the transfer of translational into the internal energy of a protein ion in multiple collisions. This collision model can be used to calculate the relative energies transferred to protein ions in tandem mass spectrometry. This model is evaluated by comparing the energy required to dissociate a given protein noncovalent complex ion at different cell pressures. In addition, the influence of inelastic collisions on apparent cross sections of protein ions also is discussed.

Chapter 5 describes the observation of highly charged hMb ions (up to +21) formed by using a two-syringe continuous-flow mixing apparatus. The conformation and stability of
these highly charged hMb ions are discussed by measuring cross sections with the method
developed in Chapter 3, and calculating the relative energies needed for removing heme, with
the new collision model proposed in Chapter 4. The results show that the protein has unfolded
appreciably in high charge states but the heme binding energy is decreased only slightly in the
more highly charged ions. Thus much of the heme-binding pocket appears to remain in this
protein as it unfolds in the gas phase.

Chapter 6 applies cross section measurements and tandem mass spectrometry to study
the noncovalent interactions of bovine liver cytochrome $b_5$ and a series of yeast iso-1-
cytochrome $c$ mutants (wild type, trimethyl-Lys72Ala, Lys73Ala, Lys79Ala and Lys87Ala).
The pathways of the fragmentation of cytochrome $c$-cytochrome $b_5$ complexes are described.
The conformation and relative stability of different charge states and different mutant
complexes are also discussed.

Chapter 7 summarizes this work and points out several possible directions for future
work.

1.6 References

2. Thomson, J. J. Rays of Positive Electricity and Their Application to Chemical Analysis,
24, 1122.


94. Recent results presented by R. D. Smith group at The 47th ASMS Conference and Allied Topics, 1999, Dallas. (a) Bruce et al. (b) Pasa-Tolic et al. (c) Smith et al.
186. Loo, J. A. Bioconjugate Chem. 1995, 6, 644.


Chapter 2  Instrumentation and Sample Preparation

This chapter describes the experimental apparatus and sample preparation used in these studies. These include a description of the ESI-triple quadrupole mass spectrometer and the delineation of the function and operation principles of major sections of this system including the ESI source, ion sampling interface, quadrupoles and detector. A homemade continuous-flow mixing setup used in the observation of highly charged holomyoglobin ions is also described briefly. In addition, the preparation of protein solutions and other relevant reagents is also described.

2.1 Electrospray ionization-triple quadrupole mass spectrometer

The electrospray ionization-triple quadrupole mass spectrometer system used in this work is an early prototype of the PE-Sciex API-300. This system is shown schematically in Figure 2-1. This apparatus includes an ESI source, ion sampling interface, RF-only quadrupole (Q0), first mass-analyzing quadrupole (Q1), RF-only quadrupole/collision cell (Q2), second mass-analyzing quadrupole (Q3) and the detector. The diameter of the sampling orifice is 0.25 mm. The diameter of the skimmer orifice is 0.75 mm. The distance from the sampling orifice to the tip of skimmer is 2 mm. The diameters of the entrance and exit apertures of the collision cell are 2.5 mm. The geometric length of the collision cell is 20.6
All quadrupole rods are 1.59 cm in diameter. The mass-to-charge ratio scan range of this quadrupole system is 10-4000 m/z. The vacuum of this apparatus was maintained by a three-stage differential pumping system. A mechanical pump was used for the interface region and turbo molecular pumps were used for the main chambers. The pressures in different regions are listed in Table 2-1. The function and operation principles of major sections are briefly described below.

Figure 2-1. Schematic of the ESI-triple quadrupole mass spectrometer system. S, electrospray ionization source; N2, nitrogen “curtain” gas; C, curtain plate; O, sampling orifice; SK, skimmer; Q0, RF-only quadrupole; PF, prefilter; Q1, mass-analyzing quadrupole; Q2, RF-only quadrupole/collision cell; Q3, mass-analyzing quadrupole; D, detector.
Table 2-1. The pressures in different regions of the triple quadrupole mass spectrometer

<table>
<thead>
<tr>
<th>Region</th>
<th>Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curtain plate-orifice</td>
<td>760 Torr</td>
</tr>
<tr>
<td>Orifice-skimmer region</td>
<td>2 Torr</td>
</tr>
<tr>
<td>Q0</td>
<td>$7 \times 10^{-3}$ Torr</td>
</tr>
<tr>
<td>Q2</td>
<td>variable</td>
</tr>
<tr>
<td>Main chamber (Q1, Q3 and detector region)</td>
<td>$\sim 2 \times 10^{-5}$ Torr</td>
</tr>
</tbody>
</table>

2.1.1 Electrospray ionization

![Figure 2-2. Pneumatically-assisted electrospray ionization.](image)
The electrospray ionization source is key to the formation of intact large biomolecular ions in the gas phase. A pneumatically-assisted electrospray ionization source, shown in Figure 2-2, was used throughout this research. A solution is pumped through a fused silica capillary (inner diameter, i. d., 75 μm and outer diameter, o. d., 150 μm), into a stainless steel capillary (i. d. 250 μm and o. d. 380 μm) by a syringe pump (model 22, Harvard Apparatus). A second stainless steel capillary (i. d. 500 μm and o. d. 760 μm) is concentric with other two capillaries. Air flow (1-1.5 liter per minute) passes between the stainless steel capillaries to assist nebulization. The assembly is floated at a high potential, typically 4-5.5 kV.

2.1.2 Ion sampling interface

Multiply charged droplets are produced and then desolvated in the gas between the curtain plate and the orifice in Figure 2-1. This gas, typically high purity nitrogen, is defined as the "curtain gas" or "drying gas". The curtain gas prevents the solvent from entering the vacuum chamber and provides a dry region for declustering solvent molecules from the ions. Small charged droplets further shrink and then split into even smaller droplets. This process is constantly repeated and finally results in the production of multiply charged single molecular ions. Ions from the ESI source arrive at the ion sampling interface region which is depicted in Figure 2-3. In this region, different voltages are applied to the curtain plate, orifice, and skimmer (Typical voltages are curtain plate 500-1000 V, orifice 140-220 V, and skimmer 110 V). This yields an axial electric field. For the positive operation mode, the voltages applied to these elements decrease along the axial direction. Positive ions and any tiny charged droplet residues pass through the sampling orifice along the direction of the electric field. Further desolvation can occur in the orifice-skimmer region. For the negative operation mode, an
opposite polarity electric field is needed. The skimmer extracts the desolvated ions from the interface. The background pressure in the orifice-skimmer region is ca. 2 Torr. The voltage difference between the orifice and the skimmer (OS) is a critical factor for the observation of intact protein ions. A voltage difference which is too low may cause incomplete desolvation of charged droplets, resulting in a poor signal and poor signal-to-noise ratio. However, if the OS voltage difference is too high, ions obtain high kinetic energy in the OS region where a series of collisions between ions and the background gas occur. This may lead to enough energy deposition to cause the breakdown of relatively weak interactions such as noncovalent complexes, or even covalent bonds, resulting in the fragmentation of molecular ions. Thus the OS voltage difference usually needs to be optimized experimentally. In most cases, mild interface conditions are required for the production of intact species in the gas phase. Sometimes, one can use the orifice-skimmer region to carry out fragmentation or dissociation experiments but the precursor ions can not be mass selected.

![Figure 2-3. Ion sampling interface.](image)
2.1.3 Operation principles of quadrupole mass filters

An ideal quadrupole mass filter is constructed of four electrically conducting parallel hyperbolic cylindrical surfaces [1]. Many mass filters employ circular rods instead of hyperbolic rods due to the difficulty of manufacturing hyperbolic rods [2, 3]. The typical design is depicted in Figure 2-4(a). The best approximation to a hyperbolic field using cylindrical rods is to space them so that \( r' = 1.148r_0 \) [4], where \( r' \) is the rod radius and \( r_0 \) is the radius of the inscribed circle tangent to the four rods [3]. Each pair of opposite rods is electrically connected. One pair of rods has an applied potential \( \Phi_0 = U - V \cos \omega t \), and the other pair has an applied potential \( -\Phi_0 = (U - V \cos \omega t) \), where \( U \) is a DC voltage and \( V \) is the zero to peak amplitude of an RF voltage at the frequency \( \omega = 2\pi f \), \( f \) being the frequency in Hz.

As shown in Figure 2-4, a quadrupole mass filter is a two-dimensional quadrupole field with a radius of the field, \( r_0 \). There is no field in the \( z \) direction, thus

\[
\Phi = \frac{\Phi_0}{r_0} \left( p_i x^2 + p_j y^2 \right) \quad (2-1)
\]

and under Laplace's condition, i.e.

\[
\nabla^2 \Phi = \frac{\partial^2 \Phi}{\partial x^2} + \frac{\partial^2 \Phi}{\partial y^2} = 0 \quad (2-2)
\]

\[
\frac{\partial \Phi}{\partial x} = \frac{\partial}{\partial x} \left( \frac{\Phi_0}{r_0^2} p_i x^2 \right) = 2p_i x \frac{\Phi_0}{r_0^2} \quad (2-3)
\]

and

\[
\frac{\partial^2 \Phi}{\partial x^2} = 2p_i \frac{\Phi_0}{r_0^2} \quad (2-4)
\]
Similarly

$$\frac{\partial^2 \Phi}{\partial y^2} = 2P_j \frac{\Phi_0}{r_0^2}$$  \hspace{1cm} (2-5)

Thus

$$\nabla^2 \Phi = \frac{\Phi_0}{r_0^2} (2P_i + 2P_j) = 0$$  \hspace{1cm} (2-6)

from which $P_i + P_j = 0$ and if we choose $P_i = -P_j = 1$, then the field expression is given by

$$\Phi(x, y) = \frac{\Phi_0}{r_0^2} (x^2 - y^2)$$  \hspace{1cm} (2-7)

Equipotential contours of a quadrupole field are shown in Figure 2-4(b). The center of the quadrupole experiences a potential of zero and the potential at any point $(x, y)$ within the quadrupole is given by equation 2-7. In this 2-dimensional quadrupole field, the forces on a particle with charge $e$, in the $x$ and $y$ directions, are given by

$$F_x = m \frac{d^2 x}{dt^2} = -e \frac{\partial \Phi}{\partial x}$$  \hspace{1cm} (2-8)

$$F_y = m \frac{d^2 y}{dt^2} = -e \frac{\partial \Phi}{\partial y}$$  \hspace{1cm} (2-9)

Substituting equation 2-3 for $\frac{\partial \Phi}{\partial x}$ in equation 2-8 and letting $P_i = 1$ and $\Phi_0 = U-V\cos \omega t$ leads to the equation of motion of an ion in the $x$ direction of a quadrupole field. That is

$$\frac{d^2 x}{dt^2} + \frac{2e}{m r_0^2} (U - V \cos \omega t)x = 0$$  \hspace{1cm} (2-10)

Similarly, the equation of motion in the $y$ direction is given by

$$\frac{d^2 y}{dt^2} - \frac{2e}{m r_0^2} (U - V \cos \omega t)y = 0$$  \hspace{1cm} (2-11)
(a) Conventional circular quadrupole rods in a quadrupole mass filter. (*Adapted from ref. [3]*)

(b) Equipotential curves in an ideal 2-dimensional quadrupole field. (*Adapted from ref. [3]*)

Figure 2-4. Quadrupole rods and equipotential curves.
Let \( a_x = -a_y = \frac{8eU}{m\omega^2 r_0^2} \) \hspace{1cm} (2-12)

\( q_x = -q_y = \frac{4eV}{m\omega^2 r_0^2} \) \hspace{1cm} (2-13)

and let \( \xi = \frac{\alpha_x}{2} \), so the equations of motion can be expressed as

\[
\frac{d^2u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0
\]

(2-14)

where \( u \) represents one of the coordinates \( x \) or \( y \). This is the Mathieu equation.

The "stability region" is an important concept in the description of ion motion in a quadrupole field based on the Mathieu equation. "Stable motion" means that the trajectory of an ion is confined to the quadrupole field. The ion will remain within the device and will be transmitted. "Unstable motion" means that the trajectory of an ion exceeds this field radius and the ion will strike a rod. In general for the Mathieu equation, the stability of ion motion depends on the constant \( \eta \), where \( \eta = \alpha_u + i\beta_u \), where \( \beta_u \) is a complex function of the Mathieu parameters, \( a_u \) and \( q_u \) [3]. Thus the regions with stable solutions of the Mathieu equation can be plotted using \( a_u \) and \( q_u \) as coordinate axes. These are depicted in Figure 2-5. Overlapping regions labeled by I, II, and III in the figure represent regions where the motion of ions is stable in both the \( x \) and \( y \) directions simultaneously and ions can be transmitted by the quadrupole mass filter. Of the most practical interest is the area nearest the origin, i.e. the stability region I in Figure 2-5. Almost all commercially available quadrupole instruments are operated in this stability region.
Figure 2-5. The 2-dimensional (x and y) stability diagram of the quadrupole. The overlapping regions for which ion motion is simultaneously stable in the x and y directions are labeled I, II, and III. (Adapted from ref. [3])

The upper half of the stability region A of Figure 2-5 is shown in detail in Figure 2-6. The operating line represents a fixed \( \frac{a}{q} \) ratio which corresponds to a series of combinations of DC voltage (\( U \)) and RF voltage (\( V \)), and mass \( m \). The slope of this operating line is determined by

\[
\frac{a}{q} = \frac{8eU}{4eV} = \frac{2U}{V}
\]

(2-15)
A constant $U/V$ ratio will maintain a constant $a/q$ ratio, thus generating an operating line. Along this operating line, as the $U$ and $V$ values are changed, the masses which lie in the stability region are also changed. Changing the ratio of $U$ to $V$ will change the slope of the operating line. Increasing the slope of the operating line will increase the resolution but decrease the sensitivity. As shown in Figure 2-6, $M_1$, $M_2$ and $M_3$ are three ions with different masses on the same operating line with the order of mass $M_3 > M_2 > M_1$. In this particular case, only the motion of $M_2$ is stable in both directions, so $M_2$ will pass through the mass filter. The motion of $M_1$ and $M_3$ are unstable so they can not be transmitted. Thus $M_2$ will be separated and detected. If a higher mass such as $M_3$ is to be transmitted, then higher values of $U$ and $V$ are required. Similarly, if one wants to transmit a lower mass such as $M_1$, then lower values of $U$ and $V$ should be applied. Although the $a/q$ ratio is maintained constant for a given operating line, by scanning the $U$ and $V$ values along this line, ions with different masses will be transmitted in sequence of mass-to-charge ratio and will reach the detector in order, producing a mass spectrum. By decreasing the slope of the operating line by varying the $U/V$ ratio, the resolution can be decreased. In this case, the transmission will increase although $M_2$ may not be separated from $M_3$ or $M_1$.

**2.1.4 Quadrupole operation**

The quadrupole $Q_0$ is an RF-only quadrupole where only an RF voltage is applied between the rods. The sample ions extracted by the skimmer enter into the $Q_0$ region through a free jet expansion. The voltages applied to the $Q_0$ rods and the prefilter, $PF$, (the short rods between $Q_0$ and $Q_1$) focus the sample ion beam into the first mass-analyzing quadrupole, $Q_1$. 


Figure 2-6. Mass analysis of the ions in the quadrupole field. \( M_1 \), \( M_2 \), and \( M_3 \) represent ions with three different masses.

By scanning the voltages on Q1, ions with different mass-to-charge (\( m/z \)) ratios will be transmitted and transported to the detector, resulting in a mass spectrum. Q1 can also act as an ion guide with no filtering action by removing the DC voltage between the quadrupole rods and operating it with RF-only mode. When an MS/MS experiment is run, Q1 filters out all ions except those of a particular \( m/z \) ratio, and the selected ions are fragmented through collisions with target gas in the Q2 collision cell. By scanning Q3 the \( m/z \) ratios of the precursor ions and fragment ions can be obtained. The second quadrupole, Q2, an RF-only quadrupole, is always maintained in a total ion transport mode. If no collision gas is present, Q2 transports all the sample ions into Q3. If collision gas is present, fragmentation may occur in Q2, and both precursors and fragments are transported to Q3. The second mass-analyzing
quadrupole, Q3, identical to Q1, can also be operated in ion guide mode to transport all the ions from Q2 to the detector. In a conventional measurement of a mass spectrum from the source, one can select either "Q1 scan" or "Q3 scan" where in each selection the other quadrupole acts as an "ion guide". However in tandem mass spectrometry, one selects precursors in Q1 and scans m/z ratios in Q3. In this triple quadrupole system, the kinetic energy of an ion at the entrance of the collision cell is determined by the rod-offset voltages applied to quadrupoles Q0 and Q2.

### 2.1.5 Ion detection

A channel electron multiplier (CEM) is used as the ion detector. The function of the detector is to convert ions to electric current pulses. As schematically shown in Figure 2-7, the ion detector is located off-axis at the exit from Q3. The CEM is a continuous dynode device which can detect either positive or negative ions. Ions of the selected m/z ratio pass through the exit lens (EL) and are deflected by the Faraday plate (FP) into the collector of the CEM. Each ion starts an electron cascade, resulting in a current pulse (~10^-6 A) which is amplified. Noise is rejected by a discriminator and the discrimination level is set to only allow the relatively strong ion signals to be transmitted. The ion signal is recorded in ion counting mode. The recorded data are the total number of ions at different m/z ratios. The computer calculates the total ion counts per second (counts/s) for each scan m/z ratio and these data are used to construct a mass spectrum over a certain m/z range. The purpose of the off-axis arrangement for the CEM is to prevent photons and neutrals, which can cause noise, from reaching the CEM.
Figure 2-7. Schematic of the channel electron multiplier (CEM) detector. EL, exit lens; FP, Faraday plate; CEM, channel electron multiplier; R, resistor; C, capacitor; PA, preamplifier.

2.2 The continuous-flow mixing apparatus

Figure 2-8. Schematic of the continuous-flow mixing apparatus for ESI-MS. A and B are two syringes. Solutions from A and B mix at "tee" T and then flow to the ESI source.
Instead of a conventional single syringe pumping system, a continuous-flow mixing apparatus was used for the formation of partially acid-denatured holomyoglobin ions. The experiments in Chapter 4 and 5 were performed with this apparatus. This setup is shown in Figure 2-8. Two syringes, A and B with the same total volume (1 ml each) are separately connected to a fused silica capillary with an inner diameter (i.d.) of 75 μm by a connector (P742, Upchurch Scientific, Oak Harbor, WA). These two capillaries are connected to a third "reaction" capillary of the same i.d. by a homemade tee. The two syringes are advanced simultaneously by a syringe pump at the same speed. The reaction of two solutions from syringes A and B is initiated by mixing them at the "T". The reaction time (τ) of two solutions, i.e. the time from mixing to spraying, can be calculated from

\[
\tau = \frac{V_t}{r_f} = \frac{\pi r^2 L}{r_f}
\]  

(2-16)

where \(V_t\) is the total volume of the "reaction" capillary (μL), \(r_f\) is the total flow rate of the solution (μL/min.), \(r\) is the inner radius of the "reaction" capillary, and \(L\) is the length of the "reaction" capillary. If \(r\) is fixed, then the reaction time can be adjusted by changing either the length of the mixing capillary or the flow rate of the solution. For example, if a pumping speed of 5.0 μL/min. is applied, then the total flow rate in the reaction capillary is 10.0 μL/min. For an 8.0-cm length of the capillary, the reaction time is calculated to be 2.1 s.
2.3 Sample preparation

2.3.1 Protein solutions

The proteins used in this work are horse heart myoglobin (m.w. 17,570), horse heart cytochrome c (m.w. 12,360), tryptic bovine cytochrome b5, yeast iso-1-cytochrome c Cys102Thr and its trimethyl-Lys72Ala (J72A), Lys73Ala (K73A), Lys79Ala (K79A) and Lys87Ala (K87A) mutants. Horse heart myoglobin and horse heart cytochrome c were purchased from Sigma (St. Louis, MO). Tryptic bovine cytochrome b5, yeast iso-1-cytochrome c Cys102Thr and its trimethyl-Lys72Ala (J72A), Lys73Ala (K73A), Lys79Ala (K79A) and Lys87Ala (K87A) mutants were provided by Professor A. G. Mauk’s group. These were prepared and purified by the procedures described in references [5-7]. Molecular weights, measured by ESI-MS, for bovine tryptic cytochrome b5, wild type yeast iso-1-cytochrome c and its mutants, and expected cytochrome c-cytochrome b5 complexes are listed in Table 2-2. The uncertainties are standard deviations obtained from three separate measurements. The observed masses were in good agreement with the calculated values from the sequences. The calculated masses are 10,079 for cyt b5, 12,709 for wild type cyt c, 12,607 for trimethyl-Lys72Ala mutant, and 12,652 for the other three mutants.

In Chapter 3, protein solutions were myoglobin 5 μM in 1/1 deionized water/acetonitrile and 0.1% acetic acid, and cytochrome c 5 μM in 1/1 deionized water/acetonitrile and 0.5% formic acid. In Chapters 4 and 5, a 40 μM holomyoglobin (hMb) aqueous solution and a 0.45% acetic acid were loaded separately in two identical syringes of the continuous-flow mixing apparatus. In Chapter 6, all sample solutions contained 10 μM
individual protein or protein mixtures were obtained by mixing equal volumes of 20 μM single protein solutions. Methanol (10%) was added to these solutions to improve the spray efficiency.

**Table 2-2. Molecular weights for cytochrome b₅, cytochrome c (wild-type and mutants) and the cytochrome c-cytochrome b₅ complexes**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein m. w.</th>
<th>Complex m. w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b₅</td>
<td>10,080 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>Wild-type yeast iso-1- cyt c</td>
<td>12,710 ± 1</td>
<td>22,790 ± 2</td>
</tr>
<tr>
<td>Trimethyl-Lys72Ala cyt c</td>
<td>12,605 ± 1</td>
<td>22,685 ± 2</td>
</tr>
<tr>
<td>Lys73Ala cyt c</td>
<td>12,648 ± 1</td>
<td>22,728 ± 2</td>
</tr>
<tr>
<td>Lys79Ala cyt c</td>
<td>12,648 ± 1</td>
<td>22,728 ± 2</td>
</tr>
<tr>
<td>Lys87Ala cyt c</td>
<td>12,648 ± 1</td>
<td>22,728 ± 2</td>
</tr>
</tbody>
</table>

**2.3.2 Other reagents**

Other reagents used included methanol, acetic acid and formic acid, all HPLC grade and from Fisher Scientific (Nepean, Ontario). Collision gases used in this work and manufacturer's stated purities were Ne, UHP grade (99.996%), Ar, Linde grade (99.9995%), and Kr, Research grade (99.995%). The curtain gas was N₂, UHP grade (99.999%). All gases were from Praxair, Richmond, B. C.
2.4 References

Chapter 3  Collision Cross Sections of Protein Ions

This chapter discusses the experimental and theoretical descriptions of collision cross sections of gas phase protein ions. The energy losses of protein ions (myoglobin and cytochrome c) in collision with Ne, Ar, and Kr were measured with a triple quadrupole mass spectrometer. The thermal motion of the target gas and inelastic collisions have been effectively modeled by introducing a drag coefficient model to correct the simple hard sphere model used previously. Comparisons of cross sections obtained with different collision gases and comparisons to literature cross sections measured by ion mobility method suggest that a "diffuse" scattering model gives the best description of collisions between protein ions and neutrals. The contribution of different polarizabilities to the collision cross sections of protein ions is also discussed. This work has been published (J. Am. Soc. Mass Spectrom. [1]).

3.1 Introduction

Collision cross sections of protein ions with neutral gases are of interest for at least two reasons: (i) they are required for modeling the transport of ions through low density gases [2-5] and (ii) they provide insights into the structures of gas phase protein ions. Two methods have been used to determine these cross sections, ion mobility [6-11] and energy loss experiments [12-15]. In mobility experiments, ions are injected into a cell where they drift
under an electric field, the drift speed is measured and related through a collision integral to the size of the ion or collision cross section [16-18]. In energy loss experiments, ions are injected into a radio frequency quadrupole and the loss of kinetic energy from collisions with a neutral gas is measured and related to the cross section through kinetic theory.

The dynamics of protein ion neutral collisions are largely unknown. The contribution of the ion-induced dipole potential to the cross section determined in energy loss experiments has not yet been assessed in detail. Initial experiments showed that collision cross sections were 2 to 5 times greater than expected from this polarization potential [12]. However, some contribution from the ion dipole attraction may remain. In addition it has also been suggested that the dipole moment of the ion may contribute significantly to the interaction potential and hence the cross section [11]. For mobility experiments with He buffer gas, it has been calculated that the contribution of the ion-induced dipole term to the cross section is very small due to the low polarizability of He and the large size of protein ions [7].

To date, the energy loss experiments have been interpreted with a simple hard sphere model that neglects the thermal motion of the collision gas and assumes an average center-of-mass scattering angle of 90° [12]. While simplistic, this model has the advantage of giving simple analytical expressions for the energy loss. Comparison of cross sections measured by the energy loss method to those obtained by mobility or to those of proposed ion structures requires a more realistic model of the collision process. An improved model based on drag coefficients has been proposed but not yet evaluated in detail [15].

Although the real cross sections for gas phase protein ions are unknown, previously reported collision cross section data [7, 12] show that significant differences exist between the energy loss measurements interpreted with the simple hard sphere model and the ion mobility measurements. For example, the cross section data of cytochrome c ions obtained from the
energy loss measurements are typically 20-50% (variation with charge state) bigger than those obtained from mobility experiments. This difference is much greater than the experimental errors between two methods. This indicates that some improvements and modifications to the above models are required. Some assumptions are over-simplified and obviously deviate from the real protein ion neutral collision case. Both methods treat experimental data based on simple hard sphere collisions and the simple model for the energy loss does not take into account thermal motion of the collision gas. An improved model would benefit our understanding of ion-neutral collisions and reduce the difference of collision cross sections measured with the energy loss method and the ion mobility method.

Here we have measured the energy losses of myoglobin and cytochrome c ions in collisions with Ne, Ar, and Kr. These gases differ significantly in their polarizabilities, and so allow an assessment of the contribution of ion-induced dipole polarization forces to the collision cross section. They also differ significantly in their thermal speeds and so allow an assessment of the effect of target gas motion on the energy loss experiments. The energy loss data are interpreted with drag coefficients from the literature (reviewed in section 3.3.1). The results show the limitations of the simple model previously used to interpret the energy loss experiments. The drag coefficients give cross sections equal to the average projection area. These cross sections for different collision gases are found to be nearly identical. No evidence is found for substantial contributions of ion-induced dipole or other polarization forces to the cross section. Comparison of the cross sections among different gases and comparison to literature cross sections from mobility experiments suggest that a "diffuse" scattering model, suitable for collisions with a rough surface, is most suitable to describe collisions between protein ions and neutrals.
3.2 Methods for collision cross section measurements

3.2.1 Energy loss

Covey and Douglas [11] first described an energy loss method for the determination of collision cross sections for gas phase protein ions with a triple quadrupole mass spectrometer system. The experimental setup is schematically shown in Figure 2-1 (Chapter 2). Ions with a given initial kinetic energy (initial energy is defined as the number of charges times the potential difference between Q0 and Q2) are injected into the collision cell Q2. In this work, a 10z eV injection energy, where z is the number of charges, was used. In Q2 the ions lose some kinetic energy through collisions with the target gas. The kinetic energy losses of ions depend on the collision cross sections of the ions, the mass of the ions, the nature of the collisions between ions and neutral target, the mass and thermal speed of the target gas etc. The energy of the ions leaving the cell can be determined by scanning the Q3 rod offset potential and Q3 was operated as a mass analyzer. The energy distributions of ions were determined by increasing the Q3 voltage in a step of 0.50 V until the signal drops by 2-3 orders of magnitude. Stopping curves were obtained at several different cell pressures over a range of 0-1.0 millitorr. The collision cross sections are derived by interpreting the energy loss data with different collision models. These models were developed under different assumptions. The simple hard sphere model proposed by Covey and Douglas [12] and the drag coefficient model proposed by Douglas [15] are briefly described in sections 3.3.2. The experimental details of the energy loss method, together with an example of the data analysis, are described in section 3.4.
3.2.2 Ion mobility

The ion mobility measurement is a classic approach which has been widely used to derive collision cross sections for small ions [16]. Recently, the mobility method has been used to measure cross sections for large biomolecules such as proteins and polypeptides [6-11]. The apparatus for conventional mobility experiments is shown in Figure 3-1. Ions are injected in a pulse through an ion gate into a drift tube where a uniform electric field is maintained by applying a potential gradient along a series of guard rings. The drift tube is filled with a buffer gas (usually helium, argon, or nitrogen) at the pressure of a few torr.

![Schematic of a drift tube for mobility experiments](image)

**Figure 3-1. Schematic of a drift tube for mobility experiments**

Ion mobility is defined as the ratio of an ion drift velocity in a bath gas to the applied electric field, that is

\[ K = \frac{V_d}{E} \]  

(3-1)
where \( K \) is the mobility, \( v_d \) is the drift speed of the ion, and \( \varepsilon \) is the electric field. Under a given electric field, the ion drift speed is constant. When ions travel through a drift tube with a length of \( l \), then

\[
K = \frac{l}{V} = \frac{l^2}{Vt_d}
\]

(3-2)

where \( V \) is the applied voltage across the drift tube and \( t_d \) is the ion drift time.

The measured mobility, \( K \), is usually converted to a "standard" or "reduced" mobility, \( K_0 \), by

\[
K_0 = K \cdot \frac{n}{n_0}
\]

(3-3)

where \( n \) is the gas number density, and \( n_0 = 2.687 \times 10^{19} \text{ cm}^{-3} \) is the gas number density at standard temperature and pressure i.e. 273.15 K and 760 torr. Combining equations 3-2 and 3-3, then the ion drift time, \( t_d \) is given by

\[
t_d = \frac{l^2 n}{K_0 n_0 V}
\]

(3-4)

The measured arrival time \( (t_a) \) is equal to the sum of the drift time through the drift cell and the detection time, \( t_{det} \). The detection time is defined as the time required to move from the exit of the drift cell to the detector.

\[
t_a = t_d + t_{det} = \frac{l^2 n}{K_0 n_0 V} + t_{det}
\]

(3-5)

Hence, a plot of arrival time vs. \( 1/V \), at a given pressure, is linear, with a slope from which the reduced mobility can be determined. The ion mobility directly relates to the ion-neutral collision cross section integral. Thus the cross sections of ions can be calculated. Hard sphere theory has been widely used to convert mobility to cross sections [16]. In this work the
concept of drag coefficient is also introduced to refine the simple model and the improved model is shown to be more suitable for the interpretation of mobility experiments with protein ions [1]. These models are described in section 3.3.3.

3.3 Theory

3.3.1 Drag coefficients

The drag coefficient, $C_D$, relates the force on an object moving through a gas with speed $v$ to the cross sectional area or projection area, $A$, through equation 1-8. In general $C_D$ varies with (i) the gas density, viscosity, and object’s speed, through the Reynolds number, (ii) the ratio of the mean free path of the collision gas $\lambda$ to the object size (the Knudsen number, $K_n = \lambda/D$ where $D$ is the diameter of the object), and (iii) the ratio of the object speed, $v$ to the thermal speed of the gas. The latter is commonly expressed by the speed ratio $s$, given by

$$ s = \frac{v}{\sqrt{2k_BT/m_2}} \quad (3-6) $$

where $k_B$ is Boltzmann's constant and $T$ the gas temperature. For all the experiments discussed here the Reynolds number is zero and the Knudsen number very high.

Scattering from the surface of an object has been modeled as “specular” where the component of momentum parallel to the surface is conserved and the component perpendicular is reversed, and “diffuse” where all memory of the incident momentum is lost and gas leaves the surface in a cosine distribution about the normal to the surface [20-24]. For
a sphere, specular scattering is equivalent to the familiar hard sphere scattering. Diffuse scattering applies where gas is accommodated on the surface before being re-emitted. It also applies to the case where the surface is rough and multiple collisions with concave regions can lead to a pseudorandom distribution of scattering angles. For small aerosol particles diffuse scattering has generally given the best fit to experimental data [22-25].

The drag coefficient can be calculated by considering the momentum transfer from incident gas striking the surface and from scattered gas leaving the surface, integrated over all scattering angles and the appropriate thermal distribution of speeds. Epstein [22] first calculated the force on a sphere in a low density gas for low speed ratios i.e. \( s \ll 1 \). Writing Epstein's results in terms of a drag coefficient defined by equation 1-8 leads for specular scattering to,

\[
C_{D_s} = \frac{16}{3\sqrt{\pi}} \frac{1}{s} \quad (3-7)
\]

and for diffuse scattering

\[
C_{D_d} = \frac{13}{9} \frac{16}{3\sqrt{\pi}} \frac{1}{s} \quad (3-8)
\]

where \( C_{D_s} \) is the drag coefficient for specular scattering and \( C_{D_d} \) the drag coefficient for diffuse scattering. Note the force on the sphere is greater where diffuse scattering applies.

Stalder and Zurick [23] calculated the drag coefficient for a sphere for the general case of all speed ratios and found, for specular scattering,

\[
C_{D_s} = \frac{2}{\sqrt{\pi}} \frac{e^{-s^2}}{s} \left( 1 + \frac{1}{2s^2} \right) + 2 \left( 1 + \frac{1}{s^2} - \frac{1}{4s^4} \right) \text{erf}(s) \quad (3-9)
\]

and for diffuse scattering,
This work has been cited in a text [20] but with a typographical error, see [25].) Henderson has given a summary of all theoretical and experimental data for drag coefficients up to 1976 [24]. Diffuse scattering was in general found to give the best fit to experimental data. Henderson proposed for $s > 1$

$$C_{Dd} = \frac{2}{\sqrt{\pi}} e^{-s^2} \left( 1 + \frac{1}{2s^2} \right) + 2 \left( 1 + \frac{1}{s^2} - \frac{1}{4s^4} \right) \text{erf}(s) + \frac{2\sqrt{\pi}}{3s} \quad (3-10)$$

and for $s < 1$

$$C_{Dd} = \frac{4.07}{s} + 0.6s \quad (3-12)$$

In equation (3-11) $T_w$ is the wall temperature of the object which can differ from the gas temperature $T$. The Henderson equations correspond to a mixture of 90% diffuse scattering and 10% specular scattering.

Tolmachev et al. have modeled ion motion with collisions in an RF only quadrupole taking into account the thermal motion of the gas [4]. Hard sphere collisions were used and all gas molecules were given the same thermal speed, but random directions of motion. The resulting equations of motion for the ion can be expressed in terms of a drag coefficient and for $s > 1$ lead to

$$C_{Ds} = \left( \frac{m_1}{m_1 + m_2} \right) \left[ \frac{16}{3\pi s^2} + 2 - \frac{32}{15\pi^3 s^4} \right] \quad (3-13)$$

and for $s < 1$

$$C_{Ds} = \frac{8}{3} \left( \frac{m_1}{m_1 + m_2} \right) \left[ \frac{2}{\sqrt{\pi}} \frac{1}{s} + \frac{s\sqrt{\pi}}{10} \right] \quad (3-14)$$
Finally, the simple hard spheres model used previously by Covey and Douglas [11], with no thermal motion of the gas, corresponds to specular scattering with $C_D = 2.0$.

Values of $C_D$ calculated from equations (3-10)-(3-12) and (3-14), are shown in Figure 3-2. For the experiments here the speed ratio varied from 2 to 7, giving values of $C_D$ from 3.1 to 2.1. The difference in $C_D$ calculated from the specular and diffuse models is 20% at $s = 2$, decreasing to 10% at $s = 7$.

Figure 3-2. Drag coefficient $C_D$ vs. speed ratio $s$ for (a) the Stalder and Zurick model with diffuse scattering (b) the Stalder and Zurick model with specular scattering (c) Henderson’s equation (d) specular scattering with the model of Tolmachev et al.
3.3.2 Energy loss: Simple and drag coefficient models

3.3.2.1 Simple model [12]

The simple hard spheres model was the first proposed to interpret the energy loss experiments on a triple quadrupole mass spectrometer and to deduce the collision cross sections of the protein ions [12]. This model requires several assumptions. These include (i) the collisions between protein ions and target gas are modeled as hard sphere collisions; (ii) the collisions are elastic; (iii) an average center-of-mass scattering angle is 90°; and (iv) the thermal motion of the target gas is neglected. Based on these approximations, the relation between the energy losses of an ion and the cross section is derived as follows.

For a single collision, the lab energy of an ion after and before a collision can be expressed by [12]

\[
\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} - \frac{m_2 \cdot E_{\text{int}}}{M \cdot E_{\text{lab}}} + \frac{2m_1m_2}{M^2} \left[ 1 - \frac{E_{\text{int}} \cdot M}{E_{\text{lab}} \cdot m_2} \right]^{1/2} \cos \theta_{\text{cm}} \tag{3-15}
\]

where \(E'_{\text{lab}}\) is the lab energy of an ion after a collision, \(E_{\text{lab}}\) is the lab energy of an ion before a collision, \(m_1\) is the ion mass, \(m_2\) the neutral collision gas mass, \(E_{\text{int}}\) the energy transferred to internal energy, \(\theta_{\text{cm}}\) the center-of-mass scattering angle, and \(M\) is the sum of the masses of the collision partners. Under the above assumptions, i.e. \(\theta_{\text{cm}} = 90°\), \(E_{\text{int}} = 0\) (elastic), equation 3-15 becomes

\[
\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} \tag{3-16}
\]
To interpret the experimental data, equation 3-16 was taken as a measure of the average energy change per collision. This is valid for a hard-sphere potential. When protein ions pass through a target gas, the ions experience many collisions. We define the average ratio of lab energy after one collision \(E_1\) to that before a first collision \(E_0\) as \(\alpha\), that is,

\[
\alpha = \frac{E_1}{E_0} = \frac{m_1^2 + m_2^2}{M^2}
\]  

(3-17)

After two collisions, the average lab energy \(E_2\) is given by

\[
E_2 = \alpha \cdot E_1 = \alpha^2 \cdot E_0
\]  

(3-18)

and after \(N\) collisions,

\[
E_N = \alpha^N \cdot E_0
\]  

(3-19)

The total number of collisions, \(N\), is determined by the cross section, \(\sigma\), the gas number density, \(n\), and the length of the collision cell, \(l\).

\[
N = \frac{l}{\lambda} = n\sigma l
\]  

(3-20)

where \(\lambda\) is the mean free path. Thus, the energy of an ion leaving the collision cell is given by equation 1-1 (Chapter 1). Let \(\alpha' = 1/\alpha\), write \(\alpha' = \exp (ln \alpha')\), and define the target thickness \(S\) by \(S = nl\), then equation 1-1 gives

\[
\frac{E}{E_0} = e^{-\alpha' S \ln \alpha'}
\]  

(3-21)
Equation 3-21 indicates that the energies of ions show an exponential decrease with increasing target thickness. Collision cross sections can be calculated from the experimental energy loss data. Equation 3-21 can be expressed in the form of natural logarithm, i.e.

\[ \ln \left( \frac{E}{E_0} \right) = -\sigma S \ln \alpha' \]

(3-22)

By measuring a series of energy losses at varied target thickness, more precise cross section data can be obtained from the slope of a plot of \( \ln \left( \frac{E}{E_0} \right) \) against \( S \ln \alpha' \). This model provides a simple analytical expression for the energy loss of the ions in collisions with neutrals although some approximations may be oversimplified.

3.3.2.2 Drag coefficient model

In practice, the collisions between protein ions and neutral targets may not meet some of the assumptions of the simple model. First, protein ions may have rough surfaces (i.e. not hard sphere); second, the collisions may be inelastic; third, the scattering from a rough surface may occur through a range of angles; and finally, the thermal motion of the collision gas also affects the collision process. A protein ion moving through a collision gas is similar to a projectile passing through a low density gas. This is a classic subject in aerodynamics [22-24, 26-37]. Based these considerations, Douglas [15] applied the drag coefficient to improve the simple hard sphere model. This improved model, i.e. the drag coefficient model and the relation between this model and the simple model are briefly described as follows.
Equation 1-8 can be written as

\[ \frac{1}{v} \frac{dv}{dx} = -C_D \frac{Anm_2}{2m_1} \]  

(3-23)

Integrating equation 3-23, then

\[ \int_{v_0}^{v} \frac{1}{v} dv = \int_{0}^{i} -C_D \frac{Anm_2}{2m_1} dx \]  

(3-24)

so

\[ (\ln v)_{v_0}^i = - \frac{C_D Anm_2}{2m_1} x_{i0} \]  

(3-25)

where \( v_0 \) is the speed of the ions entering the collision cell, \( v \) is the speed of the ions leaving the cell, and \( l \) is the length of the collision cell. (This makes the approximation that \( C_D \) is constant.)

\[ \ln \left( \frac{v}{v_0} \right) = - \frac{C_D Anm_2 l}{2m_1} \]  

(3-26)

\[ \ln \left( \frac{v}{v_0} \right)^2 = - \frac{C_D Anm_2 l}{m_1} \]  

(3-27)

Thus

\[ \left( \frac{v}{v_0} \right)^2 = \exp \left( - \frac{C_D Anm_2 l}{m_1} \right) \]  

(3-28)

The kinetic energy of the particle \( (E) \) is proportional to the square of the velocity of the particle. Hence, the energy losses of ions in the collision cell are fit to
Experimental drag coefficients include the effects of inelastic collisions, the thermal speed of the target and scattering through a range of angles from a rough surface. Thus, the drag coefficient model is expected to provide a better description of the protein ion neutral system.

For the case of protein ion neutral collisions, $m_f \gg m_i$, and a simple relation between the cross section obtained from the simple hard sphere model and the projection area obtained from the drag coefficient model was derived by Douglas [15]. That is

$$A = \frac{2\sigma}{C_D}$$

where $A$ is the projection area (=cross section) from the drag coefficient model, $\sigma$ is the cross section from the simple hard sphere model. The key factor here is the drag coefficient, $C_D$, which can be calculated from different models as reviewed in section 3.3.1. For hard sphere collisions, $C_D = 2.0$ and thus $A = \sigma$.

### 3.3.3 Ion mobility: Hard sphere and drag coefficient models

#### 3.3.3.1 Hard sphere model

Section 3.2.2 described the experimental measurements of the mobility of ions moving through a buffer gas under a uniform electric field. According to mobility theory [16], the drift time or mobility of the ions can be converted to collision cross sections by equation 1-2
(see Chapter 1). The experimentally measured collision integrals of the ions can be derived from the drift time by equation 1-2. Structural information of the ions is obtained by comparing the measured cross sections or mobilities with the theoretically calculated cross sections or mobilities. So far, three approaches have been developed to calculate the collision integrals for polyatomic ion-neutral systems. These include the projection approximation [38], the exact hard-spheres scattering model [39] and trajectory calculations [40].

In the projection approximation method [38], assuming hard sphere interactions between the ion and buffer gas, the cross section is obtained by averaging geometric projection areas over all orientations. The collision integral is approximated by

\[ \Omega_{\text{avg}} \approx \frac{1}{4\pi^2} \int_0^{2\pi} d\theta \int_0^\pi d\phi \sin \phi \int_0^{2\pi} d\gamma \frac{b_{\text{min}}^2}{2} \] (3-31)

where \( \theta, \phi, \gamma \) are three angles that define the collision geometry, \( b_{\text{min}} \) is the minimum impact parameter. This approach ignores the long-range interactions and all the details of the scattering process between the polyatomic ion and buffer gas.

The exact hard-spheres scattering model was described by Shvartsburg and Jarrold [39]. The averaged collision integral should be calculated by averaging the momentum transfer cross sections. This is related to the scattering angle, which is the angle between the trajectory before and after a collision. This model is given by

\[ \Omega_{\text{avg}} = \frac{1}{4\pi^2} \int_0^{2\pi} d\theta \int_0^\pi d\phi \sin \phi \int_0^{2\pi} d\gamma \int db 2b(1 - \cos \chi(\theta, \phi, \gamma, b)) \] (3-32)
where $\chi(\theta, \varphi, \gamma, b)$ is the scattering angle. This model considers the details of the collision process. However, the effect of the long-range potentials has not been taken into account yet.

A more accurate calculation of the collision integrals should include the contribution from the long-range interactions between the polyatomic ion and buffer gas atom. This was first done by Mesleh et al. [40]. To perform this calculation, an effective potential must be defined and trajectories are run within this potential to determine the scattering angles. Then, the collision integral is calculated by averaging over the impact parameter and relative velocity. The average collision integral is obtained by averaging the collision integral over all possible geometries. That is, the collision integral is given by

$$
\Omega_{\text{avg}} = \frac{1}{8\pi^2} \int_0^{2\pi} d\varphi \int_0^\pi d\psi \sin \psi \left[ d\gamma \frac{\mu}{8K_B T} \int_0^\infty d\epsilon e^{-\frac{\mu\epsilon}{2K_B T}} g^5 \int db 2b(1-\cos \chi(\theta, \varphi, \gamma, b)) \right]
$$

(3-33)

where $g$ is the relative velocity, $\mu$ is the reduced mass of the collision partners.

The above models have been used to calculate collision integrals for carbon clusters, BPTI and cytochrome c [39-41]. The results showed that the collision cross sections from the projection approximation were up to 20% smaller than those obtained from the other two approaches. The long-range interactions between the ion and buffer gas produced a few percent difference of collision integral for those cases studied [40]. However, all the approaches described in this section have assumed a rigid geometry and elastic collisions. Inelastic collisions have not been considered. This may contribute a substantial error for the calculation of mobility and cross sections for the protein ion-neutral cases.
3.3.3.2 Drag coefficient model

Following the discussion in section 3.3.1, the concept of the drag coefficient may also be applied to ion mobility experiments. In mobility experiments, the speed ratios are very low, typically $s \ll 1$. Thus, the drag coefficient can be written

$$C_D = \frac{c}{s}$$  \hspace{1cm} (3-34)

where $c$ is determined by the model used to calculate the drag coefficient. Values of $c$ for the models discussed here are shown in Table 3-1. These were determined by expanding the expressions for $C_D$ in powers of $s$ and keeping only the coefficient of the term in $1/s$.

<table>
<thead>
<tr>
<th>Author</th>
<th>Reference</th>
<th>Specular</th>
<th>Diffuse</th>
<th>Specular/Diffuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epstein</td>
<td>[22]</td>
<td>3.01</td>
<td>4.35</td>
<td>0.69</td>
</tr>
<tr>
<td>Stalder and</td>
<td>[23]</td>
<td>3.39</td>
<td>4.56</td>
<td>0.74</td>
</tr>
<tr>
<td>Zurich</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Henderson</td>
<td>[24]</td>
<td></td>
<td>4.07</td>
<td></td>
</tr>
<tr>
<td>Tolmachev et al.</td>
<td>[4]</td>
<td>3.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobility theory</td>
<td>[16]</td>
<td>3.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In ion mobility experiments, the drag force on an ion is balanced by the electric force

\[ q \varepsilon (q \text{ the charge on the ion, i.e. } q = ze, \varepsilon \text{ the electric field}) \text{ so that} \]

\[ q \varepsilon = \frac{C_0 A n m_2 v_d^2}{2} \tag{3-35} \]

Substituting \( C_D \) from equation 3-34 and \( s \) from equation 3-6 gives

\[ v_d = \frac{\sqrt{2}}{c} \frac{1}{\sqrt{m_2}} \frac{q \varepsilon}{1} \frac{1}{\sqrt{kT}} \frac{1}{n} \frac{1}{A} \tag{3-36} \]

where \( v_d \) is the ion drift speed. For comparison, mobility theory, which calculates the velocity distribution for the ion, not just the drift speed, gives

\[ v_d = \frac{\sqrt{18\pi}}{16} \left[ \frac{1}{m_1} + \frac{1}{m_2} \right]^{1/2} q \varepsilon \frac{1}{\sqrt{kT}} \frac{1}{n} \frac{1}{A} \tag{3-37} \]

where \( \Omega \) is a collision integral [16]. For collisions of hard spheres, \( \Omega = \pi a^2 \), where \( a \) is the sum of the radii of the spheres, and so \( \Omega \) equals the projection area \( A \). For the case where \( m_1 \gg m_2 \) (protein ions) the mobility formula for hard spheres gives the same drift speed as that derived from the drag coefficient for hard spheres (or equivalently the same \( C_D \), Table 3-1). For diffuse scattering, there is a greater average momentum transfer to the ion per collision, the value of \( c \) in equation 3-34 is increased, and the drift speed for a given projection area \( A \) is
reduced. The magnitude of the decrease is given by the ratio of the factors $c$ in Table 3-1. Thus if diffuse scattering applies to protein ions, the collision integral $\Omega$ determined from the mobility equation 3-37 should be reduced by 0.74, to give the projection area $A$, using the drag calculations of Stalder and Zurick (or $\times 0.69$ using Epstein’s drag equations). Shvartsburg and Jarrold have performed scattering calculations for He with carbon clusters and concluded that the collision integral, $\Omega$, can exceed the projection area by 20% or more [39]. The difference between $\Omega$ and the projection area was greatest for ions with concave surfaces which can lead to multiple scattering events. This difference was greatest for large clusters of C$_{60}$, which are roughly the sizes of protein ions. For smaller clusters of C$_{60}$, $\Omega$ approached the hard sphere projection area $A$. More recently the work has been extended to include calculations of He colliding with cytochrome c and bovine pancreas trypsin inhibitor [7]. For both proteins, it was found that the collision integral, $\Omega$, is ~22-25% greater than the projection area. For comparison to $\Omega$ determined in their mobility experiments, projection areas of model structures were multiplied by 1.22. Equivalently, to determine projection areas from measured mobilities, $\Omega$ should be multiplied by $1/1.22$ or 0.82. This is similar to the factor 0.74 obtained from the drag calculations of Stalder and Zurick and suggests that the diffuse scattering model may be suitable for protein ions. For small ions such as C$_{60}^+$, specular scattering (hard spheres) is more likely to apply and $\Omega$ is equal to the projection area.
3.4 Experimental procedures

3.4.1 Measurements of ion energy loss

All measurements were performed with the triple quadrupole mass spectrometer system shown in Figure 2-1. Protein solutions used were horse heart myoglobin (5 μM + 0.1% acetic acid + 50% acetonitrile) and horse heart cytochrome c (5 μM + 0.5% formic acid + 50% acetonitrile). Under these conditions, both proteins are denatured in solution and the noncovalently bound heme in myoglobin is removed. The orifice-skimmer voltage difference was 110 V. Ions were cooled by collisions in Q0 (pressure 7 × 10⁻³ torr) to energy spreads of 1 eV or less [2]. Therefore at Q2, the ions appear to be formed at a potential close to the Q0 rod offset. Because the difference between the Q0 and the collision cell Q2 rod offset voltages was 10 V, ions entered the collision cell with an energy of approximately 10z eV, where z is the number of charges on the ion (center-of-mass energies were typically 0.1-0.8 eV). With no collision gas added, stopping potentials were typically 10-10.5 V, in accord with this interpretation. Under these conditions, no collision-induced dissociation of the ions was observed. Quadrupole Q3 was operated in mass-resolving mode. Energy distributions of ions leaving Q2 were determined from stopping curves obtained by increasing the Q3 rod offset voltage in steps of 0.5 V until the ion signal was attenuated by at least two orders of magnitude. Ion energy spreads (at 10%) were generally approximately 1 eV. Stopping curves were obtained for several values of target thicknesses, i.e. several different cell pressures. The collision cell length was 20.6 cm. The pressure in the cell, typically 0.1-1 millitorr, was measured with a precision capacitance manometer (MKS type 120 high accuracy pressure
transducer, manufacturer's stated accuracy 0.12% of reading). The potentials applied to the system are shown in Table 3-2. The potentials of the ion path are schematically shown in Figure 3-3. The target gases used in this work were neon (Ne), argon (Ar), and krypton (Kr).

**Table 3-2. Voltages used in energy loss experiments**

<table>
<thead>
<tr>
<th>Component</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprayer needle</td>
<td>4500</td>
</tr>
<tr>
<td>Curtain plate</td>
<td>1000</td>
</tr>
<tr>
<td>Orifice</td>
<td>220</td>
</tr>
<tr>
<td>Skimmer</td>
<td>110</td>
</tr>
<tr>
<td>Q0 rod offset</td>
<td>103</td>
</tr>
<tr>
<td>Prefilter</td>
<td>83</td>
</tr>
<tr>
<td>Q1 rod offset</td>
<td>102</td>
</tr>
<tr>
<td>Q1/Q2 lens</td>
<td>83</td>
</tr>
<tr>
<td>Q2 rod offset</td>
<td>93</td>
</tr>
<tr>
<td>Q2/Q3 lens</td>
<td>80</td>
</tr>
<tr>
<td>Q3 rod offset</td>
<td>Varied</td>
</tr>
<tr>
<td>Exit</td>
<td>80</td>
</tr>
</tbody>
</table>
3.4.2 Data analysis of energy loss experiments

In interpreting the experimental data, the nominal ion energy was approximated as the charge times the stopping potential at which the ion intensity was reduced to one-tenth of the value with no stopping potential. This value was interpolated from the stopping curves (a plot of the peak intensity vs. the potential difference between Q3 and Q2) and is referred to as $E_{1/10}$. The validity of this procedure has been demonstrated previously by comparing the cross section data derived from this procedure and a Monte Carlo simulation of the experiment [12]. It was concluded that the procedure of taking $E_{1/10}$ as a measure of the ion energy does
not introduce errors of more than 5% in the derived cross sections. It has also been shown that protein ions are a favorable case for this procedure because the ion energy distributions remain narrow throughout the energy loss process [12].

An example of the data analysis of the cross section measurements is given here. The stopping curves for hMb+10 ions at cell pressures of 0, 0.263, 0.546, 0.778, and 1.049 millitorr of Ne are shown in Figure 3-4. Values of the stopping potentials \( E_{1/10} \) from the stopping curves were 10.40, 9.34, 8.25, 7.40, and 6.50 V for hMb+10 ions at these pressures, respectively. Taking the stopping potential with no collision gas as \( E_0 \), i.e. \( E_0 = 10.40 \) V, the other \( E/E_0 \) ratios can be calculated accordingly. The target thickness \( S \) and \( \ln \alpha' \) can be easily calculated from the cell pressure, the cell length and the masses of the protein ion and target gas. A plot of \(- \ln(E/E_0) \) against \( S \ln \alpha' \) produces a line with a slope that gives the collision cross section of the ion from the simple model. As shown in Figure 3-5, the collision cross section for hMb+10 ions with Ne determined by the simple model is 3104 Å\(^2\) with a very good linear correlation \( (R^2 = 0.9995) \). In this work, the linear correlation coefficients are typically better than 0.996 for all measurements. Collision cross sections for myoglobin ions measured on eight separate days were reproducible to ± 2.2% (relative standard deviation).

If these data are interpreted with the drag coefficient model, then the cross sectional area of the protein ion from the drag coefficient model equals two times the cross section from the simple model divided by corresponding drag coefficient \( C_D \) (section 3.3). For the example discussed here, hMb+10 ions with Ne, the \( C_D \) value based on diffuse scattering for the above experimental conditions is 2.9802. Thus the collision cross section of hMb+10 from the diffuse scattering model is \( 2 \times 3104/2.9802 = 2083 \) Å\(^2\).
Figure 3-4. The stopping curves of hMb+10 ions at different cell pressures of Ne. The curves from right to left correspond to the cell pressures of 0, 0.263, 0.546, 0.778, and 1.049 millitorr of Ne, respectively. “0” represents no added collision gas.

Figure 3-5. The plot of $-\ln(E/E_0)$ against $S\ln\alpha'$ for hMb+10 in collision with Ne.
3.5 Collision cross sections of protein ions with Ne, Ar, and Kr

3.5.1 Mass spectra of myoglobin and cytochrome c

Two proteins, myoglobin and cytochrome c, were employed to evaluate the proposed drag coefficient model. Both myoglobin and cytochrome c are globular proteins and their three dimensional crystal structures are shown in Figure 3-6 and Figure 3-7. Myoglobin contains 153 amino acid residues and a noncovalently bound heme group. When myoglobin is denatured by acid or organic solvent in solution, the heme group is removed from the protein. As a result, only apomyoglobin ions were observed in mass spectrometry. Cytochrome c contains 104 amino acid residues and a covalently bound heme group. For cytochrome c, the addition of acid and organic solvent can cause the denaturation of the protein but no heme removal. ESI-mass spectra of myoglobin (5 μM + 0.1% acetic acid + 50% acetonitrile) and cytochrome c (5 μM + 0.5% formic acid + 50% acetonitrile) at an orifice-skimmer voltage difference of 110 V are shown in Figure 3-8 and Figure 3-9, respectively. Under the above experimental conditions, both protein solutions produced highly charged ions. For myoglobin, the 14 charge states, +9 - +22 of the apomyoglobin (aMb) ions were produced (corresponding to m/z: 1884 <+9>, 1696 <+10>, 1541 <+11>, 1412 <+12>, 1304 <+13>, 1211 <+14>, 1130 <+15>, 1059 <+16>, 997 <+17>, 942 <+18>, 892 <+19>, 848 <+20>, 807 <+21>, 771 <+22>). Also seen is the heme$^{+1}$ ion (m/z: 616). For cytochrome c, the 14 charge states, +7 - +20 (corresponding to m/z: 1767 <+7>, 1546 <+8>, 1374 <+9>, 1237 <+10>, 1124 <+11>, 1031 <+12>, 951 <+13>, 884 <+14>, 825 <+15>, 773 <+16>, 728 <+17>, 687 <+18>, 651 <+19> and 619 <+20>) are observed. The observation of high charge states is due to the fact
that the denatured proteins have more open conformations and more basic sites become available for the protonation.

Figure 3-6. The 3-dimensional structure of myoglobin. (adapted from the web site <http://www.pdb.bnl.gov>, File name: 1WLA, Resolution: 1.70 Å, Author: Maurus et al.)
Figure 3-7. The 3-dimensional structure of cytochrome c. (adapted from the web site <http://www.pdb.bnl.gov>, File name: 1CCR, Resolution: 1.50 Å, Author: Ochi et al.)
Figure 3-8. ESI-mass spectrum of myoglobin. (5μM + 0.1%HAc + 50%CH₃CN, OS = 110 V)

Figure 3-9. ESI-mass spectrum of cytochrome c. (5 μM + 0.5% formic acid + 50% CH₃CN and OS = 110 V)
### 3.5.2 Drag coefficients of myoglobin and cytochrome c ions

The drag coefficients calculated for specular scattering (equation 3-9) and diffuse scattering (equation 3-10) for myoglobin ions are shown in Table 3-3 and for cytochrome c ions in Table 3-4. The kinetic energy of the ion at the entrance of the collision cell was $10z$ eV where $z$ is the number of charges on the ions.

**Table 3-3. Drag coefficients of myoglobin ions with Ne, Ar, and Kr**

<table>
<thead>
<tr>
<th>Mb ion</th>
<th>Specular scattering ($C_{DS}$)</th>
<th>Diffuse scattering ($C_{Da}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ne</td>
<td>Ar</td>
</tr>
<tr>
<td>+9</td>
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<td>2.235</td>
</tr>
<tr>
<td>+10</td>
<td>2.408</td>
<td>2.212</td>
</tr>
<tr>
<td>+11</td>
<td>2.373</td>
<td>2.193</td>
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</tr>
<tr>
<td>+22</td>
<td>2.193</td>
<td>2.098</td>
</tr>
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</table>
### Table 3-4. Drag coefficients of cytochrome c ions with Ne, Ar, and Kr

<table>
<thead>
<tr>
<th>Cyt c ion</th>
<th>Specular scattering ($C_{DS}$)</th>
<th>Diffuse scattering ($C_{DD}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ne</td>
<td>Ar</td>
</tr>
<tr>
<td>+7</td>
<td>2.428</td>
<td>2.220</td>
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<tr>
<td>+8</td>
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</tr>
<tr>
<td>+19</td>
<td>2.162</td>
<td>2.083</td>
</tr>
<tr>
<td>+20</td>
<td>2.154</td>
<td>2.079</td>
</tr>
</tbody>
</table>

#### 3.5.3 Comparison of cross sections calculated from different models

The collision cross sections of all the aMb ions and the cyt c ions of Figures 3-8 and 3-9 with different collision gases Ne, Ar, and Kr were measured by the energy loss method.
The experimental data were interpreted with the simple model [12], and the drag coefficient model. The resulting cross sections of myoglobin and cytochrome c are shown in Figure 3-10 and Figure 3-11, respectively. Figures 3-10a and 3-11a show cross sections for myoglobin (Mb) and cytochrome c ions in collision with Ne, Ar, and Kr, calculated from the simple model of Covey and Douglas [12]. It is seen that for a given charge state the cross sections apparently decrease in the order Ne > Ar > Kr. Because both the polarizabilities and van der Waals radii of the gases increase in the reverse order i.e. Kr > Ar > Ne, it is reasonable to expect the cross sections to decrease in the order Kr > Ar >> Ne irrespective of the details of the collision dynamics. The apparently non-physical results of Figures 3-10a and 3-11a derive from neglecting the thermal motion of the target gas. Cross sections for Mb and cytochrome c calculated using drag coefficients with the specular scattering model (equation 3-9) are shown in Figures 3-10b and 3-11b. For a given charge state the cross sections are nearly equal. The cross sections are reduced from those obtained in the simple model because $C_{DS}$ is greater than 2.0. For a given ion energy this increase in $C_{DS}$ is greater for lighter gases which have higher thermal speeds and therefore a lower speed ratio $s$. This gives a corresponding greater drag force for a given cross section or projection area (for a given mass of the collision gas). Cross sections calculated using the drag coefficients with the diffuse scattering model (equation 3-10) are shown in Figures 3-10c and 3-11c. The cross sections are again reduced from those of the specular model because of an increased $C_D$. The cross sections now appear in the order Kr > Ar > Ne.
Figure 3-10. Collision cross sections for Mb ions vs. charge state calculated from the energy loss with (a) the simple model of Covey and Douglas (b) drag coefficients with specular scattering (c) drag coefficients with diffuse scattering, ○ Ne, ● Ar, ◊ Kr.
Figure 3-11. Collision cross sections for cytochrome c ions vs. charge state calculated from the energy loss with (a) the simple model of Covey and Douglas (b) drag coefficients with specular scattering (c) drag coefficients with diffuse scattering, ○ Ne, ● Ar, ◊ Kr.
If the collision of the protein (radius $r_1$) and gas (radius $r_2$) is modeled as collisions of spheres, the cross section is $\pi(r_1^2 + r_2^2)$. Using van der Waals radii of 1.60, 1.92 and 1.97 Å for Ne, Ar, and Kr respectively [42] and calculating the protein radius from the cross sections of Figures 3-10c and 3-11c with different collision gases gives the same protein radius for each charge state within 3%. The calculated radii from the collision cross sections for myoglobin and cytochrome $c$ ions are shown in Tables 3-5 and 3-6, respectively. These results suggest that the diffuse scattering model with a small contribution from the size of the target gas may be the most appropriate.

3.5.4 Effects of atomic polarizabilities on cross sections

Three different gases, Ne, Ar, and Kr were used as the collision gas for the cross section measurements in this study. These gases have significant differences in polarizability. This allows us to evaluate the influence of ion-induced dipole-dipole interactions on the collision cross sections. Polarizabilities for the collision gases are shown in Table 3-7 [43].

In the Langevin model [44] the collision cross section is given by equation 1-9 (see Chapter 1). The cross sections of myoglobin and cytochrome $c$ ions with Ne, Ar, and Kr calculated from the Langevin model are shown in Table 3-8. These cross sections are 3~5 times smaller than the observed cross sections. This suggests that the Langevin theory is not suitable to describe collisions between protein ions and the neutral gas. In the Langevin model, the differences in the polarizabilities and masses of the gases lead to cross sections in the ratio 0.70:1.00:0.85 for Ne, Ar, and Kr respectively for any given charge state.
Table 3-5. Radii (Å) of myoglobin ions calculated from the collision cross section data

<table>
<thead>
<tr>
<th>Mb ion</th>
<th>( r_p^{Ne} ) (Å) (^a)</th>
<th>( r_p^{Ar} ) (Å) (^a)</th>
<th>( r_p^{Kr} ) (Å) (^a)</th>
<th>( r_{ave} \pm S ) (Å) (^b)</th>
<th>r.s.d. (%) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+9</td>
<td>23.06</td>
<td>23.39</td>
<td>23.70</td>
<td>23.38 ± 0.32</td>
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<td>+10</td>
<td>23.67</td>
<td>23.97</td>
<td>24.64</td>
<td>24.09 ± 0.50</td>
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<tr>
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<td>24.29</td>
<td>24.59</td>
<td>25.22</td>
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<td>25.53</td>
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<td>+13</td>
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<td>25.50</td>
<td>25.91</td>
<td>25.49 ± 0.42</td>
<td>1.6</td>
</tr>
<tr>
<td>+14</td>
<td>25.29</td>
<td>25.71</td>
<td>26.04</td>
<td>25.68 ± 0.38</td>
<td>1.5</td>
</tr>
<tr>
<td>+15</td>
<td>25.52</td>
<td>25.85</td>
<td>26.18</td>
<td>25.85 ± 0.33</td>
<td>1.3</td>
</tr>
<tr>
<td>+16</td>
<td>25.73</td>
<td>25.95</td>
<td>26.31</td>
<td>26.00 ± 0.29</td>
<td>1.1</td>
</tr>
<tr>
<td>+17</td>
<td>26.12</td>
<td>26.10</td>
<td>26.46</td>
<td>26.23 ± 0.20</td>
<td>0.77</td>
</tr>
<tr>
<td>+18</td>
<td>26.28</td>
<td>26.47</td>
<td>26.68</td>
<td>26.48 ± 0.20</td>
<td>0.76</td>
</tr>
<tr>
<td>+19</td>
<td>26.47</td>
<td>26.78</td>
<td>26.92</td>
<td>26.72 ± 0.23</td>
<td>0.86</td>
</tr>
<tr>
<td>+20</td>
<td>26.67</td>
<td>26.95</td>
<td>27.15</td>
<td>26.92 ± 0.24</td>
<td>0.90</td>
</tr>
<tr>
<td>+21</td>
<td>26.92</td>
<td>27.07</td>
<td>27.47</td>
<td>27.15 ± 0.28</td>
<td>1.0</td>
</tr>
<tr>
<td>+22</td>
<td>27.04</td>
<td>27.21</td>
<td>27.60</td>
<td>27.28 ± 0.29</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\(^a\) \( r_p^{Ne} \), \( r_p^{Ar} \), and \( r_p^{Kr} \) represent the radius of protein ion calculated from collision cross sections of protein ion with Ne, Ar, and Kr respectively.

\(^b\) \( r_{ave} \) and \( S \) represent average radius and standard deviation over three different gases.

\(^c\) r.s.d. is the relative standard deviation.
Table 3-6. Radii (Å) of cytochrome c ions calculated from the collision cross section data

<table>
<thead>
<tr>
<th>Cyt c ion</th>
<th>$r_{p}^{Ne}$ (Å)$^a$</th>
<th>$r_{p}^{Ar}$ (Å)$^a$</th>
<th>$r_{p}^{Kr}$ (Å)$^a$</th>
<th>$r_{ave} \pm S$ (Å)$^b$</th>
<th>r.s.d.(%)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>+7</td>
<td>16.26</td>
<td>16.73</td>
<td>17.20</td>
<td>16.73 ± 0.47</td>
<td>2.8</td>
</tr>
<tr>
<td>+8</td>
<td>17.85</td>
<td>18.17</td>
<td>18.43</td>
<td>18.15 ± 0.29</td>
<td>1.6</td>
</tr>
<tr>
<td>+9</td>
<td>19.08</td>
<td>19.16</td>
<td>19.77</td>
<td>19.34 ± 0.38</td>
<td>2.0</td>
</tr>
<tr>
<td>+10</td>
<td>20.01</td>
<td>19.91</td>
<td>20.39</td>
<td>20.10 ± 0.25</td>
<td>1.3</td>
</tr>
<tr>
<td>+11</td>
<td>20.79</td>
<td>20.82</td>
<td>20.95</td>
<td>20.85 ± 0.09</td>
<td>0.41</td>
</tr>
<tr>
<td>+12</td>
<td>21.18</td>
<td>21.11</td>
<td>21.14</td>
<td>21.14 ± 0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>+13</td>
<td>21.62</td>
<td>21.78</td>
<td>21.57</td>
<td>21.66 ± 0.11</td>
<td>0.51</td>
</tr>
<tr>
<td>+14</td>
<td>21.93</td>
<td>22.11</td>
<td>21.81</td>
<td>21.95 ± 0.15</td>
<td>0.69</td>
</tr>
<tr>
<td>+15</td>
<td>22.28</td>
<td>22.36</td>
<td>22.13</td>
<td>22.26 ± 0.12</td>
<td>0.52</td>
</tr>
<tr>
<td>+16</td>
<td>22.64</td>
<td>22.56</td>
<td>22.41</td>
<td>22.54 ± 0.12</td>
<td>0.52</td>
</tr>
<tr>
<td>+17</td>
<td>23.07</td>
<td>22.76</td>
<td>22.97</td>
<td>22.93 ± 0.16</td>
<td>0.69</td>
</tr>
<tr>
<td>+18</td>
<td>23.22</td>
<td>22.90</td>
<td>23.35</td>
<td>23.16 ± 0.23</td>
<td>1.0</td>
</tr>
<tr>
<td>+19</td>
<td>23.37</td>
<td>23.27</td>
<td>23.47</td>
<td>23.37 ± 0.10</td>
<td>0.43</td>
</tr>
<tr>
<td>+20</td>
<td>23.64</td>
<td>23.42</td>
<td>23.74</td>
<td>23.60 ± 0.16</td>
<td>0.69</td>
</tr>
</tbody>
</table>

---

*a.* $r_{p}^{Ne}$, $r_{p}^{Ar}$ and $r_{p}^{Kr}$ represent the radius of protein ion calculated from collision cross sections of protein ion with Ne, Ar, and Kr respectively.

*b.* $r_{ave}$ and S represent average radius and standard deviation over three different gases.

*c.* r.s.d. is the relative standard deviation.
Table 3-7. Atomic polarizabilities

<table>
<thead>
<tr>
<th>Atomic Polarisabilities (units of $10^{-24}$ cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>He</td>
</tr>
<tr>
<td>Ne</td>
</tr>
<tr>
<td>Ar</td>
</tr>
<tr>
<td>Kr</td>
</tr>
<tr>
<td>Xe</td>
</tr>
</tbody>
</table>

The cross sections determined from the drag model do not show these differences. Any residual contribution of polarization forces to the cross sections must be small. Cross sections determined with the diffuse scattering model for the drag coefficients show a slight increase from Ne to Ar to Kr. While this increase may derive from polarization forces it can equally be attributed to a small contribution to the collision cross section from the size of the collision gas.
Table 3-8. The cross sections ($\text{Å}^2$) of myoglobin and cytochrome $c$ ions with Ne, Ar, and Kr, calculated from Langevin model (equation 1-9)

<table>
<thead>
<tr>
<th>Charge state</th>
<th>Myoglobin</th>
<th>Cytochrome $c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ne</td>
<td>Ar</td>
</tr>
<tr>
<td>+7</td>
<td>257</td>
<td>372</td>
</tr>
<tr>
<td>+8</td>
<td>274</td>
<td>398</td>
</tr>
<tr>
<td>+9</td>
<td>291</td>
<td>422</td>
</tr>
<tr>
<td>+10</td>
<td>307</td>
<td>445</td>
</tr>
<tr>
<td>+11</td>
<td>322</td>
<td>466</td>
</tr>
<tr>
<td>+12</td>
<td>336</td>
<td>487</td>
</tr>
<tr>
<td>+13</td>
<td>350</td>
<td>507</td>
</tr>
<tr>
<td>+14</td>
<td>363</td>
<td>526</td>
</tr>
<tr>
<td>+15</td>
<td>376</td>
<td>545</td>
</tr>
<tr>
<td>+16</td>
<td>388</td>
<td>563</td>
</tr>
<tr>
<td>+17</td>
<td>400</td>
<td>580</td>
</tr>
<tr>
<td>+18</td>
<td>412</td>
<td>597</td>
</tr>
<tr>
<td>+19</td>
<td>423</td>
<td>613</td>
</tr>
<tr>
<td>+20</td>
<td>434</td>
<td>629</td>
</tr>
<tr>
<td>+21</td>
<td>445</td>
<td>644</td>
</tr>
<tr>
<td>+22</td>
<td>455</td>
<td>660</td>
</tr>
</tbody>
</table>
3.6 Comparison of the results from energy loss and ion mobility

More persuasive evidence in favor of the diffuse scattering model comes from comparison of these cross sections to those obtained from mobility experiments. Shelimov et al. have reported collision cross sections for cytochrome c ions obtained in mobility experiments [7]. The mobility cross sections interpreted using the hard sphere formula with $\Omega$ equal to the cross section (equation 3-37) are shown in Figure 3-12a and compared to cross sections determined here, interpreted with the hard sphere model for the drag coefficient, $C_{DS}$. It is seen that the mobility cross sections exceed the energy loss cross sections by ca. 30% except for the lowest charge states +8, +9. For these charge states several conformations were detected in the mobility experiments. The energy loss experiments have difficulty detecting multiple conformations except in favorable cases. The mobility cross sections can be converted to those that would be found if a diffuse scattering model were suitable by reducing them by a factor of 0.74 as described in section 3.3.3 (Stalder and Zurick’s model [23]). These can be compared to cross sections from the energy loss experiments interpreted with drag coefficients based on diffuse scattering. The data are shown in Figure 3-12b. The agreement between the cross sections is much improved, with the mobility cross sections being slightly greater.
Figure 3-12. Comparison of cross sections for cytochrome c ions determined here to those determined by ion mobility [7], (a) with specular scattering models (b) with diffuse scattering models. \( \Delta \) ion mobility at low injection energy, \( \bullet \) ion mobility at high injection energy, \( \bigcirc \) Ne this work, \( \bigstar \) Ar this work, \( \Diamond \) Kr this work.
Figure 3-13. Comparison of cross sections for Mb ions determined here with those from ion mobility [11]. (a) with specular scattering models (b) with diffuse scattering models. ○ mobility data (N₂ collision gas), ● this work, for clarity only the Ar data are shown.
Javahery and Thomson have reported mobility cross sections for positive myoglobin ions in charge states 8, 9, 11, 13, 15 and 17 using nitrogen as the collision gas [11]. In Figure 3-13a these data, interpreted with a hard spheres model (Ω equal to projection area), are compared to cross sections determined here from the energy loss experiments interpreted with specular scattering drag coefficients. The mobility cross sections consistently exceed those from the energy loss experiments. The average difference between cross sections determined by the two methods is 21%. Mobility cross sections of Mb ions, corrected for diffuse scattering (i.e. × 0.74) are compared to energy loss cross sections determined here with the drag coefficient calculated for diffuse scattering in Figure 3-13b. The agreement is improved. The average difference is about 5% for Ne, Ar, and Kr.

The agreement between cross sections obtained by the energy loss method and by mobility methods when both are interpreted with diffuse scattering models suggests this model is preferred for protein ion scattering. The improved agreement over specular models derives from the fact that the difference in cross section between the two models is ca. 10-20% for the energy loss experiments and ca. 20-30% for the mobility experiments. A greater correction factor applies to the mobility experiments. Somewhat better agreement between cross sections determined by the two methods could be forced by using different models for the drag coefficients (e.g. 90% diffuse scattering plus 10% specular scattering [22, 24]) but, given the approximations in the models and uncertainties in the experiments, this has not been done. In addition, the collision dynamics may differ somewhat between the different energy ranges of mobility and energy loss experiments.
3.7 Comparison of protein radii in the gas phase and in solution

The radii of gyration of native, molten globule and acid-denatured cytochrome c and myoglobin have been measured with solution X-ray scattering by Kataoka et al. [45, 46]. For the solution phase cytochrome c, the radii of gyration are 13.5 Å (native), 17.0 Å and 17.4 Å (molten globule), and 24.2 Å (acid-denatured), respectively [45]. For myoglobin, the radii of gyration are 17.5 Å (native hMb), 19.7 Å (native aMb), 23.1 Å (molten globule aMb), and 30.2 Å (acid-denatured aMb), respectively [46]. For comparison, the radius of gyration has to be converted to the radius of a sphere by \( r_0 = \sqrt[3]{\frac{5}{3}R_g} \), where \( R_g \) is the radius of gyration of the protein [47]. The results are listed in Table 3-9. The radii of gas phase cytochrome c and myoglobin ions for the lowest and highest charge states, calculated from the collision cross sections with different collision gases, are also included in Table 3-9. The radii of the gas phase ions in low charge states are similar to those of the native proteins in solution, indicating that these gas phase proteins remain a relatively compact structure. For those ions with the highest charge states, the radii of the gas phase ions are similar to the radii of the molten globule states in solution but significantly smaller than that of fully acid-denatured proteins in solution. The molten globule state is defined as a compact denatured state with a significantly native-like secondary structure but a largely flexible and disordered tertiary structure and the molten globule has been proposed to be a common folding intermediate of globular proteins [48-53]. These gas phase radii suggest that, compared to solution phase proteins, protein ions may have more compact conformations in the gas phase. Shelimov and Jarrold [54] estimated a cross section of 4944 Å\(^2\) for aMb if the protein is stretched out into a "string". The cross section for the highest charge state (+22) measured here is ~2500 Å\(^2\). This
is only about half of the estimated value based on a "string" conformation. This suggests the gas phase protein does not have a "string" structure.

Table 3-9. Radii of proteins in solution and in the gas phase

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of residues</th>
<th>Solution phase $r_0^s$ (Å)$^a$</th>
<th>Gas phase $r_0^g$ (Å)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Native</td>
<td>Molten globule</td>
</tr>
<tr>
<td>Cyt c</td>
<td>104</td>
<td>17.4</td>
<td>21.9, 22.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mb</td>
<td>153</td>
<td></td>
<td>22.6</td>
</tr>
<tr>
<td>holo-</td>
<td></td>
<td>25.4</td>
<td>29.8</td>
</tr>
<tr>
<td>apo-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a.* The radii of solution phase proteins, $r_0^s$, are calculated from $r_0 = \sqrt[3]{5} R_g$ where $R_g$ is the radius of gyration [47]. The $R_g$ values are, for cytochrome c, from ref. [45], and, for myoglobin, from ref. [46].

*b.* The radii of gas phase proteins, $r_0^g$, are from this work [Tables 3-5 and 3-6].

### 3.8 Summary

Drag coefficients were successfully used to interpret both energy loss experiments and ion mobility experiments. The good agreement between cross sections obtained from the energy loss method and from mobility methods when both are interpreted with diffuse
scattering models suggests that a “diffuse” scattering model, suitable for collisions with a rough surface, gives the best description of collisions between protein ions and neutral targets. This model has (i) effectively corrected for the effect of the thermal motion of collision gas; (ii) corrected for the influence of inelastic collisions; (iii) overcome the assumption of an average center-of-mass scattering angle of 90°. This model has also reasonably explained the order of protein collision cross sections with different collision gases, i.e. Ne < Ar < Kr. The results indicate that there is little contribution of the polarizabilities of the target gases to the collision cross sections of protein ions. The drag coefficient model has bridged the gap for the first time between the different speed ratios, $s$, of the energy loss method and the ion mobility method. When this is done, similar cross sections are obtained with these two different methods. The method established here has been used to study the conformation of native, reduced bovine pancreatic trypsin inhibitor and its mutants [55]. Cross section measurements are also used in the studies of noncovalent protein complexes by tandem mass spectrometry, which will be discussed in Chapters 4-6.

The discussion of drag coefficients in this chapter has been for spheres. Highly charged ions may well have extended, approximately cylindrical, shapes. Calculations of drag coefficients for cylinders show the drag coefficient is largely independent of the ratio of the length to diameter and is close to that for a sphere if the average projection area is used [56]. Thus cross sections derived from the drag model give the projection area for the ion averaged over all orientations. It is of interest that the drag coefficients can be used to model motion of a particle that has a different temperature from the bath gas (equation 3-11). The drag force is increased for a hot ion in a cooler gas. This may be useful as a first approximation to model the motion of excited protein ions in energy loss or other experiments.
3.9 References


Chapter 4  Collisional Activation of Protein Ions

This chapter evaluates a new collision model for the description of the transfer of translational into internal energy of a protein ion in tandem mass spectrometry. The model can be used to calculate the relative energies transferred to protein ions in multiple collisions. The model is evaluated by comparing the energy required to dissociate a given protein noncovalent complex ion at different cell pressures. In addition, the influence of inelastic collisions on the apparent cross sections of protein ions also is discussed.

4.1 Introduction

Tandem mass spectrometry (MS/MS) is widely used for sequencing biomolecules and analyzing complex mixtures [1-5]. Collisional activation with a neutral gas has played an essential role in the development of MS/MS techniques [6,7]. Collision of ions with a target gas was the first activation method to be employed and continues to be the most widely used [3]. This fragmentation process is usually called collision induced dissociation (CID) or collisionally activated dissociation (CAD). In a triple quadrupole MS/MS system, ions of interest i.e. precursor ions are mass selected by a first quadrupole (Q1 in Figure 2-1) and enter a collision cell where CAD of the selected ions with gases occurs. Fragment ions exiting from the collision cell are mass analyzed by a second mass analyzer, i.e. quadrupole Q3 in Figure
2-1. Applications of collision dynamics in quadrupole mass spectrometry have recently been reviewed by Douglas [8].

In ESI-MS/MS experiments, protein ions are activated by collisions with a neutral gas, typically argon or nitrogen. Because of the very many degrees of freedom of a protein ion, the internal energy of the ion is distributed over a large number of vibration modes and is less concentrated in the reaction coordinate. According to the Rice-Rampsberger-Kassel (RRK) theory [9-11], the rate constant \( \kappa(E) \) of a fragmentation reaction is given

\[
\kappa(E) = v \left( \frac{E - E_0}{E} \right)^{N_f - 1}
\]

(4-1)

where \( v \) is the frequency factor, \( E_0 \) is the critical energy for fragmentation, \( E \) is the internal energy of the ion and \( N_f \) is the number of internal degrees of freedom of the ion [9]. For ions with the same \( v \) and \( E_0 \), larger \( N_f \) leads to lower rate constants. For a macromolecule like a protein, comparatively high internal energies are required to dissociate even a weak bond on the millisecond scale [9, 10]. Depositing these high energies in triple quadrupole mass spectrometers, where center-of-mass collision energies are in the 0.1-10 eV range, will be most effective if the transfer of translational energy to internal energy is efficient. Some evidence suggests that this is the case. Small organic ions show transfer efficiencies on the order of 70% [8]. A study of peptide ions showed that about 55% of the center-of-mass (CM) energy was transferred to internal energy [12] and that the efficiency of this energy transfer increased as the size of the peptide was increased from about 40 to 200 internal degrees of freedom. As discussed in chapter 3, we have demonstrated that a diffuse scattering model gives the best description of collisions between protein ions and neutrals in energy loss.
experiments [13]. For the 0.1-10 eV CM collision energies of triple quadrupole experiments, diffuse scattering corresponds to highly inelastic collisions [8].

This chapter describes the use of a triple quadrupole MS/MS system to study the dissociation of the globin-heme complex, holomyoglobin (hMb), to produce apomyoglobin (aMb) and heme. The complex has 2486 atoms or 7452 internal degrees of freedom. The kinetics of this dissociation have been measured by Gross et al. who trapped hMb ions with charge states +9-+12 in an ICR cell and measured rate constants, \( k(T) \), for the unimolecular loss of heme at temperatures between 119°C and 179 °C, to give activation energies, \( E_a \), and pre-exponential factors, \( A \) [14]. This chapter discusses a new collision model which accounts for differences in cross sections and energy losses of the ions as they pass through the collision cell. This collision model can be applied to calculate the relative energies required to dissociate ions. The internal energy of an ion at dissociation is equal to the sum of the energy from the ion source and the energy from collisional activation processes in the collision cell. The calculated energy from the model here is only the energy received from CAD in Q2. This model can effectively reduce a wide variation of dissociation voltages (the "dissociation voltage" is defined as the voltage difference between Q0 and Q2 at which gives a 50% yield of fragment ions) at different pressures (~250% change over a cell pressure range of 0.50-1.50 millitorr) to calculated energies in agreement within 10%. As an example of testing this new model, highly charged holomyoglobin ions have been studied. The details of the stability of different charge states of hMb ions will be discussed in Chapter 5. This chapter also discusses the effect of inelastic collisions on the apparent cross sections of protein ions. The results show that a 100% inelastic collision leads to only a ca. 0.1% change in cross section for the case of collisions between hMb ions and argon.
4.2 Experimental methods

4.2.1 Tandem mass spectrometry of hMb ions

Experiments were done with the triple quadrupole system shown in Figure 2-1. Solutions were introduced to the electrospray source with the fast-mixing apparatus described in section 2.2. Two syringes (1 ml total volume each) were simultaneously advanced by a syringe pump. The first syringe contained 40 μM hMb in water and the second 0.45% acetic acid (v/v). The two solutions mixed in a "tee" to initiate unfolding of hMb in solution and then flowed to the electrospray source through an 8 cm 75 μm i.d. silica capillary. The flow rate of each syringe was 5 μL/min. and the time from mixing to ionization was 2.1 s. This procedure produces a mixture of low and high charge states of hMb ions [15], which allows the study of a broad charge state distribution of hMb ions. A detailed study of cross sections and heme binding to higher charge states of hMb ions is given in Chapter 5.

In the MS/MS experiments, ions from the electrospray source pass through a dry nitrogen "curtain" gas, a sampling orifice, a skimmer and enter a radio frequency (RF) only quadrupole, Q0, which acts as an ion guide. Collisions in Q0 cool ions to translational energies of 1-2 eV [13, 16]. An orifice-skimmer voltage difference of 30 V was used in the MS/MS experiments. In the collision cell, ions experience a series of collisions with a neutral gas. Each collision converts part of the translational energy to internal energy of the ions. Deposition of enough internal energy will cause fragmentation of the ions. The initial kinetic energy of an ion entering the collision cell depends on the voltage difference between Q0 and Q2 and the number of charges on the ion. Thus the dissociation voltage of an ion can be obtained by measuring the change of relative yield of product ions with the voltage difference
between Q0 and Q2. The dissociation voltages (giving 50% relative yield of product ion) of each ion were determined at 5 different pressures over 0.50-1.5 millitorr. The collision gas was argon.

4.2.2 Collision cross section measurements

Collision cross sections of hMb ions were determined by the energy loss method [13] and the data were interpreted with the drag coefficient model described in Chapter 3. Drag coefficients were calculated from the diffuse scattering model. The collision gas was neon in cross section measurements. To observe the effect of the interface voltage on collision cross sections, four different orifice-skimmer voltages 30 V, 70 V, 110 V, and 150 V were used.

4.3 Collision cross sections and the number of collisions

ESI-mass spectra of myoglobin at orifice-skimmer (OS) voltage differences of 30, 70, 110, and 150 V are shown in Figure 4-1. Over a relatively wide range of the OS voltage differences, this solution produced a spectrum of a mixture of holomyoglobin (hMb) and apomyoglobin (aMb) ions. A lower OS voltage difference favors the formation of highly charged hMb ions. A higher OS voltage difference may lead to the dissociation of hMb ions to aMb ions and singly charged heme. When the OS voltage difference was 30 V, high charge states of hMb ions, up to +21, were observed. At an OS voltage difference of 150 V, only aMb ions could be detected. This indicates that the interface conditions are also very important for the observation of intact protein noncovalent complex ions.
Figure 4-1. ESI-mass spectra for myoglobin at 4 different orifice-skimmer voltage differences produced by the continuous-flow mixing apparatus with a reaction time of 2.1 s. (a) 30 V, (b) 70 V, (c) 110 V, (d) 150 V. Notation: h8 is hMb\(^{8+}\), a8 is aMb\(^{8+}\), etc.
Cross sections determine the number of collisions involved in the ion activation process. Collision cross sections for hMb ions at orifice-skimmer voltage differences of 30 V, 70 V, 110 V, and 150 V were measured. The results are shown in Table 4-1. Cross sections for hMb ions were found to depend on the orifice-skimmer voltage. Higher voltage differences gave larger cross sections and the cross sections approximately reached their maximum at about 110 V. This increase in cross section likely results from unfolding of the ions caused by collisional heating in the interface and has been seen previously for hMb ions on this apparatus [17] and for other protein ions in ion mobility experiments [18]. For the MS/MS experiments, the orifice skimmer voltage difference was 30 V. In the MS/MS experiments, ions were injected into the collision cell at laboratory energies of the order of 800 eV. It is likely that they are rapidly heated to structures with large cross sections and then undergo further activating collisions. Therefore the larger cross sections seen for ions heated in the interface should be more reasonably applied to the calculation of energy transferred into internal energy in the MS/MS experiments. However, due to the lack of cross section data for higher charge states at 110 V OS voltage difference, as an approximation, cross section data at OS = 30 V are used in the calculations below.

These cross sections indicate that the ions have many collisions in the collision cell. The number of collisions (N) that an ion has in passing through the collision cell can be calculated from

\[ N = \frac{l}{\lambda} \]  

(4-2)

where \( l \) is the length of the collision cell, 20.6 cm here, and \( \lambda \) is the mean free path in cm given by
Table 4-1. Collision cross sections (Å²) for hMb ions at orifice-skimmer voltage differences of 30 V, 70 V, 110 V, and 150 V

<table>
<thead>
<tr>
<th>hMb ion</th>
<th>Collision cross sections (Å²)</th>
<th>30 V</th>
<th>70 V</th>
<th>110 V</th>
<th>150 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>+8</td>
<td>1350</td>
<td>1800</td>
<td>1854</td>
<td>2034</td>
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<td>2553</td>
<td>2667</td>
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</tr>
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</table>
\[
\lambda = \frac{1}{n \cdot \sigma}
\]  

(4-3)

where \( n \) is the gas number density (cm\(^{-3}\)), and \( \sigma \) is the cross section of the ion. For example hMb\(^{10+} \) at 110 V (\( \sigma = 2180 \text{ Å}^2 = 2.18 \times 10^{-13} \text{ cm}^2 \)) has 145 collisions at a cell pressure of 1\( \times 10^{-3} \) torr of argon. At 5 different pressures over a range of 0.50-1.50 millitorr, the numbers of collisions for different charge states of hMb ions calculated from the cross sections at an OS voltage difference of 30 V are shown in Figure 4-2. The number of collisions is proportional to the cross section of the ion, in this work, typically 40-270.

Figure 4-2. The number of collisions for different charge state hMb ions at 5 different cell pressures: 0.50, 0.75, 1.00, 1.25, and 1.50 millitorr. Collision cross sections of hMb ions at 30 V orifice-skimmer voltage difference were used in this calculation.
4.4 Collision model

4.4.1 Fragment yield vs. collision energy

In MS/MS experiments, the plot of the relative yield of fragment ions versus initial translational energy or voltage difference between Q0 and Q2 forms a "dissociation curve", from which the threshold of the dissociation of an ion ($V_{th}$), the energy or voltage at which ion is completely dissociated ($V_M$), and the "dissociation voltage" ($V_D$) can be obtained. The voltage difference between Q0 and Q2 rod offset that gives 50% yield of fragment ions is defined as the "dissociation voltage" of that ion. Dissociation of hMb$^{k+}$ leads to loss of singly charged heme to produce aMb$^{(k-1)+}$. In these experiments some loss of neutral heme (~10%) was also seen, as described by others [19-21]. The ion energy necessary to dissociate the complex was determined by increasing the collision energy and recording the yields of aMb ions and the loss of hMb ions. Because the ion transmission can change slightly with collision energy, the precursor and product ion intensities were normalized to the sum of these ions, i.e. $[hMb^{k+}/(hMb^{k+} + aMb^{(k-1)+})]$ and $[aMb^{(k-1)+}/(hMb^{k+} + aMb^{(k-1)+})]$. A typical example of this, for hMb$^{10+}$ in collision with argon at 1.0×10$^3$ torr, is shown in Figure 4-3. There is a threshold at a laboratory energy of about 70×10 eV followed by a rapid increase in the yield of aMb$^{9+}$, reaching near 100% dissociation of the complex at about 100×10 eV. Similar behavior was observed at other collision cell pressures and also for all other hMb ions. The $V_{th}$, $V_D$, $V_M$ values for all charge state hMb ions at 1.0×10$^3$ torr Ar are listed in Table 4-2. The maximum dissociation voltages are typically 1.5 ± 0.3 times threshold values. For hMb$^{10+}$ ions the dissociation voltage is 83.7 V at 1.0 millitorr argon collision gas. Under these conditions, ions of hMb$^{10+}$ enter the collision cell with 837 eV and a speed of 3.0×10$^5$ cm/s.
Figure 4-3. The relative abundance of aMb$^9+$ and hMb$^{10+}$ vs. the injection voltage of hMb$^{10+}$ into Q2 (argon 1.0x10$^{-3}$ torr).

The rapid increase in fragment yield of Figure 4-3 is unusual. For smaller organic ions the yield rises more slowly and reaches a maximum at 2 or 3 times the threshold energy [8, 22]. By contrast, the yield in Figure 4-3 reaches a maximum at approximately 1.4 times the threshold energy. This means that every ion must receive enough energy to dissociate when the total energy is only 1.4 times the threshold and that therefore the internal energy distribution must be very narrow and correspond to efficient transfer of translational to internal energy. This is at least qualitatively consistent with the conclusions of Marzluff et al.
who argued that for large ions, the energy transfer efficiency \( \phi \) (the fraction of maximum translational energy transferred to internal energy) is close to 1.0 [12].

Table 4-2. Threshold voltages (\( V_{\text{th}} \)), dissociation voltages (\( V_D \)), maximum dissociation voltages (\( V_M \)) and \( V_M/V_{\text{th}} \) values for hMb +8--+19 ions at 1.0 millitorr Ar

<table>
<thead>
<tr>
<th>hMb ion</th>
<th>( V_{\text{th}} ) (V)</th>
<th>( V_D ) (V)</th>
<th>( V_M ) (V)</th>
<th>( V_M/V_{\text{th}} )</th>
</tr>
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<tbody>
<tr>
<td>+8</td>
<td>117.2</td>
<td>130.5</td>
<td>143.9</td>
<td>1.23</td>
</tr>
<tr>
<td>+9</td>
<td>88.5</td>
<td>102.4</td>
<td>116.9</td>
<td>1.32</td>
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<td>55.7</td>
<td>69.0</td>
<td>82.5</td>
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<td>53.0</td>
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<td>1.61</td>
</tr>
<tr>
<td>+14</td>
<td>36.4</td>
<td>47.0</td>
<td>57.9</td>
<td>1.59</td>
</tr>
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<td>+15</td>
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<td>40.9</td>
<td>50.8</td>
<td>1.62</td>
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<tr>
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<td>43.2</td>
<td>1.55</td>
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<tr>
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<td>20.7</td>
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<td>37.1</td>
<td>1.79</td>
</tr>
<tr>
<td>+19</td>
<td>18.2</td>
<td>26.4</td>
<td>34.7</td>
<td>1.91</td>
</tr>
</tbody>
</table>
4.4.2 Collision model

The model can be described as follows. The maximum possible energy that the ions can acquire in the collision cell, Q2, is the center-of-mass (CM) energy of each collision summed over all collisions. This is determined by several factors. Ions were dissociated with cell pressures between $5 \times 10^{-4}$ torr and $1.5 \times 10^{-3}$ torr. Higher dissociation voltages are required at lower pressures because there are fewer collisions. Different charge states may have different cross sections and thus at a given cell pressure have different numbers of collisions. Higher charge states are injected at higher absolute energies. Finally, ions lose energy as they pass through Q2 and this can reduce the total energy available for activation. A reasonable collision model can effectively convert the observed dissociation voltage into the internal energy required for the dissociation, i.e. the "dissociation energy". Here a model is proposed which considers the effects of different cross sections for different ions and of different energy losses.

![Figure 4-4. Collision model for ion activation. The ion of mass $m_1$ at a distance $z$ from the cell inlet has a CM collision energy $E_{CM}(z)$. The gas number density is $n$, the target gas mass $m_2$ and cell length $l$.](image)
The motion of an ion in a target gas in a collision cell is schematically illustrated in Figure 4-4. Calculation of the total energy that can be transferred to the ion was done as follows. Considering the motion of an ion at a distance \( z \) from the cell entrance, the LAB energy at this point is given by

\[
E_{LAB} = E_0 e^{-\frac{C_D n m_2 \sigma z}{m_1}}
\]  

(4-4)

where \( E_0 \) is the initial kinetic energy, \( C_D \) is a drag coefficient [15], \( n \) is the gas number density, \( m_2 \) is the collision gas mass, \( \sigma \) is the collision cross section and \( m_1 \) is the ion mass. In traveling a distance \( \Delta z \) the number of collisions the ion experiences is \( \Delta z / \lambda \) where \( \lambda \) is the mean free path given by equation 4-3.

Let \( \phi \) be the average fraction of energy transferred to internal energy in a single collision. Then the increase in internal energy \( \Delta E_{int} \) in traveling \( \Delta z \) is

\[
\Delta E_{int} = \phi \frac{m_2}{M} E_0 e^{-\frac{C_D n m_2 \sigma z}{m_1}} n\sigma \Delta z
\]  

(4-5)

where \( M = m_1 + m_2 \). Writing this equation in differential form and integrating over the cell length, \( l \), gives

\[
\int dE_{int} = \int_0^l \phi \frac{m_2}{M} E_0 e^{-\frac{C_D n m_2 \sigma z}{m_1}} n\sigma dz
\]  

(4-6)

or
\[ E_{\text{int}} = \phi \frac{m_2}{M} \varepsilon_0 \frac{m_1}{m_2} C_D \left[ 1 - e^{-\frac{C_D n m_2 \sigma l}{m_1}} \right] \] (4-7)

For small values of \( \frac{C_D n m_2 \sigma l}{m_1} \), where the ion energy is constant and equal to the initial injection energy, and where \( \phi = 1 \), this reduces to

\[ E_{\text{int}} = \left( \frac{m_2}{M} \varepsilon_0 \right) (n \sigma l) \] (4-8)

which is just the center-of-mass energy per collision multiplied by the number of collisions. This model is similar to that of Marzluff et al. who considered the total energy transfer integrated over many collisions in an ICR cell [12].

### 4.4.3 Evaluation of the collision model

A group of dissociation curves for hMb\(^{10+}\) ions at different cell pressures is shown in Figure 4-5. The dissociation voltage differs significantly at different pressures. In this case, dissociation voltages for hMb\(^{10+}\) ions were 160.4 V (0.50 millitorr), 108.7 V (0.75 millitorr), 83.7 V (1.00 millitorr), 71.7 V (1.25 millitorr) and 62.7 V (1.50 millitorr), respectively. The increase of cell pressure by a factor of 3 leads to the decrease of the dissociation voltage by a factor of \( \sim 2.5 \). For a given ion, the internal energy required for dissociation should be independent of cell pressure. Thus MS/MS experiments with different cell pressures can be used to test the collision model.
Figure 4-5. The relative yield of aMb\textsuperscript{9+} ions from dissociation of hMb\textsuperscript{10+} ions vs. injection voltage for five different cell pressures. The pressure of Ar in millitorr is shown beside each curve.

The example shown here is for hMb\textsuperscript{10+} \rightarrow aMb\textsuperscript{9+} + heme\textsuperscript{+}. The dissociation voltages for hMb\textsuperscript{10+} ions at these pressures obtained from Figure 4-5 are shown in Figure 4-6. The dissociation voltage is strongly dependent on the collision cell pressure. The collision model described here can be applied to the data of Figure 4-6 where dissociation voltages vary by a factor of \sim 2.5 for different pressures. For each experiment the maximum possible energy transfer can be calculated from equation 4-7. The results are shown in Figure 4-7 (here $\phi = 1$). Despite the wide variation of dissociation voltages at different pressures, the calculated energies transferred to the ions at different pressures agree reasonably well. If simple statistics is applied to these data, a relative standard deviation of $\pm 3.1\%$ is found for the energies. More careful examination shows that the calculated energies at different cell pressures have a
systematic decrease at higher pressure. This can be qualitatively explained as follows. Ions move more slowly at higher pressure because lower injection voltages are used. They therefore have longer times to react and so require a lower energy to dissociate (equation 4-1). In addition, the remaining variations in Figure 4-7 may partially be the result of approximating $C_D$ as constant or approximating the cross section as constant as the ion moves through the cell. Similar behavior has also been seen for all other hMb ions. More data and further discussion are given in Chapter 5 and 6. This result shows that these large ions are activated by accumulating increasing internal energy over a large number of collisions (ca. 40-270 collisions for all experiments here). The center-of-mass energy is small in each collision (e.g. 1.9 eV for 837 eV hMb$^{10+}$ incident on argon) but the effects of multiple collisions are cumulative. In the above calculations, we have assumed the efficiency of CM energy transfer to internal energy was 100%, i.e. $\phi = 1$ for all ions.

Figure 4-6. The dissociation voltages of hMb$^{10+}$ ions at pressures of $5 \times 10^{-4}$ to $1.5 \times 10^{-3}$ torr.
Figure 4-7. Energies transferred to the ions to cause dissociation at five different pressures, calculated from equation 4-7. The relative standard deviation of the calculated energies over this pressure range is ± 3.1%.

The above example gives evidence that the collision model can successfully convert the dissociation voltage of an ion to the relative internal energy deposition from the collisions with a neutral target gas. Similar observations were found for all other charge states. Typically, this model can reduce a ~250% change of dissociation voltage over a pressure range of 0.50-1.50 millitorr to relative energies in agreement within 10%. This approach can be used to compare the relative stabilities of different charge state protein ions, as well as the relative binding strengths of various protein noncovalent complexes. Two applications of this collision model are discussed in the next two chapters.
4.5 Influence of inelastic collisions on apparent cross sections of protein ions

As discussed above, collisions between protein ions and a target gas in the collision cell appear to be highly inelastic. The increase of ion internal energy may cause the unfolding of protein ions and finally lead to dissociation of ions. Here we discuss the influence of inelastic collisions on apparent cross sections of protein ions. A 100% inelastic collision corresponds to a high efficiency of transfer of CM energy into internal energy.

For a single collision, the ratio of Lab energy of an ion after collision with a neutral \( E_{\text{Lab}}' \) to that before collision \( E_{\text{Lab}} \) is given by equation 3-15 [23] (see Chapter 3). In the equation 3-15, \( \theta_{\text{cm}} \) is the scattering angle in center-of-mass coordinates and \( E_{\text{int}} \) is the energy transferred to internal energy in a single collision. For a hard sphere collision, \( \theta_{\text{cm}} \) is distributed between 0° to 180° and an average energy transfer corresponds to \( \theta_{\text{cm}} = 90° \). Thus

\[
\frac{E_{\text{Lab}}'}{E_{\text{Lab}}} = \frac{m_1^2 + m_2^2}{M^2 - \frac{m_2 \cdot E_{\text{int}}}{M \cdot E_{\text{Lab}}}}
\] (4-9)

For multiple collisions,

\[
\left( \frac{E_{\text{Lab}}'}{E_{\text{Lab}}} \right)_m = \left[ \left( \frac{E_{\text{Lab}}'}{E_{\text{Lab}}} \right)_s \right]^N
\] (4-10)
where $N$ is the number of collisions and $N = \sigma n l$, the footnotes $m$ and $s$ represents multiple collisions and single collision, respectively. So

$$\left(\frac{E'_{\text{lab}}}{E_{\text{lab}}}\right)_m = \left(\frac{E'_{\text{lab}}}{E_{\text{lab}}}\right)_s^{\sigma n l} \tag{4-11}$$

In a single collision, if the collision is elastic, $E_{\text{int}} = 0$ and $\theta_{\text{cm}} = 90^\circ$, then equation 4-9 becomes

$$\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} \tag{4-12}$$

If the collision is 100\% inelastic, $E_{\text{int}} = E_{\text{cm}} = \frac{m_1}{M} E_{\text{lab}}$, then equation 4-9 is expressed as

$$\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} - \frac{m_2}{M} \cdot \frac{E_{\text{int}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} - \frac{m_2^2}{M^2} \tag{4-13}$$

That is

$$\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2}{M^2} \tag{4-14}$$

Let $\alpha = \frac{E'_{\text{lab}}}{E_{\text{lab}}} \tag{4-15}$

In many collisions, as described in reference [29],

$$\frac{E}{E_0} = \alpha^{\sigma n l} = \exp(-\sigma n l \ln \alpha') \tag{4-16}$$
where $E$ is the lab energy after $N$ collisions, $E_0$ is the lab energy before any collisions, and

$$\alpha' = \frac{1}{\alpha}.$$ 

The collision cross section is determined from $\ln \left( \frac{E}{E_0} \right) = -\sigma n l (\ln \alpha')$, therefore

$$\sigma = -\frac{1}{n l (\ln \alpha')} \ln \left( \frac{E}{E_0} \right) \quad (4-17)$$

Considering the case of a hMb ion in collision with argon, $m_1 = 17,500$, $m_2 = 40$. If the collision between a hMb ion and argon is elastic, then

$$\alpha = \frac{m_1^2 + m_2^2}{M^2} = \frac{17,500^2 + 40^2}{(17,500 + 40)^2} = 0.995449$$

So $\ln \alpha' = 4.56099 \times 10^{-3}$

If the collision between a hMb ion and argon is 100% inelastic, then

$$\alpha = \frac{m_1^2}{M^2} = \frac{17,500^2}{(17,500 + 40)^2} = 0.995444$$

and $\ln \alpha' = 4.56621 \times 10^{-3}$

Thus, from equation 4-18, the ratio of cross section calculated assuming elastic collisions ($\sigma_e$) to that assuming inelastic collisions ($\sigma_{\text{ine}}$) is $\frac{\sigma_e}{\sigma_{\text{ine}}} = 1.00114$. That is, the difference in cross section is only 0.114%. A 100% of inelastic collision for hMb-argon system leads to an apparent decrease in cross section of ~ 0.1%. This is because the mass of the target gas argon is small compared to the mass of a protein ion.
4.6 Summary

In this chapter, we have proposed a collision model which can be applied to calculate quantitatively relative energies required to dissociate noncovalent complex ions. Because ions from the source already contain some energy before entering the collision cell, the relative energy calculated from this model is the energy must be added to the ion to cause it to dissociate. This collision model considers the effect of different collision cross sections of different ions and different energy losses. Ions with higher charge states may have larger cross sections and they have more collisions when they pass through the collision cell at a given pressure. Thus more CM energy is deposited in the ions. In the MS/MS experiment, the relative abundance of product ions increases very quickly as the collision energy is increased. This provides indirect evidence that transfer of translational energy to internal energy in collisions of protein ions with neutrals is highly efficient for CM energies of 0.1-10 eV. The accuracy of this collision model is ~10%. Thus it should be possible to measure relatively small differences in dissociation energies of gas phase complexes by tandem quadrupole mass spectrometry. One such example shows the binding energy of heme observed in low charge states of hMb is maintained in high charge states where the protein has unfolded significantly [24]. This is discussed in detail in Chapter 5.
4.7 References

Chapter 5  Heme binding in Highly Charged Holomyoglobin Ions

The heme-myoglobin complex is a text-book example of a protein-ligand noncovalent complex. The heme binding in myoglobin is of particular interest. This chapter describes the observation of highly charged holomyoglobin (hMb) ions (up to +21) formed by using a two-syringe continuous-flow mixing apparatus coupled with ESI-MS. The conformation of these highly charged hMb ions are discussed with measurements of cross sections by the energy loss method coupled with the interpretation of the data by the drag coefficient model developed in Chapter 3. The heme binding strengths of different charge states are evaluated with measurements of the relative internal energies needed for removing heme from hMb ions with the new collision model described in Chapter 4. The results show that the protein has unfolded appreciably in high charge states but the heme binding energy is decreased only slightly in the more highly charged ions. Thus much of the heme-binding pocket appears to remain in this protein as it unfolds in the gas phase. Collisionally activated dissociation of the hMb ions in the orifice-skimmer region is also briefly discussed.

5.1 Introduction

Electrospray is a sufficiently soft ionization process that it allows transfer of noncovalent protein-ligand or protein-protein complexes into the gas phase [1-3]. These
noncovalent complexes are of particular interest. If ion source and sampling conditions can be found where the complexes retain the specific interactions that they have in solution it may be possible to use mass spectrometry to measure at least relative binding energies of complexes that are relevant to solution proteins. In holomyoglobin (hMb), a heme group is bound noncovalently to the protein by van der Waals interactions, hydrogen bonds and iron coordination to a histidine residue [4-5]. This noncovalent complex can be observed in ESI-MS [6-10] and provides a model system for gas phase noncovalent complexes. One focus of previous studies of gas phase hMb has been the strength of the binding of the heme to the protein. Previous work has examined the kinetics of the unimolecular dissociation of hMb into apomyoglobin (aMb) and heme. McLuckey and Ramsey [11] reported that hMb +8 and +9 ions could retain a heme group at approximately room temperature for at least 200 ms by trapping these ions in a Paul trap with a gas bath of 1 millitorr helium. In a later experiment the +9 charge state was studied via proton transfer reactions [12] and it was concluded that the heme must have an activation energy for dissociation of at least 0.6 eV. A more detailed and direct study of the kinetics of the decomposition of hMb was reported by Gross et al. [13], who investigated the +9 to +12 charge states by the Blackbody Infrared Radiative Dissociation (BIRD) technique in an ion cyclotron resonance (ICR) cell. This study provided values for unimolecular rates of the dissociation of hMb into aMb and heme (pre-exponential, \(A\), and Arrhenius activation energies, \(E_a\)), with the \(E_a\) values ranging from 0.7 to 1.0 eV. In related work Hunter et al. [14] studied the binding of heme to a series of mutant myoglobins where individual hydrogen bonds between the heme and protein were systematically removed to lower the heme binding energy in solution. Dissociation of +11 to +14 ions of the holomyoglobin complexes in the orifice-skimmer region of an electrospray mass spectrometer showed a strong correlation between the voltages required to induce dissociation of the
complexes and the heme binding energies in solution. It was concluded that the individual hydrogen bonds known to be present in solution remain in the gas phase complex and that therefore the heme-binding pocket in gas phase holomyoglobin retains much of its solution structure.

All previous studies were limited to hMb in relatively low charge states. High charge states of a protein are normally formed in ESI after unfolding the protein in solution with acid or by other means [15-17]. This cannot normally be done with hMb because when the protein unfolds in solution the heme-protein interactions are disrupted [18-21] and only aMb peaks are seen in the mass spectrum [6, 22]. In this chapter, hMb ions in charge states up to +21 were formed with a novel continuous-flow mixing apparatus [22]. The collision cross sections of these ions and the corresponding aMb ions were measured by the ion energy loss method and the results were interpreted with the diffuse scattering model described in Chapter 3. The cross sections show that the highly charged hMb ions are unfolded (ca. up to 90% increase in area or up to 40% increase in average radius) in comparison to low charge states but still retain a considerable degree of folding. The stability of these hMb ions was studied by tandem mass spectrometry and the relative energies needed to dissociate heme from these ions were calculated with the new collision model described in Chapter 4. This allows a comparison of the relative internal energies required to dissociate heme from different charge states. The results show that the required energies for highly charged ions are only slightly lower (ca. 15-20%) than those for hMb in low charge states. These results suggest that in highly charged gas phase hMb ions the heme-protein interactions remain relatively unperturbed, even though the protein has somewhat unfolded. Additional supporting evidence comes from trapping experiments in our lab by J. Campbell [23] which show that the highly charged ions can bind heme for 0.5 seconds or more, similar to ions in low charge states, and thus also show that
energies needed to dissociate heme from the highly charged ions are about equal to those of ions in low charge states. The dissociation of ions in the orifice-skimmer region has also been studied. Comparison to MS/MS experiments in the collision cell shows that there is a good correlation between the voltage required to induce dissociation of an ion in the interface and the voltage required to dissociate this ion in the collision cell, Q2.

5.2 Experimental procedures

5.2.1 Collision cross section measurements

The continuous-flow mixing setup described in section 2.2 was used to produce a mixture of low and high charge states of hMb [22]. Collision cross sections for both hMb ions and the corresponding aMb ions at four different orifice-skimmer voltage differences (30 V, 70 V, 110 V, and 150 V) were determined by the energy loss method and the experimental data were interpreted with the drag coefficient model based on diffuse scattering. The collision gas was neon. The detailed experimental procedures for cross section measurements are described in Chapter 3.

5.2.2 Tandem mass spectrometry

The procedure for tandem mass spectrometry here is the same as that described in section 4.2.1. The orifice-skimmer voltage difference was 30 V and the collision gas was argon. Five different pressures, i.e. 0.50, 0.75, 1.00, 1.25 and 1.50 millitorr, were employed in the MS/MS experiments of all charge state hMb ions.
5.2.3 Collisionally activated dissociation in the interface

In the ESI triple quadrupole mass spectrometer system, protein ions formed by electrospray pass through a dry nitrogen "curtain" gas, a sampling orifice, a skimmer and then enter an RF only quadrupole, Q0 (Figure 2-1). The background pressure of the orifice-skimmer region was 2 torr (nitrogen that flowed in from the curtain gas). When a very low orifice-skimmer (OS) voltage is applied, almost no aMb ions are produced. When a relatively high OS voltage is applied, collisionally activated dissociation of hMb ions occurs. With an increase of the orifice-skimmer voltage, the relative abundance of product ions, i.e. aMb ions and singly charged heme, increase. Further increases of the interface voltage difference lead to the dissociation of all hMb ions to aMb ions and heme. Both precursor ions and product ions enter Q0 and then are mass analyzed in either Q1 or Q3. In this work, Q3 scans were used. Mass spectra at a series of different OS voltage differences were recorded. According to the dissociation reaction: hMb$^{k+}$ → aMb$^{(k-1)+}$ + heme$^+$, the relative yield for each ion can be calculated as $\frac{aMb^{(k-1)+}}{hMb^{k+} + aMb^{(k-1)+}}$. The plot of relative yield of product ion against OS voltage difference produces a dissociation curve from which the "dissociation voltage" where the relative abundance of product ion is 50%, can be obtained. The CAD experiment in the interface is different from the MS/MS experiment in the collision cell, Q2. In the interface measurement, precursor ions cannot be mass selected. All ions formed in the source are dissociated at the same time and the mass spectrum records all fragments and the remaining precursor ions. More details are given in section 5.7.
5.3 Formation of highly charged holomyoglobin ions

Figure 5-1. Schematic diagram of a continuous-flow mixing apparatus coupled with the ESI-triple quadrupole mass spectrometer system.

Electrospray ionization from solution which contains a protein in its native state produces protein ions in comparatively low charge states. High charge states are commonly produced by acidifying the solution to denature the protein [15-17]. This method cannot produce high charge states of hMb ions or other noncovalent complexes because denaturing the protein disrupts the complex [18-22]. Here, the continuous-flow mixing apparatus described in section 2.2, coupled with the triple quadrupole mass spectrometer system was used to produce highly charged hMb ions. In this two-syringe continuous-flow mixing apparatus, one syringe was loaded with a 40 μM hMb aqueous solution (pH = 7.3) and the other was filled with a 0.45% (v/v) acetic acid solution (pH = 3.1). The two syringes were simultaneously advanced at the same speed (5 μL/min for each) and the two solutions were mixed in a “tee” where unfolding of the hMb complex was initiated. The mixed solution then flowed to the electrospray source through an 8-cm long 75 μm i.d. capillary. The pH of the mixed solution was 3.4. The total flow rate was 10 μL/min. and the time from mixing to ionization was 2.1 s. The mass spectrum of the solution at a 30V orifice-skimmer voltage difference and 2.1 s after mixing is shown in Figure 5-2. This spectrum consists of a bimodal
distribution of hMb ions and a unimodal distribution of aMb ions. Ions of hMb in low charge states (+7-+10) derive from the native conformation in solution, ions of hMb in high charge states (+11-+21) are produced by hMb which has partially unfolded in solution, and aMb ions in high charge states (+9-+25) are formed from hMb which has unfolded and lost heme in solution [22].

Figure 5-2. Mass spectrum of myoglobin obtained with a continuous-flow mixing apparatus combined with ESI. The orifice-skimmer voltage difference (OS) was 30 V. Notation: h8 is hMb8+, a9 is aMb9+, etc.

Higher solution acidity, longer reaction time and higher orifice-skimmer voltage difference favor the formation of denatured aMb ions. As shown in Figure 4-1, when the
orifice-skimmer voltage difference is higher than 110 V, almost no high charge states of hMb ions are seen. The solution reaction time is determined by the length of mixing capillary and the flow rate of solutions. Mass spectra were produced from the above solutions at several different flow rates and are shown in Figure 5-3. The flow rates of 2 ×1.0 μL/min., 2 ×3.0 μL/min. and 2 ×15 μL/min. correspond to reaction times of 10.5 s, 3.5 s and 0.7 s respectively (8 cm 75 μm i.d. capillary). A 90 V orifice-skimmer voltage difference was used. The results show that the reaction time of 0.7 s is too short to produce highly charged hMb ions, and a 10.5 s reaction time leads to a mixture of highly charged aMb ions and low charge states of hMb. Thus the reaction time of two solutions is critical for the observation of this intermediate. From the observation of highly charged hMb ions, it was proposed that the mechanism of the acid-induced denaturation of hMb follows the sequence (heme-protein)$_\text{native}$ → (heme-protein)$_\text{unfolded}$ → heme + (protein)$_\text{unfolded}$ [22]. The generation of this transient species allows us to study the stability of heme binding in high charge states.
Figure 5-3. Mass spectra of myoglobin obtained with a continuous-flow mixing apparatus combined with ESI at different reaction times: (a) 0.7 s; (b) 3.5 s; (c) 10.5 s. The orifice-skimmer voltage difference (OS) was 90 V.
5.4 Collision cross sections of hMb ions and aMb ions

In the cross section measurements, the injection energy of ions is $10^z$ eV where $z$ is the number of charges, the collision gas is neon, and the cell pressure range is 0 to 1.0 millitorr. Cross sections for hMb and aMb ions measured at orifice-skimmer voltage differences of 30 V, 70 V, 110 V and 150 V are shown in Figures 5-4a and 5-4b. The cross sections for both hMb and aMb ions were found to depend on the orifice-skimmer voltage difference, especially for those charge states below +14. Higher orifice-skimmer voltages give greater cross sections presumably because protein ions are activated and unfold. This is similar to the unfolding of protein ions seen in mobility experiments when ions are injected into the mobility cell at high ion energies [24-28]. Here, ions in high charge states show a smaller voltage dependence of the cross section, possibly because these ions are already unfolded significantly when formed. The cross sections increase substantially with charge state for both hMb and aMb ions. For a given charge state at the same orifice-skimmer voltage difference, hMb and aMb ions have similar cross sections.

For comparison to cross sections determined by mobility experiments [28], the mobility collision integrals must be reduced by a factor of 0.74 [29]. For low charge states, when a 30 V orifice-skimmer difference is applied, the cross sections here are significantly less than those of the ion mobility experiments. However, as shown in Figure 5-4b, for all charge states cross sections measured here at an orifice-skimmer voltage difference of 110 V agree within about 5% with those measured by ion mobility. This suggests that in the ion mobility experiments aMb ions were unfolded somewhat during ion injection or ion sampling. An increase in cross section with charge is commonly seen for protein ions and is usually attributed to Coulombic repulsion in the ions [25, 30].
Figure 5-4. (a) Collision cross sections of hMb ions at different orifice-skimmer voltages, 30 V (○), 70 V (●), 110 V (▼), and 150 V (▲), respectively. (b) Collision cross sections of aMb ions at different orifice-skimmer voltages, 30 V (○), 70 V (●), 110 V (▼), and 150 V (▲), respectively. Cross sections from ion mobility measurements, recalculated using the drag coefficient model, are also shown (□).
Figure 5-4a shows that, provided a low orifice-skimmer voltage difference is used, increasing the number of charges causes hMb ions to unfold from structures with minimum cross sections of ca. 1300 Å² to structures with cross sections up to 2500 Å² (an increase of up to 90% in area) and still retain the heme group. This corresponds to an increase in average radius of up to 40%. A cross section of 4944 Å² has been estimated for aMb stretched out into a “string” [28]. The cross sections for aMb and hMb are substantially less than this even for the highest charge states and show that the ions have structures which retain a considerable degree of folding.

5.5 Dissociation of holomyoglobin ions

Typically, fragmentation of hMb ions in the collision cell, Q2, follows the reaction hMb\(^{k^+}\) → aMb\(^{k-1^+}\) + heme\(^+\). Loss of 10-20% neutral heme, i.e. the reaction hMb\(^{k^+}\) → aMb\(^{k^+}\) + heme, was also observed for all charge states of hMb and over a wide range of injection energies. This phenomenon is similar to a previous report [10]. Tandem mass spectra of several typical hMb ions (+9, +12, +15, and +17) are shown in Figure 5-5. All other charge states have similar tandem mass spectra.
Figure 5-5. Tandem mass spectra for different charge states of hMb ions. The injection voltage is defined as the voltage difference between Q0 and Q2.
5.6 Relative dissociation energies of holomyoglobin ions

5.6.1 Influence of neutral heme loss on the dissociation voltage of hMb

The loss of neutral heme from hMb ions may introduce a systematic error for the measurements of dissociation voltages or dissociation energies of these hMb ions. If we take into account the product ions of neutral heme loss from hMb\(^{k+}\) ions, i.e. aMb\(^{k+}\) ions, then the relative abundance of precursor ions, hMb\(^{k+}\), can be calculated from \([\text{hMb}^{k+} / (\text{hMb}^{k+} + \text{aMb}^{(k-1)+} + \text{aMb}^{k+})]\). If neutral heme loss is ignored, then we calculate the relative abundance of precursor ions or product ions for the reaction of hMb\(^{k+}\) \(\rightarrow\) aMb\(^{(k-1)+}\) + heme\(^+\) by \([\text{hMb}^{k+} / (\text{hMb}^{k+} + \text{aMb}^{(k-1)+})]\) or \([\text{aMb}^{(k-1)+} / (\text{hMb}^{k+} + \text{aMb}^{(k-1)+})]\). Thus the influence of neutral heme loss on the dissociation voltage for this fragmentation reaction can be evaluated by comparing dissociation voltages extracted from two different data analysis methods. Dissociation voltages \((V_D)\) for all charge states of hMb ions at 0.50 millitorr and 1.5 millitorr of argon are shown in Table 5-1.

In Table 5-1, dissociation voltages without consideration of neutral heme loss are listed in the left half and dissociation voltages taking into account neutral heme loss are shown in the right half. The results show that only a very minor variation of \(V_D\) is contributed by the loss of neutral heme. Thus, in the discussion below, loss of neutral heme is ignored.
Table 5-1. Comparison of dissociation voltages for all charge states of hMb ions without \( (V_D) \) and with \( (V_D') \) consideration of neutral heme loss at 0.50 and 1.5 millitorr Ar

<table>
<thead>
<tr>
<th>hMb ion</th>
<th>Without neutral heme loss</th>
<th>With neutral heme loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_D ) (V) (&lt;0.50)</td>
<td>( V_D ) (V) &lt;1.5)</td>
</tr>
<tr>
<td>+8</td>
<td>250.0</td>
<td>96.0</td>
</tr>
<tr>
<td>+10</td>
<td>160.4</td>
<td>62.7</td>
</tr>
<tr>
<td>+12</td>
<td>118.0</td>
<td>45.6</td>
</tr>
<tr>
<td>+14</td>
<td>90.3</td>
<td>36.5</td>
</tr>
<tr>
<td>+15</td>
<td>79.6</td>
<td>31.3</td>
</tr>
<tr>
<td>+16</td>
<td>70.4</td>
<td>27.9</td>
</tr>
<tr>
<td>+17</td>
<td>59.9</td>
<td>24.4</td>
</tr>
<tr>
<td>+18</td>
<td>54.8</td>
<td>22.1</td>
</tr>
<tr>
<td>+19</td>
<td>50.6</td>
<td>20.7</td>
</tr>
<tr>
<td>+20</td>
<td>31.3</td>
<td>-</td>
</tr>
</tbody>
</table>

5.6.2 Dissociation voltages for hMb ions

The abundances of hMb\(^{k+}\) ions and the corresponding aMb\(^{(k-1)+}\) ions were measured for each charge state pair at different injection voltages (Q0-Q2). For each charge state pair, five different cell pressures from 0.50 to 1.5 millitorr were used. The relative yields of product ions were calculated as aMb\(^{(k-1)+}\)/[aMb\(^{(k-1)+}\) +hMb\(^{k+}\)] for all fragmentation reactions. A plot of relative yield of product ions vs. injection voltage produces a series of dissociation curves from which the dissociation voltage of each hMb ion can be obtained. A group of
typical dissociation curves for hMb $+8$-$+19$ ions at 1.0 millitorr of argon are shown in Figure 5-6. The yield of product ions shows a rapid increase with increasing injection energy and reaches a maximum at approximately 1.2-1.8 times the threshold energy. Dissociation voltages for all hMb ions at five different cell pressures are shown in Figure 5-7. For a given charge state, the dissociation voltage decreases by a factor of $\sim 2.5$ over a pressure range of 0.50 to 1.5 millitorr. At a given cell pressure, the dissociation voltage changes by a factor of

![Graph](image)

Figure 5-6. The relative yield of aMb$^{(k-1)+}$ ions from dissociation of hMb$^k+$ ions against injection voltage (Q0-Q2) at 1.0 millitorr argon.
Figure 5-7. Dissociation voltages for hMb +8–19 ions at 5 different pressures from 0.50 to 1.5 millitorr argon.

~5 for charge states of hMb ions from +8 to +19. The collision model described in Chapter 4 was employed to calculate the energy required to induce heme loss in high and low charge states of hMb ions.

### 5.6.3 Energy Transfer in the Collision Cell

Equation 4-7 was used to calculate the energy transferred to a hMb ion to induce dissociation. Here $\phi = 1.0$ was used. It is relative binding energies that are of interest.
Provided different ions have the same efficiency $\phi$, relative energies will be calculated correctly. The total energies transferred to induce dissociation were calculated for hMb ions in charge states +8-+19 for five different cell pressures between $5 \times 10^{-4}$ and $1.5 \times 10^{-3}$ torr (Ar gas). The cross sections of Figure 5-4a (an orifice-skimmer voltage 30V) were used. This calculation is approximate because collision cross sections may change somewhat if ions unfold while moving through the cell [31]. Although dissociation voltages for each charge state differed by up to a factor of ~2.5 between different pressures, the energies transferred to the ions, calculated from equation 4-7 for different pressures, agreed typically within 10% for all charge states. This method can therefore distinguish differences in the relative energies that hMb acquire through collisional activation of about 10%. Measured energies for all hMb ions at five different cell pressures (and with $\phi = 1$), are shown in Table 5-2. For any charge state, generally a slight decrease of the calculated energies is seen with increasing cell pressure. This is because lower injection energies are used with the higher pressure gas and an ion has a longer reaction time, resulting in a slightly lower energy requirement. The average energy for five different pressures plotted against the charge state is shown in Figure 5-8. For the +9 to +12 charge states the calculated energies here are very similar. This is in agreement with the BIRD experiments that show the activation energies for heme loss from these ions are the same within experimental uncertainty (±10% in those experiments) [13]. This agreement further supports the validity of the collision model. It also illustrates the importance of correcting for the different collision cross sections of different ions. If the same cross section is assumed for the +9 to +12 ions, the calculated internal energy for the +12 ion is 20% less than that of the +9 ion. For complexes in higher charge states between +12 and +19 there is only a relatively small (ca. 15-20%) decrease in the energy necessary to
dissociate the complex. The energies required to dissociate heme in the higher charge states appear to be similar to those of the lower charge states. The ICR experiments with trapped hMb ions [13] show that the activation energy for removing heme in low charge states is about 0.7-1.0 eV. It follows that the energies for the high charge states are approximately 0.6-0.8 eV.

Table 5-2. Energies, $E_{\text{int}}$, of hMb ions at 5 different pressures calculated from equation 4-7 ($\phi=1$)

<table>
<thead>
<tr>
<th>hMb ion</th>
<th>0.50 x 10$^3$ Torr</th>
<th>0.75 x 10$^3$ Torr</th>
<th>1.0 x 10$^3$ Torr</th>
<th>1.25 x 10$^3$ Torr</th>
<th>1.5 x 10$^3$ Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td>+8</td>
<td>185.0</td>
<td>183.4</td>
<td>175.8</td>
<td>174.2</td>
<td>176.1</td>
</tr>
<tr>
<td>+9</td>
<td>175.3</td>
<td>170.3</td>
<td>165.0</td>
<td>162.9</td>
<td>163.7</td>
</tr>
<tr>
<td>+10</td>
<td>178.3</td>
<td>170.8</td>
<td>165.6</td>
<td>167.3</td>
<td>166.1</td>
</tr>
<tr>
<td>+12</td>
<td>181.2</td>
<td>172.4</td>
<td>166.1</td>
<td>164.6</td>
<td>160.9</td>
</tr>
<tr>
<td>+14</td>
<td>180.2</td>
<td>167.5</td>
<td>161.5</td>
<td>161.8</td>
<td>162.1</td>
</tr>
<tr>
<td>+15</td>
<td>176.1</td>
<td>169.2</td>
<td>154.6</td>
<td>153.8</td>
<td>152.5</td>
</tr>
<tr>
<td>+16</td>
<td>170.4</td>
<td>163.6</td>
<td>148.3</td>
<td>145.3</td>
<td>147.2</td>
</tr>
<tr>
<td>+17</td>
<td>156.5</td>
<td>153.0</td>
<td>142.0</td>
<td>141.5</td>
<td>138.3</td>
</tr>
<tr>
<td>+18</td>
<td>154.3</td>
<td>147.1</td>
<td>137.2</td>
<td>134.9</td>
<td>134.3</td>
</tr>
<tr>
<td>+19</td>
<td>152.7</td>
<td>148.9</td>
<td>131.0</td>
<td>131.0</td>
<td>133.9</td>
</tr>
</tbody>
</table>

Unit: eV
Figure 5-8. Energy transferred to hMb ions to induce dissociation calculated from equation 4-7 with $\phi = 1$. The error bars are the standard deviation of experiments at 5 different pressures.

### 5.6.4 Effect of dissociation length on the calculated relative internal energy

In the above discussion, the geometric length of the collision cell, 20.6 cm, was used in the calculation of relative energies required to dissociate hMb ions. A more realistic choice for $l$ would be the average distance ions travel before dissociating. However, the actual dissociation length is unknown. Here the effects of using different dissociation lengths on the calculated relative internal energy is investigated by calculating the relative internal energies transferred to induce dissociation from equation 4-7 with $l = 10.0$, 15.0 and 20.6 cm. Cross sections of Figure 5-4a (orifice-skimmer 30 V) were used. With smaller lengths the agreement
between energies at different pressures is improved. For example, for hMb\textsuperscript{10+}, relative standard deviations of calculated energies at five pressures are ±3.1\% with \( l = 20.6 \) cm, ±1.4\% with \( l = 15.0 \) cm, and ±1.9\% with \( l = 10.0 \) cm, respectively. Thus the model can account for relative energies quite well. The results show that the relative energies are insensitive to dissociation length, \( l \). However absolute energies calculated depend strongly on \( l \) and cannot be calculated with this model. Normalized relative energies calculated from equation 4-7 with three different lengths are compared in Figure 5-9. The maximum possible value of \( l \), 20.6 cm, gives the greatest decrease.

![Figure 5-9. Influence of dissociation length on relative internal energy transferred to the hMb ions calculated using equation 4-7 with \( \phi =1 \).](image)
5.7 Collisionally activated dissociation of hMb ions in the interface

In the interface region of the ESI triple quadrupole system, the gas expanding through the ion sampling orifice forms a free jet where the gas number density decreases from $2.5 \times 10^{19}$ cm$^{-3}$ at the sampling orifice to $3.5 \times 10^{16}$ cm$^{-3}$ at the skimmer tip. The terminal flow speed, reached within a few orifice diameters downstream of the sampling orifice, is $7.8 \times 10^4$ cm/s. In the absence of any electric fields, the ions are carried by the gas flow from the orifice to the skimmer in 2.6 μs. The voltage difference across the orifice-skimmer region accelerates the ions through this region to reduce the transit time below 2.6 μs. Collisionally activated dissociation of protein ions can also be done in the ion-sampling interface [14, 32-35]. Hunter et al. [14] have studied the binding of heme to a series of myoglobin mutants by dissociating the +11 to +14 holomyoglobin ions in this way. Here higher charge states up to +21 were formed. Thus the study of the dissociation of these ions in the interface becomes feasible. In this experiment, mass spectra of myoglobin were recorded at different orifice-skimmer (OS) voltage differences. The relative abundances of hMb ions decreased with increases of the OS voltage difference. Higher charge states of hMb ions disappear at lower OS voltage differences and lower charge states of hMb ions dissociate at higher OS voltage differences. A group of mass spectra at different OS voltage differences was shown in Figure 4-1. The dissociation curve for each charge state of hMb$^{k+}$ ions can be obtained by plotting the relative yield of aMb$^{(k-1)+}$ ions or the corresponding hMb$^{k+}$ ions against the OS voltage difference, from which a "dissociation voltage" (giving 50% product yield) for each charge state of hMb ions can be acquired. As an example, the dissociation curve of hMb$^{9+} \rightarrow$ aMb$^{8+}$ + heme$^+$ is shown in Figure 5-10. The dissociation voltage for hMb$^{9+}$ ions here is 138 V. Dissociation
voltages for all charge state hMb ions are shown in Figure 5-11. The dissociation voltages from three measurements on three separate days for each charge state agreed within ±1.2% (a typical error bar is shown in the figure). Figure 5-11 shows that the dissociation voltage decreases approximately linearly from +8 to +10, has a flatter region from +10 to +13, and then decreases approximately linearly again from +13 to +20. The dissociation voltage decreases by a factor of ~2 from charge state +8 to +20.

Figure 5-10. The relative yield of aMb^{8+} from dissociation of hMb^{9+} in the orifice-skimmer region vs. the orifice-skimmer voltage difference.
Figure 5-11. The dissociation voltages of hMb +8→20 ions in the orifice-skimmer region.

Recently, Harrison [36] and van Dongen et al. [37] have made systematic comparisons between CID in the interface region and in the collision cell for a series of the protonated peptides. They found that the instrumental settings necessary for obtaining comparable product ion spectra from the two methods are correlated. They concluded that the CID in the interface is also a low-energy multiple collision process similar to that in the collision cell. The dissociation voltages for the hMb ions in the orifice-skimmer interface and the MS/MS experiments in the collision cell, compared in Figure 5-12, also show good correlations. However, because of the gas density between the sampling orifice and the skimmer is not uniform, the description of the collisions between the ions and the background gas in the interface region is more complicated than that in the collision cell. This will require a more comprehensive understanding of the gas and ion dynamics in the interface.
Figure 5-12. Comparison of dissociation voltages of the hMb ions in the collision cell and in the orifice-skimmer interface. Curves (a), (b), (c), (d), and (e) represent the cell pressures of 0.50, 0.75, 1.00, 1.25, and 1.50 millitorr Ar. The background pressure in the orifice-skimmer region was 2 Torr nitrogen.

5.8 Structure of holomyoglobin

The tandem mass spectrometry shows that the binding energies of heme in high charge states of hMb are similar to those of low charge states. The experiments here and the earlier ICR experiments [13] do not show any evidence of a Coulomb barrier to dissociation.
It is the difference in Coulomb energy between the transition state and heme-protein complex that contributes to the barrier in the exit channel [38]. The difference can be small if breaking short range interactions (hydrogen bonds, van der Waals forces, etc.) makes the major contribution to the energy required to dissociate the complex. However it is also possible that there is a fortuitous cancellation of effects with reduced intrinsic binding in the higher charge states being offset by an increased Coulomb contribution to the dissociation energy. There is some indirect evidence that this is not the case. The MS/MS experiments show primarily loss of singly charged heme with about 10% neutral loss for all charge states. If a Coulomb barrier was the dominant contribution to the dissociation energy, neutral heme loss might be expected to be the major dissociation channel, particularly for higher charge states. This is not observed.

The small decrease in heme dissociation energy for higher charge states is surprising because the cross section data show that the protein has unfolded somewhat. Dissociation studies of hMb ions in charge states +11 to +14 have indicated that the individual hydrogen bonds between the heme and the protein that are present in solution persist in the gas phase, and suggest that much of the specific binding of the heme pocket is preserved in the gas phase ion [14]. The dissociation energies measured here suggest that the heme binding pockets of the lower charge states may also be partially retained in the charge states up to +19. This observation and the cross section data show that ions of hMb in high charge states cannot be a string, as is sometimes used as a limiting model for highly charged gas phase proteins [24-28, 39-41]. However it is conceivable that the specific interactions of low charge states have been replaced by myriad nonspecific interactions that happen to give a similar binding energy. Future experiments with specific residues altered by site directed mutagenesis could give a detailed picture of which of the individual protein-heme interactions remain in the highly
charged ions. The observations here for gas phase hMb ions are analogous to the solution behavior of hMb which can unfold significantly (as demonstrated by the formation of high charge states in ESI-MS) yet still retain a largely undisturbed heme pocket [20, 22]. Gas phase hMb ions also show other parallels to solution hMb: (i) the mechanism of heme loss is similar (hMb unfolding followed by heme loss) [22, 42], (ii) the binding energy of the heme is similar (ca. 0.5-1.1 eV solution [14, 43], ca. 0.9 eV gas phase [13]) and (iii) individual hydrogen bonds between heme and the protein persist in the gas phase, at least for ions in low charge states [14]. Thus there is increasing evidence that gas phase hMb appears to retain at least some of its solution properties.

5.9 Summary

A novel continuous-flow mixing apparatus was used to produce highly charged hMb ions. Collision cross sections of these hMb ions and the corresponding aMb ions were measured by the energy loss method. Highly charged hMb ions have cross sections up to ~90% greater than hMb in low charge states, indicating that these hMb ions have unfolded significantly. The energies required to induce dissociation of heme from these hMb ions in tandem mass spectrometry experiments, calculated with the new collision model, show that the heme binding in high charge states is similar to that in low charge states. Additional evidence for this comes from trapping experiments of these hMb ions by J. Campbell in our lab. Ion trapping experiments show that highly charged hMb ions can be stable for 0.5 s or longer, similar to low charge states, indicating that high charge states of hMb ions have stability similar to ions with low charge states. Collision cross section measurements and the new collision model on the calculation of energy transfer are further applied to study noncovalent protein-protein complexes in Chapter 6.
5.10 References

Chapter 6  Protein-Protein Noncovalent Interactions:  
Cytochrome c-Cytochrome b$_5$ Complexes

This chapter applies cross section measurements and tandem mass spectrometry to study the noncovalent interactions of bovine liver cytochrome $b_5$ with a series of yeast iso-1-cytochrome $c$ mutants (wild type, trimethyl-Lys72Ala, Lys73Ala, Lys79Ala and Lys87Ala). The pathways of the fragmentation of cytochrome $c$-cytochrome $b_5$ complexes are described. The conformation and relative stability of different charge states and different mutant complexes are compared. The effect of different collision gases on the calculation of energy transfer in the collision process is discussed. The influence of the continuum background of mass spectra on the dissociation voltage and the relative internal dissociation energy of an ion is also addressed.

6.1 Introduction

Protein-protein noncovalent complexes are of central importance to many biological processes. Cytochrome $c$ and cytochrome $b_5$ are two well-characterized proteins. Their
structure, properties and mutagenesis have been extensively studied [1-6]. The noncovalent complex of cytochrome c and cytochrome b₅ is considered a model system for studying electron-transfer complexes of proteins. The structure, stability and electron-transfer properties of this complex have been characterized by various approaches such as spectrophotometric methods [7, 8], fluorescence quenching methods [7, 9], NMR [7, 10-12], potentiometric titrations [7, 12, 13], electrostatic analysis [7, 13, 14], electrochemical measurements [7, 15], and kinetic and thermodynamic methods [7, 16, 17]. The effects of chemical modifications and mutations of cytochrome b₅ or cytochrome c on the formation of the cytochrome c-cytochrome b₅ complex have also been reported [4, 12]. Mauk et al. [7] have given a comprehensive review of experimental and theoretical investigations of the interaction of cytochrome c and cytochrome b₅. The noncovalent interaction of cytochrome c and cytochrome b₅ forms a specific complex with the stoichiometry of 1:1 [7]. The stability of the complex in solution was found to be strongly dependent on ionic strength and pH [8]. The association constant of this complex at an ionic strength of 0.001 M (25°C, pH = 7.0 phosphate buffer) was found to be $4 \times 10^6$ M⁻¹ [8].

In last chapter, ESI-MS was applied to study a particular case of a protein-ligand noncovalent complex – the heme binding in highly charged holomyoglobin ions. Compared to myoglobin, the binding constant of cytochrome c-cytochrome b₅ in solution is almost 9 orders of magnitude lower. An interesting question is whether ESI can also produce the intact cytochrome c-cytochrome b₅ complex ions in the gas phase. If so, does the binding of these complexes in the gas phase reflect that found in solution? This chapter describes the application of tandem mass spectrometry to these less stable noncovalent protein complexes. Noncovalent complexes of yeast iso-1-cytochrome c and cytochrome b₅ were successfully
detected by ESI-MS. Charge state distributions and collision cross section measurements show that ions of these complexes have relatively compact conformations. Tandem mass spectrometry reveals the pathway of fragmentation of the complex ions and the possible charge state distribution in the complex. Relative internal energies required for the dissociation of these complex ions have been calculated with the collision model described in Chapter 4. A series of cytochrome c mutants such as trimethy-Lys72Ala, Lys73Ala, Lys79Ala, and Lys87Ala have also been used to form noncovalent complexes with cytochrome b_5. These mutations make only small changes to the binding energies of the complex in solution [18]. These mutations were found to make no substantial perturbations to the formation, conformation and stability of cytochrome c-cytochrome b_5 complexes in the gas phase (within the 10% uncertainty). To further examine the collision model, three different gases, Ne, Ar, and Kr, were used as the collision gas in the MS/MS experiments. Comparison of the relative internal energies, calculated from equation 4-7, suggests that further improvements of the collision model are needed when using this model to interpret data with different collision gases.

6.2 Experimental methods

6.2.1 Production of cytochrome c-cytochrome b_5 complex ions

The cytochrome c-cytochrome b_5 complex was formed by simply mixing two protein solutions (each 20 μM in deionized water) at a ratio of 1:1. Methanol (10%) was added to this
mixed solution to improve the efficiency of the ion spray process. The flow rate was 1.0 μL/min. Mass spectra of cytochrome c-cytochrome b₅ complexes and the corresponding individual proteins were recorded at an orifice-skimmer voltage difference of 50 V. Mass spectra of the c-b₅ complex at an orifice-skimmer voltage difference of 100 V were also recorded. Complexes of cytochrome b₅ and other cytochrome c mutants can also be observed by this procedure.

6.2.2 Collision cross section measurements

Collision cross sections of cytochrome b₅, cytochrome c and their complex ions were measured by the energy loss method as described in Chapter 3 and reference [19]. The collision gas was neon. The orifice-skimmer voltage difference was 50 V. Collision cross sections for the complex ions +10 and +11 at a 100 V orifice-skimmer voltage difference were also measured.

6.2.3 Tandem mass spectrometry

Tandem mass spectrometry of cytochrome c-cytochrome b₅ complex was performed in the collision cell, Q2, of the triple quadrupole mass spectrometer as described in section 4.2.1. The collision gases were Ne, Ar, and Kr. The orifice-skimmer voltage difference was 50 V. The injection energies of the ions (i.e. the number of charges times the voltage
difference between Q0 and Q2) were between one hundred electron volts and three thousand electron volts.

6.3 Observation of cytochrome c-cytochrome b₅ complexes

Mass spectra of cytochrome c, cytochrome b₅ and the c-b₅ complex are shown in Figure 6-1. In Figure 6-1c, four charge states of the complex ions, i.e. +10 (m/z, 2280), +11 (m/z, 2073), +12 (m/z, 1900) and +13 (m/z, 1754) were produced. The charge state +9 (m/z, 2533) of the complex could not be resolved from the overlapping peak of charge state +5 (m/z, 2542) of cytochrome c and probably +4 (m/z, 2520) of cytochrome b₅ as well. Figures 6-1a and 6-1b are mass spectra of cyt c (wild type iso-1-cytochrome c) and cyt b₅ (cytochrome b₅), respectively. Deconvolution of these mass spectra gave molecular weights: cytochrome c, m.w. 12,710 ± 1; cytochrome b₅, m.w. 10,080 ± 1 and cytochrome c-cytochrome b₅, m.w. 22,790 ± 2. These molecular weights were obtained by averaging three separate measurements on three separate days. Figure 6-1c shows that the noncovalent complex between cytochrome c and cytochrome b₅ can be detected by ESI-MS. This indicates that electrospray can be used to characterize relatively weak noncovalent interactions in biomolecules. Based upon the equilibrium constant ($K_a \sim 10^6 \text{M}^{-1}$) of this complex [8], ca. 99% of both proteins have formed the complex in solution. However, the relative abundance of the complex ions in the mass spectrum from a 10% methanol solution was less than 15%,
much lower than expected from the equilibrium constant for a 100% aqueous solution. This suggests that the ionization or ion sampling processes dissociate the complexes.

Comparison of mass spectra at different orifice-skimmer voltages (see Figures 6-1c and 6-1d) shows that when a 50 V orifice-skimmer voltage difference was applied, the charge state distribution from +10 to +13 for the complex could be observed and all four charge states showed considerable intensities. An OS voltage lower than 50 V resulted in low signal intensity and poor signal-to-noise (S/N) ratio, indicating that the voltage was not sufficient for complete desolvation of charged droplets. When the orifice-skimmer voltage difference was increased to 100 V, only the charge states +10 and +11 survived and with low abundance. The peak at \( m/z \) 1893 in Figure 6-1d is apo-cytochrome \( b_5^{5+} \) produced from the dissociation of holo-cytochrome \( b_5 \). This indicates that ion sampling interface conditions play a very important role for the detection of the noncovalent complexes. Thus, the observation of intact noncovalent complexes by ESI-MS requires a balance between interface conditions which can provide sufficient energy for ion desolvation, while ensuring that interface conditions are gentle enough to prevent the dissociation of the complexes.

The cytochrome \( c \)-cytochrome \( b_5 \) complexes (including those mutants studied here) have 4-5 charge states from +10 (or +9) to +13. Compared to the individual proteins, these complex ions show relatively low charge states and narrow charge state distributions. This may be a common attribute for all protein-protein complexes [20, 21]. As Loo pointed out in a recent review [22], multiply charged ions of intact noncovalent complexes such as protein-protein complexes exhibit relatively low charge states; furthermore the distribution of charge states is typically rather narrow; with 3-5 charge states.
Figure 6-1. Mass spectra of cytochrome c, cytochrome b₅, and the c-b₅ complex. The protein was 10 μM in 10% methanol and 90% water. The orifice-skimmer voltage difference was 50 V for (a), (b), and (c), and 100 V for (d). (a) cytochrome c, (b) cytochrome b₅, (c) and (d) the c-b₅ complex. Ions of the complex are marked with a symbol "*". (a) and (b) share a common m/z scale; (c) and (d) share another m/z scale.
6.4 Collision cross sections

Collision cross sections of cytochrome c, cytochrome $b_5$ and the cytochrome $c$-cytochrome $b_5$ complex ions with neon were measured with the energy loss method and the data were interpreted with the diffuse scattering model. The results are shown in Figure 6-2. The results show that, for both single proteins and their complex, collision cross sections increase with the charge on the ion. This is usually attributed to Coulombic repulsion in the ions [23, 24]. The collision cross sections for the cytochrome $c$-cytochrome $b_5$ complex ions were found to be dependent on the orifice-skimmer voltage difference. A higher orifice-skimmer voltage difference gives greater cross sections because the complex ions are activated and partially unfolded. This activation and unfolding destabilizes these noncovalent complex ions. Thus when a 100 V orifice-skimmer voltage difference was applied, higher charge states could not be detected. The collision cross sections of cytochrome $c$-cytochrome $b_5$ ions at 50 V orifice-skimmer voltage difference are used below.

For a given charge state, the cross section of the $c$-$b_5$ complex is only slightly larger (5-25%) than that of the individual proteins cytochrome $c$ and cytochrome $b_5$ of the same charge state. This is much smaller than that estimated from the same charge state single proteins. For example, the measured cross section for the complex ion of $+11$ is $1773 \text{ Å}^2$ which is only 5% larger than that of cytochrome $c$ $+11$ ($1696 \text{ Å}^2$) and 23% larger than that of cytochrome $b_5$ $+11$ ($1446 \text{ Å}^2$). Simple estimations of cross sections for the complex ions can be made from the hard spheres model shown in Figure 6-3. The maximum possible cross section (see Figure 6-3a) for the $c$-$b_5$ complex ion ($+11$) calculated based on the cyt $c$ and the cyt $b_5$ of the same charge states is $\sigma = \pi r_1^2 + \pi r_2^2 = 3142 \text{ Å}^2$. The measured value ($1773 \text{ Å}^2$) is
Figure 6-2. Collision cross sections of cytochrome c, cytochrome b₅ and the cyt c-cyt b₅ complex. The collision gas was Ne. The voltage shown on the figure refers to the orifice-skimmer voltage difference. c-b₅ represents the cytochrome c-cytochrome b₅ complex.

Figure 6-3. Estimation of cross sections of protein-protein complexes. (a) Maximum possible cross section, and (b) Minimum possible cross section. The $r_1$ and $r_2$ are radii of two proteins, respectively.
only 56% of this number. Considering the reaction \( \text{cyt} \, c^7^+ + \text{cyt} \, b_5^4^+ \to \{ \text{cyt} \, c \text{-cyt} \, b_5 \}^{11^+} \) (A better calculation should use the reaction \( \text{cyt} \, c^8^+ + \text{cyt} \, b_5^3^+ \to \{ \text{cyt} \, c \text{-cyt} \, b_5 \}^{11^+} \), but the cross section for \( \text{cyt} \, b_5^3^+ \) is not available here), the cross sections of \( \text{cyt} \, c^7^+ \) and \( \text{cyt} \, b_5^4^+ \) are 1179 \( \AA^2 \) and 714 \( \AA^2 \), respectively. The maximum possible cross section, estimated based on Figure 6-3a, i.e. \( \sigma_{\text{max}} = \pi r_1^2 + \pi r_2^2 \), for the complex +11 is 1893 \( \AA^2 \). The experimental cross section for this ion (1773 \( \AA^2 \)) is only slightly smaller than this estimated cross section. If the proteins are orientated as in Figure 6-3b, then this complex ion has the minimum projection area, i.e. \( \sigma_{\text{min}} = \sigma_1 = 1179 \, \AA^2 \) in this case. The above calculations are simplistic. More reasonable and realistic estimation can be obtained by averaging projection areas over different orientations. Compared to these estimated cross sections, the experimental value (1773 \( \AA^2 \)) is in between the maximum value (like Figure 6-3a) and the minimum value (like Figure 6-3b), but closer to that in Figure 6-3a. Usually in ESI, \( \text{cyt} \, c^7^+ \) and \( \text{cyt} \, b_5^4^+ \), or \( \text{cyt} \, c^8^+ \) and \( \text{cyt} \, b_5^3^+ \) are formed from the native proteins. These ions have compact conformations. Thus, cross section results of the complex ions suggest that the cytochrome \( c \)-cytochrome \( b_5 \) complex has a relatively compact conformation.

### 6.5 Pathways of fragmentation of noncovalent complexes

Tandem mass spectra of different charge states of cytochrome \( c \)-cytochrome \( b_5 \) complex ions are shown in Figure 6-4. The results show that each charge state of the complex ions was fragmented to several pairs of cytochrome \( c \) and cytochrome \( b_5 \) ions. In these ion-
pairs, one daughter ion, cytochrome $b_5$, basically has a charge state distribution from +1 (+1 and +2 can not be seen because of the limited mass range of the mass spectrometer) to +5 with the maximum at the charge state +3. The other daughter ion, cytochrome $c$, shows a charge state distribution from +5 to +10 with the maximum at the charge state of $+(k-3)$, where $k$ is the charge state of the precursor ion. That is, with an increase of the charge state of the precursor ions, the charge state of the cytochrome $b_5$ ions with maximum abundance remains unchanged, while the charge state of the cytochrome $c$ ions with the maximum abundance shifts from +7 to +10. Therefore, the pathway of fragmentation of cytochrome $c$-cytochrome $b_5$ complex ions is dominated by the reaction

$$\{\text{cyt } c \text{-cyt } b_5\}_k^+ \rightarrow \text{cyt } c^{(k-3)+} + \text{cyt } b_5^{3+}$$

Green et al. [25] suggested that the charge states of fragments give insights to the charge site in the complex. The above fragmentation reaction suggests that, if charges do not move in the CAD process, the charges in this complex are distributed mainly on the cytochrome $c$, whereas the cytochrome $b_5$ surface maintains a relatively low and unperturbed charge state distribution. The appearance of apo-cyt $b_5$ ions and heme$^+$ in tandem mass spectra shows that the daughter ions of the $c$-$b_5$ complex, holo-cyt $b_5^{k+}$ can be fragmented further to apo-cyt $b_5^{(k-1)+}$ and singly charged heme.
Figure 6-4. Tandem mass spectra of cyt c-cyt bs complex ions. c, bs, ab5, and c-bs or cb5 represent cyt c, cyt bs, apo-cyt bs and cyt c-cyt bs noncovalent complexes, respectively. The collision gas was 0.50 millitorr krypton. The collision energies are shown in the figures.
6.6 Dissociation energies of noncovalent complex ions of different charge states

6.6.1 Dissociation curves and dissociation voltages

From tandem mass spectra of different charge states of the c-b₅ complex at different voltage differences between Q0 and Q2, the relative abundance of the precursor ion can be calculated by \( \frac{I_p}{I_p + \sum_{i=1}^{\text{frag}} I_{\text{frag}}} \), where \( I_p \) is the area of the peak of the precursor ion, i.e. the complex ion with subtraction of the continuum background; \( \sum_{i=1}^{\text{frag}} I_{\text{frag}} \) is the sum of areas of the peaks of all fragments. The influence of the background of the mass spectrum on the dissociation voltages and calculations of energy transfer is described in section 6.8. A plot of these relative abundances against the Q0-Q2 voltage difference gives a curve, which is defined as the “dissociation curve”. The dissociation curves for the cytochrome c-cytochrome b₅ +10 at 0.50, 0.75, 1.00, and 1.50 millitorr of argon collision gas are shown in Figure 6-5. Dissociation curves for all other charge states +11, +12, and +13 at above four cell pressures are also similar (data not shown).

The “dissociation voltage” was defined as the voltage difference between Q0 and Q2 at which the relative intensity of the precursor ions drops to 50%. Dissociation voltages for the cytochrome c-cytochrome b₅ complex ions +10 to +13, at four different cell pressures of Ar between 0.50 and 1.50 millitorr are shown in Figure 6-6. Figure 6-6 shows that the
dissociation voltage for each charge state of the ions decreases by a factor of ca. 2.5 when the pressure is increased by a factor of 3 (from 0.50 to 1.50 millitorr). The dissociation voltage cannot be directly used to compare the relative stability of different ions because these ions have different charge states and different cross sections (corresponding to different number of collisions) at a given cell pressure.

Figure 6-5. Relative abundance of the cytochrome c-cytochrome b₅ complex +10 at four different cell pressures from 0.50 to 1.50 millitorr Ar vs. injection voltage (Q0-Q2). The number beside each curve is the cell pressure in millitorr.
Figure 6-6. The dissociation voltages of the cytochrome c-cytochrome $b_5$ complex ions with charges from +10 to +13 at four different cell pressures over a range of 0.50-1.50 millitorr. The collision gas was Ar.

### 6.6.2 Relative internal energies required to dissociate the complex ions

The energy transferred to an ion to cause dissociation, $E_{\text{int}}$, is calculated from equation 4-7 [also see references 26, 27]. For comparison of the relative internal energies required by different ions, here we assume $\phi = 1.0$ and $l = 20.6$ cm. Calculated internal energies for each charge state of the complex ions at different pressures from the dissociation voltages in Figure 6-6 are shown in Table 6-1.
Table 6-1. Relative internal energy ($E_{\text{int}}$) required for the dissociation of cytochrome $c$-cytochrome $b_5$ complex ions at different pressures of argon

<table>
<thead>
<tr>
<th>$P_{\text{Ar}}$ (mTorr)</th>
<th>+10</th>
<th>+11</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>151.5</td>
<td>111.3</td>
<td>104.3</td>
<td>100.0</td>
</tr>
<tr>
<td>0.75</td>
<td>142.9</td>
<td>110.8</td>
<td>99.6</td>
<td>96.5</td>
</tr>
<tr>
<td>1.00</td>
<td>140.4</td>
<td>106.0</td>
<td>92.4</td>
<td>90.0</td>
</tr>
<tr>
<td>1.50</td>
<td>133.1</td>
<td>95.6</td>
<td>86.6</td>
<td>84.9</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>142.0</td>
<td>105.9</td>
<td>95.7</td>
<td>92.9</td>
</tr>
</tbody>
</table>

Unit: eV

For a given charge state, the internal energies calculated from equation 4-7 at four different pressures agree within 10%, indicating that this collision model works reasonably well over this pressure range. More careful comparison shows that there is a systematic decrease of $E_{\text{int}}$ at higher cell pressures. As for hMb, this can be qualitatively explained as follows: The ions travel more slowly in the higher pressure gas and they have longer residence times. Thus they require relatively lower internal energies to dissociate. Comparison of the internal energies for different charge states shows that the +10 ion requires the largest energy for dissociation and other three charge states +11, +12 and +13 require similar energies.
6.7 Collisionally activated dissociation of the complex ions with different gases

As a further test of the collision model, Ne, Ar, and Kr were used as collision gases in MS/MS experiments of cytochrome c-cytochrome $b_5$ complex ions. The dissociation curves of the cytochrome c-cytochrome $b_5$ complex +10 ions at 1.00 millitorr of Ne, or Ar, or Kr are shown in Figure 6-7. From these dissociation curves, dissociation voltages can be extracted. For the charge state +10, the dissociation voltages are 175 V for Ne, 87 V for Ar, and 41 V for Kr. The CM energies of collisions between a given ion and different collision gases are different. A collision with a heavier collision gas has larger CM energy. However, the internal energy required to induce dissociation of an ion is not dependent on the collision gas. Thus, to compare the relative stabilities of these complex ions, the dissociation voltages must be converted to internal energies.

The energies needed for the dissociation of the complex ions when Ne, Ar, and Kr were used as the collision gas can be calculated from equation 4-7. Here the total length of the collision cell, $l = 20.6$ cm, was used in all calculations. The drag coefficient for each case was considered as constant. Assuming $\phi = 1$ for all cases, the calculated internal energies required for the dissociation of the complex +10 ions in collisions with Ne, Ar, and Kr are 158 eV, 140 eV and 116 eV, respectively. Simply taking the average, the mean internal energy over the three collision gases is 138 eV with a relative standard deviation of ±15%. The ratio of the internal energies calculated from equation 4-7 is 1.13 : 1.00 : 0.83 for Ne, Ar, and Kr, respectively. It is clear that there is a systematic decrease with increasing mass of the collision gas. An ion moves more slowly in a heavier gas and it has longer time to react, so a smaller
internal energy is required to induce dissociation. For a given ion and for a given reaction time, the internal energy requirement for the dissociation of the ions should be constant. This collision model gives a ca. 30% spread in internal energy for collisionally activated dissociation experiments with different gases. This illustrates that, besides the above described systematic decrease, some assumptions such as (i) the dissociation length = the geometric length of the collision cell (ii) constant $C_D$ when the ions pass through the cell and (iii) the energy transfer efficiency $\phi = 1$ for all collision systems could be oversimplified. Further refinements of the collision model are required when it is used to describe collisions with different gases. Additional evidence is given in section 6.9.3.
6.8 Influence of background on dissociation voltage and relative internal energy

As shown in Figure 6-1c, mass spectrum of the $c$-$b_5$ complex contains a strong background, typically ca. 25% of the signal intensity of the complex ions. In the previous data analysis, the background was subtracted from the signal intensity of peaks. If the background is not deducted when calculating relative abundance of a precursor ion, then a higher "dissociation voltage" is obtained. The dissociation curves for the $c$-$b_5$ complex ion $+10$ at four different pressures, without the background subtraction, are shown in Figure 6-8. The dissociation voltages for the $+10$ $+13$ ions of the complex, without and with background subtraction, are compared in Table 6-2. For a given charge state at a given pressure, the dissociation voltage obtained from mass spectra without background subtraction is about 10%
higher than that obtained from spectra with background subtraction. The trend of dissociation voltage with cell pressure remains the same with and without background subtraction.

Table 6-2. Effect of background on dissociation voltage of the c-b5 complex ions

<table>
<thead>
<tr>
<th>P_Ar (mr) Tor</th>
<th>Without background subtraction</th>
<th>With background subtraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+10</td>
<td>+11</td>
</tr>
<tr>
<td>0.50</td>
<td>182</td>
<td>116</td>
</tr>
<tr>
<td>0.75</td>
<td>125</td>
<td>82</td>
</tr>
<tr>
<td>1.00</td>
<td>93</td>
<td>62</td>
</tr>
<tr>
<td>1.50</td>
<td>68</td>
<td>43</td>
</tr>
</tbody>
</table>

The relative internal energies required for the dissociation of the complex of different charge states, calculated by equation 4-7, are shown in Table 6-1 (with background subtraction) and in Table 6-3 (without background subtraction). Comparisons of the $E_{int}$ data in Table 6-3 and in Table 6-1 show that the background of the spectrum does make a significant contribution to the calculated internal energy (absolute value) but does not change the order of the relative energies of different charge states. Thus, for a comparison of relative energies of the ions from the same source, the influence of the background is less critical. However, to compare the relative energies of different complexes or ions formed from different sources, the subtraction of the background is necessary. For the CAD of the c-b5 complex with different collision gases, the effect of the background of the spectrum on the dissociation voltage and the internal energy are also similar (data not shown here). Except for
this section of the thesis, the background of mass spectra has been subtracted for all other data analyses.

Table 6-3. Relative internal energy ($E_{\text{int}}$) required for the dissociation of cytochrome c-cytochrome $b_5$ complex ions at different pressures of argon
(without background subtraction)

<table>
<thead>
<tr>
<th>$P_{\text{Ar}}$ (mTorr)</th>
<th>+10</th>
<th>+11</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>163.2</td>
<td>118.5</td>
<td>100.1</td>
<td>106.9</td>
</tr>
<tr>
<td>0.75</td>
<td>159.5</td>
<td>118.8</td>
<td>105.5</td>
<td>103.3</td>
</tr>
<tr>
<td>1.00</td>
<td>150.1</td>
<td>113.3</td>
<td>98.7</td>
<td>97.3</td>
</tr>
<tr>
<td>1.50</td>
<td>148.4</td>
<td>105.4</td>
<td>92.1</td>
<td>92.9</td>
</tr>
<tr>
<td>Average</td>
<td>155.3</td>
<td>114.0</td>
<td>99.1</td>
<td>100.1</td>
</tr>
</tbody>
</table>

Unit: eV

6.9 Effects of mutations of cytochrome $c$ on the stability of the complex ions

6.9.1 Mutations of iso-1-cytochrome $c$ and the corresponding complexes

All of protein samples used in this work were provided by the group of Professor A. G. Mauk in the Department of Biochemistry, UBC. The yeast iso-1-cytochrome $c$ was prepared as described in reference [2, 3]. All yeast cytochrome $c$ samples carried a Cys102Thr substitution. It has been shown that this substitution prevents intermolecular disulphide bond
formation and greatly decreases the rate of autoreduction without affecting the structural or functional properties of the protein [28]. Chemical modifications of surface residues on cytochrome c and the effect of these modifications on the reaction of cytochrome c derivatives with other proteins have been reviewed by Millet and Durham [29]. The surface lysine residues on the cytochrome c such as Lys73, Lys79, Lys86, Lys87 and trimethyl-Lys72 are of structural and functional significance. The lysine residues at these positions are located in the binding domain of cytochrome c. They play an important role in the electron transfer reaction with other proteins such as cytochrome c oxidase and cytochrome b₅ [29, 30]. The replacement of one or more of these lysine residues on the surface of cytochrome c by another amino acid can in principle reveal the exact binding sites or the contribution of individual lysines to the binding to cytochrome b₅. The trimethyl-Lys72Ala (J72A), Lys73Ala (K73A), Lys79Ala (K79A) and Lys87Ala (K87A) mutants of yeast iso-1-cytochrome c have been described elsewhere [1, 3, 31, 32]. These mutants were found to have similar acidity ($pK_a$: wild type, 8.70; Lys73Ala, 8.82; Lys79Ala, 8.44; Lys87Ala, 8.47) and similar reduction potentials and thermal stability [31]. Bovine liver cytochrome b₅, i.e. cytochrome b₅ used in this study was prepared by Professor Mauk's group as described in references [13, 33].

Complexes of cytochrome b₅ with the trimethyl-Lys72Ala, Lys73Ala, Lys79Ala, or Lys87Ala mutants of yeast iso-1-cytochrome c were produced in the gas phase with the same procedures used to produce the complex of cytochrome b₅ and wild type iso-1-cytochrome c (section 6.2.1). ESI-mass spectra of these mutant complexes at an orifice-skimmer voltage difference of 50 V are shown in Figure 6-9. Similar to the wild type protein complex, all of these mutant complexes have the same charge state distribution, +10 to +13. The deconvolution of these spectra gave molecular weights of 22,685 ± 2 for trimethyl-Lys72Ala-cyt b₅ complex and 22,728 ± 2 for Lys73Ala-cyt b₅, Lys79Ala-cyt b₅ and Lys87Ala-cyt b₅,
indicating that all of these cytochrome c mutants react with cytochrome \( b_5 \) to form the noncovalent complex with a stoichiometry of 1:1. The relative abundance of the complex ions is ca. 15\%. This is much lower than expected from the solution equilibrium.

Figure 6-9. ESI-mass spectra for noncovalent complexes of cytochrome \( b_5 \) and cytochrome \( c \) mutants (a) trimethyl-Lys72Ala, (b) Lys73Ala, (c) Lys79Ala, and (d) Lys87Ala. The orifice-skimmer voltage difference was 50 V. Ions of the mutant complexes are marked with the symbol "*" in the figures.
6.9.2 Collision cross sections of cytochrome c mutants and complexes

Collision cross sections for the trimethyl-Lys72Ala, Lys73Ala, Lys79Ala, and Lys87Ala mutants of yeast iso-1-cytochrome c and the corresponding cytochrome c-cytochrome b₅ complexes with Ne are shown in Figure 6-10a and 6-10b. For comparison, collision cross sections for wild type cytochrome c ions and wild type c-b₅ complex ions are also shown in the figures. The differences in cross sections between wild type cyt c and mutant cyt c for a given charge state are less than 6%, and less than 4% for the complexes. The precision of cross section measurements is typically ±3%. Thus, there are no significant effects of mutations on collision cross sections for all cytochrome c mutants and the corresponding noncovalent complexes within the precision of the data. This indicates that the replacement of trimethyl lysine residue at position 72 and lysine residues at positions 73, 79 and 87 by alanine residues does not have a large effect on the conformation of ions of cytochrome c and cytochrome c-cytochrome b₅ complexes. Similar to the complex formed by cytochrome b₅ with wild type cytochrome c, the complexes of these mutants have relatively compact conformations. Recently, Pollock et al. [3] studied the trimethyl-Lys72Ala mutation in yeast iso-1-cytochrome c and found that trimethylation of Lys72 has no effect on the structural stability of cytochrome c. The dimethyl derivatives of horse and yeast cytochrome c, i.e. N, N-dimethyl-lysine cytochrome c, were characterized for use as NMR probes of the complexes formed by cytochrome c with cytochrome b₅ and yeast cytochrome c oxidase by Moore et al. [12]. They concluded that the electrostatic properties and structures of the derivatized cytochromes are not significantly perturbed by the dimethylation of lysine residues; neither are the electrostatics of protein-protein complex formation or rates of inter-
Figure 6-10. Collision cross sections of cytochrome c mutants and the corresponding noncovalent cyt c-cyt b₅ complexes. The collision gas was Ne. The orifice-skimmer voltage difference was 50 V. (a) wild-type cytochrome c and several mutants; (b) the corresponding noncovalent cyt c-cyt b₅ complexes. The same legend is used for both (a) and (b): “■”, wild-type; “○”, trimethyl-Lys72Ala; “□”, Lys73Ala; “Δ”, Lys79Ala and “▴”, Lys87Ala.
protein transfer. The same charge state distribution for wild type and mutant complexes and the similar cross sections for wild type and different mutants in the present work show that the conformation of the cytochromes and the corresponding complexes in the gas phase are not greatly affected by those mutations.

6.9.3 Comparison of dissociation voltages and internal energies required for the dissociation of complexes of cytochrome c mutants

Dissociation voltages for all charge states of the complexes of various cytochrome c mutants with cytochrome b5 in collisions with Ne, Ar and Kr, measured by tandem mass spectrometry, are shown in Table 6-4. For a given charge state in collisions with a given target gas, all mutant complexes have similar dissociation voltages. This indicates that these complexes have similar binding energies. For a given charge state, the dissociation voltages change by a factor of 2 ~ 4.5 for different collision gases.

The collision model described in Chapter 4 was used to calculate the maximum possible energy transferred into internal energy of the ions from the dissociation voltages of Table 6-4. Calculated internal energies for all complex ions in collisions with Ne, Ar and Kr are listed in Table 6-5. For each type of ion, the ratio of the calculated internal energies for different collision gases is also shown in this table. The average ratio over all 24 cases is 1.21:1.00:0.81 for Ne, Ar, and Kr. Typically, a ca. 30 ~ 40% difference between Ne and Kr exists in each case. This collision model has corrected a 200 ~ 450% difference in dissociation voltage to ca. ±15 ~ ±20% spread in internal energy when different gases were used as collision gas. In each case, the $E_{\text{int}}$ values show a systematic decrease with increasing
mass of the collision gas. As discussed before, this is because an ion moves more slowly in a heavier gas and the ion has a longer reaction time in the collision cell and thus requires a lower internal energy for dissociation. Besides this systematic change, further refinement may also be needed for the application of this model to different collision gases.

Table 6-4. Dissociation voltages of the complexes of cytochrome c mutants with cytochrome b₅ in collisions with Ne, Ar, and Kr (P=1.0 millitorr)

<table>
<thead>
<tr>
<th>Cyt c-cyt b₅</th>
<th>Collision gas</th>
<th>+10</th>
<th>+11</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>J72A-cyt b₅</td>
<td>Ne</td>
<td>184</td>
<td>118</td>
<td>92</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>96</td>
<td>60</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>42.5</td>
<td>28</td>
<td>20</td>
<td>17.5</td>
</tr>
<tr>
<td>K73A-cyt b₅</td>
<td>Ne</td>
<td>188</td>
<td>126</td>
<td>96</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>92</td>
<td>59</td>
<td>44</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>42</td>
<td>28</td>
<td>22</td>
<td>17.5</td>
</tr>
<tr>
<td>K79A-cyt b₅</td>
<td>Ne</td>
<td>176</td>
<td>128</td>
<td>106</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>94</td>
<td>59</td>
<td>45</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>43.5</td>
<td>29</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>K87A-cyt b₅</td>
<td>Ne</td>
<td>178</td>
<td>126</td>
<td>104</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>90</td>
<td>60</td>
<td>45</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>46</td>
<td>30</td>
<td>23</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 6-5. Internal energies ($E_{\text{int}}$) of the complexes of cytochrome $b_5$ and cytochrome $c$ mutants in collisions with Ne, Ar, and Kr (calculated from equation 4-7)

\( (l = 20.6 \text{ cm} \quad \phi = 1) \)

<table>
<thead>
<tr>
<th>Cyt c-cyt $b_5$</th>
<th>Collision gas</th>
<th>+10</th>
<th>+11</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>J72A-cyt $b_5$</td>
<td>Ne</td>
<td>159.4</td>
<td>118.2</td>
<td>106.7</td>
<td>101.5</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>149.3</td>
<td>107.2</td>
<td>92.2</td>
<td>82.8</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>112.9</td>
<td>84.2</td>
<td>67.9</td>
<td>67.5</td>
</tr>
<tr>
<td>Ne:Ar:Kr</td>
<td>1.07:1.00:0.76</td>
<td>1.10:1.00:0.79</td>
<td>1.16:1.00:0.74</td>
<td>1.23:1.00:0.82</td>
<td></td>
</tr>
<tr>
<td>K73A-cyt $b_5$</td>
<td>Ne</td>
<td>166.2</td>
<td>127.8</td>
<td>111.6</td>
<td>118.2</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>145.7</td>
<td>106.5</td>
<td>90.3</td>
<td>85.4</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>113.1</td>
<td>84.9</td>
<td>74.9</td>
<td>67.5</td>
</tr>
<tr>
<td>Ne:Ar:Kr</td>
<td>1.14:1.00:0.78</td>
<td>1.20:1.00:0.80</td>
<td>1.24:1.00:0.83</td>
<td>1.38:1.00:0.79</td>
<td></td>
</tr>
<tr>
<td>K79A-cyt $b_5$</td>
<td>Ne</td>
<td>159.3</td>
<td>131.8</td>
<td>123.6</td>
<td>120.0</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>152.1</td>
<td>107.9</td>
<td>92.6</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>119.2</td>
<td>88.9</td>
<td>75.1</td>
<td>74.7</td>
</tr>
<tr>
<td>Ne:Ar:Kr</td>
<td>1.05:1.00:0.78</td>
<td>1.22:1.00:0.82</td>
<td>1.33:1.00:0.81</td>
<td>1.37:1.00:0.85</td>
<td></td>
</tr>
<tr>
<td>K87A-cyt $b_5$</td>
<td>Ne</td>
<td>160.8</td>
<td>129.9</td>
<td>122.0</td>
<td>117.2</td>
</tr>
<tr>
<td></td>
<td>Ar</td>
<td>145.3</td>
<td>109.9</td>
<td>93.1</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>Kr</td>
<td>125.9</td>
<td>92.2</td>
<td>78.9</td>
<td>74.9</td>
</tr>
<tr>
<td>Ne:Ar:Kr</td>
<td>1.11:1.00:0.87</td>
<td>1.18:1.00:0.84</td>
<td>1.31:1.00:0.85</td>
<td>1.34:1.00:0.86</td>
<td></td>
</tr>
</tbody>
</table>

Unit: eV
6.9.4 Relative stability of complexes of various mutants

As demonstrated in the last chapter and section 6.6, when the same collision gas is used, the internal energy calculated from the collision model can be used for the comparison of the relative stability of these noncovalent complexes. The results for all complexes in collisions with 1.00 millitorr of argon are shown in Table 6-6.

Table 6-6. Comparison of internal energy ($E_{\text{int}}$) required for the dissociation of cytochrome c mutant-cytochrome $b_5$ complexes

(Collision gas: 1.00 millitorr Ar; \ $l = 20.6$ cm)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>+10</th>
<th>+11</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type-Cyt $b_5$</td>
<td>150.1</td>
<td>113.3</td>
<td>98.7</td>
<td>97.3</td>
</tr>
<tr>
<td>Trimethyl Lys72Ala-Cyt $b_5$</td>
<td>157.1</td>
<td>119.7</td>
<td>96.3</td>
<td>91.1</td>
</tr>
<tr>
<td>Lys73Ala-Cyt $b_5$</td>
<td>152.8</td>
<td>117.3</td>
<td>96.5</td>
<td>92.5</td>
</tr>
<tr>
<td>Lys79Ala-Cyt $b_5$</td>
<td>160.2</td>
<td>118.9</td>
<td>98.8</td>
<td>94.7</td>
</tr>
<tr>
<td>Lys87Ala-Cyt $b_5$</td>
<td>155.0</td>
<td>113.5</td>
<td>97.3</td>
<td>89.8</td>
</tr>
</tbody>
</table>

The results show that, for all cytochrome c-cytochrome $b_5$ complexes, the charge state +10 is more stable than higher charge states, and the other three charge states show similar stability. However, for a given charge state of different mutant complexes, the energies required for the dissociation of complexes in the gas phase were found to be similar. This
demonstrates that the mutations do not cause significant perturbation to the formation, conformation and stability of the cytochrome c-cytochrome $b_5$ complex ions in the gas phase. This is similar to these mutant complexes in solution. These mutations were found to only slightly destabilize the cytochrome c-cytochrome $b_5$ complex in solution [18]. More recently, Döpner et al. [30] studied the complexes of these cytochrome c mutants with bovine cytochrome c oxidase in solution by Resonance Raman measurements and the results showed that the dissociation constant is increased by only a factor of 1.5 ~ 3 for binding of trimethyl-Lys72Ala, Lys73Ala, Lys79Ala, and Lys87Ala mutants to the cytochrome c oxidase. The present method used here, with a 10% uncertainty, can not recognize such a slight change.

6.10 Summary

In this chapter, noncovalent protein-protein complexes of cytochrome $b_5$ with wild-type iso-1-cytochrome c and a series of its mutants have been successfully detected by electrospray ionization-mass spectrometry. The protein pairs used in this study, i.e. cytochrome $b_5$ and cytochrome c have similar sizes and comparable molecular weights. Previous reports on protein-protein complexes have been limited to those noncovalent interactions in quaternary structures or in protein-inhibitor complexes. The successful detection of these complexes in the gas phase illustrates that ESI-MS is a potentially important tool for studying noncovalent interactions of biomolecules. The CAD experiments reveal the pathway of the fragmentation of these cyt c-cyt $b_5$ complex ions. In MS/MS experiments, the charge state of one fragment ion, cytochrome c, is increased with an increase
of the charge state of the precursor ions, while the other fragment ions, cytochrome $b_5$, maintain an unchanged charge state distribution. This suggests that protonation mostly occurs in the cytochrome $c$ part of the complex. Calculation of the energies needed to dissociate these complex ions shows that the +10 charge state of the complex ions is more stable than higher charge states +11-+13, and that the other three charge states have similar stabilities. That is, the stability of the complex ions is in order $+10 > +11 = +12 = +13$. The estimation of the internal energy required for dissociation of the complex ions at different cell pressures indicates that the collision model suggested in Chapter 4 can effectively correct a 250 - 300% difference of dissociation voltage over a pressure range of 0.50-1.50 millitorr, to a better than 10% spread in relative internal energy. Considering the systematic decrease of $E_{\text{int}}$ at higher pressures, this suggests that the model can be used to calculate relative energies to reasonable accuracy. However, comparisons of the results obtained with different collision gases suggest that further refinement of this collision model may be necessary. At least three factors in equation 4-7 can be further modified. These are the drag coefficient, the dissociation length, and the energy transfer efficiency. In addition, the complexes of these mutants and cyt $b_5$ were found to have similar binding strengths in the gas phase. These mutant complexes also have similar binding properties in solution [18]. To better examine whether the structure of gas phase protein complexes preserve their nature in solution, protein mutants with substantial differences in binding strength for the formation of noncovalent complexes in solution should provide the best case.
6.11 References


Chapter 7  Concluding Remarks and Future Work

7.1 Concluding remarks

This research mainly focused on fundamental aspects of the structure and energetics of gas phase proteins and noncovalent protein complexes studied with electrospray ionization-triple quadrupole mass spectrometry. The major conclusions are summarized here.

First, collision cross sections of myoglobin and cytochrome c ions with different target gases, Ne, Ar, and Kr, were measured by the energy loss method and the data were interpreted with the simple hard sphere model previously suggested by Covey and Douglas [1]. The results showed an unreasonable trend for both proteins i.e. cross sections with Ne > Ar > Kr. Also there was a clear difference between the cross sections obtained from energy loss experiments and from ion mobility experiments. This required a more reasonable model to describe energy loss experiments. An improved model - the drag coefficient model [2] was proposed to interpret energy loss experiments. Drag coefficients can effectively correct for the effects of thermal motion of the target gas, a range of scattering angles, different shapes of the objects and inelastic collisions. These factors were neglected in the simple hard sphere model. Systematic comparisons of different drag coefficient models suggest that diffuse scattering is the most suitable model for collisions between protein ions and neutral targets. The drag coefficient model also suggests the collision integrals obtained from the ion mobility should
be reduced by a factor of about 0.74 to give projection areas. The results obtained from the energy loss method and the ion mobility method agree within 3% when both are interpreted with the diffuse scattering drag coefficient model. The drag coefficient model also gives a reasonable cross section order with different gases, i.e. Ne < Ar < Kr. The results also show that, for a given charge state, collision cross sections with Ne, Ar, and Kr are very similar. No evidence is found for substantial contributions to the cross section from ion-induced dipole interactions. This work has successfully for the first time unified two important approaches to cross section measurements, energy loss and ion mobility [3].

Second, a simple collision model has been proposed to calculate the relative energy required to dissociate ions of noncovalent protein complexes in MS/MS experiments. This model has considered the effect of cross sections of the ions and energy loss as the ions move through the collision cell. Ions with larger cross sections experience more collisions and thus deposit more energy into internal energy than ions with smaller cross sections. This model can effectively reduce a large difference (~ 250%) in dissociation voltage for a given ion over a range of pressure from 0.50 to 1.5 millitorr Ar to a ca. 10% difference in internal energy. This collision model was applied to calculate the relative energies needed for the dissociation of noncovalent complexes [4-6].

Third, highly charged holomyoglobin ions, which were produced by coupling ESI-MS with a novel continuous-flow mixing apparatus, were studied through cross section measurements and tandem mass spectrometry [5]. Cross section measurements show that the highly charged hMb ions unfold significantly in comparison to low charge states. The relative internal energies required for the dissociation of the heme-protein complex, calculated from
the above collision model, show that the heme-binding in the highly charged ions is similar to those of low charge states. The heme-binding pocket appears to be preserved in the highly charged unfolded hMb ions.

Finally, noncovalent complexes of iso-1-cytochrome c and cytochrome b₅ were characterized by ESI-triple quadrupole mass spectrometry [6]. The noncovalent complex ions between cytochrome c and cytochrome b₅ with charge states from +10 to +13 were observed for the first time by ESI-MS. Cross section measurements, tandem mass spectrometry and corresponding internal energy calculations were used in this study. Tandem mass spectrometry revealed that the dominant fragmentation pathway of these protein-protein complex ions is \( \text{cyt c-cyt b₅}^{k+} \rightarrow \text{cyt c}^{(k-3)+} + \text{cyt b₅}^{3+} \) (\( k = 10, 11, 12, \) or 13). The relative binding energies of these complexes have been estimated using the above collision model. The results show that the +10 ions are more stable than higher charge states which have similar stability. The study of a series of cytochrome c mutants (trimethyl-Lys72Ala, Lys73Ala, Lys79Ala, and Lys87Ala) showed that noncovalent complexes of these mutants with cytochrome b₅ were found to require similar energies to dissociate the gas phase ions of these complexes. This illustrates that these mutations do not cause substantial perturbation for the formation, structure and stability of cytochrome c-cytochrome b₅ noncovalent complexes in the gas phase.
7.2 Future work

Based upon the present research, possible future work of interest in several areas is suggested.

A. Collision cross section measurements

Cross sections of proteins and other biomolecules provide insight into the conformation of these ions. Collision cross section measurements of protein ions are of increasing importance. The drag coefficient model has been successfully used to interpret the energy loss experiments. The drag coefficient has bridged the gap between the energy loss method and the ion mobility method. However, all data we used in comparisons were from different research groups and different setups. Experimental errors from different laboratories could limit a more thorough and precise comparison and further evaluation to the suggested model. If energy loss experiments and ion mobility experiments can be performed in the same apparatus, then a more precise comparison and a deeper understanding of these two methods will be possible since possible differences from solution conditions and sampling interface conditions will be eliminated. Thus, one future direction could be to design a collision cell/drift tube switchable mass spectrometer system to meet this requirement. The general idea is to modify the Q2 cell in the present quadrupole system by setting up a multiply segmented RF only quadrupole collision cell, which can be used for ion mobility experiments. The original Q2 cell can be used for energy loss measurements. In the modified Q2 cell, each segment will be supplied with the same RF voltage and a constant electric field along the axis will be maintained by applying a constant voltage difference between segments. Since the RF
quadrupole field provides effective radial confinement, this modified system will possess the advantage of reducing or even completely eliminating ion losses due to diffusion. It has been proven that this is feasible by Javahery and Thomson [7]. In addition, by comparing the experimental results obtained from this modified apparatus (mobility measurements) with the results obtained from the original apparatus (energy loss measurements), further discussion of the drag coefficient model will be possible. In the present research, comparisons of diffuse scattering, specular scattering, Henderson's, and Tomalchev's models suggest that diffuse scattering is most suitable to describe collisions between protein ions and neutrals. The diffuse scattering model applies to collisions with a rough surface. For most proteins in native states and relatively low charge states this is the case. However, highly charged protein ions may have well extended, approximately cylindrical, shapes. Further detailed consideration of $C_D$ for different shapes may further improve the present model and provide a more complete understanding of the behavior of proteins in the gas phase.

B. Improvements of the collision model used to calculate energies required for the dissociation of noncovalent protein complexes

In chapter 4, we proposed a collision model which can be used to calculate the energies needed to dissociate ions of noncovalent protein complexes. This model considered the effect of cross sections of the ions and is suitable for the case of multiple collisions. This model has improved our understanding of the collisional activation of protein ions in tandem mass spectrometry. In this model, as an approximation, we assumed $C_D$ as constant while ions pass through the collision cell. In addition, the geometric length of the collision cell was used as the dissociation length in all calculations and the same dissociation lengths were used for
different collision gas cases (see Chapter 6). The actual dissociation length is unknown. Future work on the improvements of the present model could include: (i) using variable $C_D$ values instead of a constant; (ii) using a more realistic dissociation length for each particular case. To determine a realistic dissociation length, Monte Carlo simulation of the collision process may be able to provide an average dissociation length for each case. These improvements will allow this model to give a better description of the internal energy distribution and the transfer of translational energy into internal energy in MS/MS experiments. The efficiencies of CM energies transferred to internal energies for different collision gas systems could be further evaluated. Some better application cases, such as the relative stability of heme-binding in gas phase in a series of myoglobin mutants which have significant differences in solution, will provide more persuasive evidence to evaluate the sensitivity and reliability of the collision model.

C. Noncovalent protein interactions

In this research, it was demonstrated that ESI-MS can be used to study noncovalent interactions in protein-ligand and protein-protein complexes. For those noncovalent complexes with a strong affinity ($K_A \sim 10^{12}$ or bigger), electrospray is gentle enough to produce the intact complex ions. However, for those noncovalent complexes with a relatively weak affinity ($K_A < 10^6$), it is difficult to detect the intact complex ion by ESI-MS. A more gentle and more sensitive ionization technique such as micro- or nano- electrospray [8] would favor the observation of these noncovalent complexes in gas phase. This will provide a more powerful tool for the study of biomolecules.
7.3 References