Abstract

Acid and base-induced unfolding of myoglobin in solution is monitored by electrospray ionization mass spectrometry. It is shown that acid-induced Mb unfolding causes the appearance of different charge state distributions in positive and negative ion modes compared to base-induced unfolding, suggesting different protein conformations in solution. Collision cross sections of both apomyoglobin and holomyoglobin ions are measured at various orifice-skimmer voltage differences (ΔVOS). The results show that at low ΔVOS, apomyoglobin ions have greater collision cross sections than holomyoglobin ions, indicating that heme, when attached to the globin, helps maintain a more compact myoglobin structure. Coulomb effects in binding of heme in gas phase holomyoglobin ions are studied. Positive and negative ions are formed from solutions of Fe$^{+2}$ (ferromyoglobin) and Fe$^{+3}$ (ferrimyoglobin). The energy that must be added to the resulting holomyoglobin ions to cause heme loss is measured with a triple quadrupole MS/MS system. With negative ions, neutral heme is lost, regardless of the charge state of Fe in solution. It is likely that the Fe$^{+3}$ is reduced to Fe$^{+2}$ in the negative electrospray process. With positive ions, predominantly neutral heme loss is observed for ions formed from ferromyoglobin in solution, and positive heme loss for ions formed from ferrimyoglobin in solution. The energies required to induce neutral heme loss are similar for positive and negative ions. The energies required to induce charged heme loss from positive holomyoglobin ions are significantly less. Coulomb repulsion between the charged heme and charged protein appears to lower the barrier for heme loss. These
results are consistent with a simple model of a long range Coulomb repulsion and a short range attraction between the heme and protein.
# Table of Contents

Abstract ...........................................................................................................ii  
Table of Contents ..............................................................................................iv  
List of Tables .....................................................................................................viii  
List of Figures .....................................................................................................x  
List of Symbols and Abbreviations ...................................................................xiii  
Acknowledgements ...........................................................................................xx  

**Chapter 1: Introduction** .............................................................................1  
  1.1 Electrospray Ionization Mass Spectrometry ...........................................2  
  1.2 Studies of Non-Covalent Interactions in Myoglobin ...............................5  
    1.2.1 Non-Covalent Interactions in Myoglobin .........................................6  
    1.2.2 Application of Various Analytical Techniques to Study Myoglobin ...7  
    1.2.3 Application of ESI-MS to Study Myoglobin ......................................9  
  1.3 Studies of Myoglobin Conformations by Mass Spectrometry ...............11  
    1.3.1 Myoglobin Conformations in Solution ............................................11  
    1.3.2 Myoglobin Conformations in the Gas Phase ....................................14  
  1.4 Tandem Mass Spectrometry of Myoglobin ..........................................18  
  1.5 Coulomb Energy and The Dissociation of Heme From Myoglobin ........19  
  1.6 Goals of This Work ..................................................................................22
1.7 Outline of This Work ................................................................. 25

Chapter 2: Experimental Methods .................................................. 27

2.1 Electrospray Ionization Mass Spectrometer ................................ 27
2.2 Electrospray Ionization ............................................................. 29
2.3 Interface Region ..................................................................... 29
2.4 Quadrupole Potentials .............................................................. 30
2.5 Ion Motion in a Quadrupole ...................................................... 32
2.6 Instrument Operation ............................................................... 34
2.7 Ion Detection ......................................................................... 35
2.8 Myoglobin Solutions and Reagents ......................................... 37

Chapter 3: Myoglobin Spectra ......................................................... 38

3.1 Negative and Positive Ion Mass Spectra .................................. 38
3.2 Myoglobin Conformations in Solution Monitored in Negative Ion Mode ................................. 42
3.3 Summary ............................................................................... 47

Chapter 4: Collision Cross Sections of Holomyoglobin Ions ............ 49

4.1 Drag Model ............................................................................ 49
4.2 Energy Loss Measurements .................................................... 53
List of Tables

Table 2.1 Pressures in the different regions of the mass spectrometer

Table 2.2 Voltages (V) used in positive ion mode MS/MS and collision cross section measurements. For negative ions, the polarity of these voltages was reversed

Table 4.1a) Collision cross sections (Å²) of negative hMb ions produced from solutions of ferri and ferromyoglobin

Table 4.1b) Collision cross sections (Å²) of positive hMb ions produced from solutions of ferri and ferromyoglobin

Table 5.1 Collision cross sections (Å²) of aMb ions formed in solution at AVOS of 30, 110 and 180 V

Table 5.2 Collision cross sections (Å²) of hMb ions at AVOS of 30 V and 110 V, and aMb ions formed in the gas phase by dissociating hMb ions, at AVOS of 110 and 180 V

Table 6.1 Dissociation voltages, pressures, collision cross sections and ΔE_int values for different charge states of holomyoglobin: a) negative ions from ferriMb solution, b) negative ions from ferroMb solution, c) positive ions from ferriMb solution, and d) positive ions from ferroMb solution

Table 6.2 ΔE_int values for a constant reaction time of 18 μs for a) negative
ions from ferriMb solution, b) negative ions from ferroMb solution, c) positive ions from ferriMb solution, and d) positive ions from ferroMb solution.
## List of Figures

| Figure 1.1 | Schematic of ESI and a mass spectrometer interface region | 3 |
| Figure 1.2 | Structure of heme [39] | 6 |
| Figure 2.1 | Diagram of the ESI-MS system. ESI is the Electrospray ionization source, Q0 is the RF only quadrupole, SRO are short rods, Q0/Q1, Q1/Q2, and Q2/Q3 are ion lenses, Q1 is the first mass analyzer, Q2 is an RF only quadrupole, Q3 is the second mass analyzer, EXIT is an aperture plate and CEM is the detector | 27 |
| Figure 2.2 | Electrospray source used in Mb experiments | 29 |
| Figure 2.3 | Schematic of the quadrupole rods. The distance from the center to the rods is $r_0$ | 30 |
| Figure 2.4 | First stability region of ion motion in a quadrupole field based on the Mathieu equation. Different masses ($m_1$, $m_2$, and $m_3$) lie on the operating lines labeled A and B | 33 |
| Figure 3.1 | Negative ion ESI-MS spectra of a) $1 \times 10^{-5}$ M ferrimyoglobin, and b) $5 \times 10^{-6}$ M ferromyoglobin, in a solution of 50% methanol, at pH 7.1. The orifice-skimmer voltage difference was 170 V. Notation: -5h is -5 holomyoglobin (hMb) and -5a is -5 apomyoglobin (aMb) | 39 |
| Figure 3.2 | Positive ion mode mass spectra of a) $1 \times 10^{-5}$ M ferriMb, and b) $5 \times 10^{-6}$ M ferroMb, in a solution of 50% MeOH, at pH 7.1. The $\Delta$VOS was 170 V | 41 |
Figure 3.3  Negative ion mass spectra of ferrimyoglobin ions formed from a solution containing 50% methanol, at pH: a) 3.2, and b) 10.1. ΔVOS was 170V.

Figure 4.1  The stopping curves of +6 ferriMb ions at different cell pressures of Ar. The stopping curves from right to left correspond to added argon pressures of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mtorr.

Figure 4.2  A plot of $-\ln \frac{E}{E_0}$ versus $\frac{C_p n m^2}{m_i}$ for the +6 ferriMb ion.

Figure 5.1  Mass spectra of apomyoglobin ions, formed from a solution containing 10% MeOH. The concentration of aMb was $1.2 \times 10^{-5}$ M. The ΔVOS was a) 30 V, and b) 110 V.

Figure 5.2  Mass spectra of apomyoglobin ions, formed from a solution containing 10% MeOH. The concentration of aMb was $1.2 \times 10^{-5}$ M. The ΔVOS was 180 V.

Figure 5.3  Collision cross sections versus charge state of positive apomyoglobin ions formed in solution at ΔVOS (●) 30 V, (○) 110 V, and (▼) 180 V.

Figure 5.4  Collision cross sections vs. charge state for hMb and aMb ions at ΔVOS of 30 V.

Figure 6.1  Tandem mass spectra of -7 holomyoglobin ions from a solution of ferrimyoglobin at a collision gas pressure of 1.7 mtorr. The collision energy $E$ is a) 840 eV, b) 1085 eV, c) 1190 eV, and d) 1260 eV.
Figure 6.2  Tandem mass spectra of +7 holomyoglobin ions formed from a solution of ferrimyoglobin, at a collision gas pressure of 1.7 mtorr. The collision energy $E$ is a) 490 eV, b) 630 eV, c) 770 eV, and d) 875 eV.

Figure 6.3  Precursor and fragment ion intensities versus Q0-Q2 voltage difference for the -7 holomyoglobin ions formed from a solution of ferrimyoglobin.

Figure 6.4  Dissociation voltage vs. pressure for negative ferroMb ions, for charges -4h to -7h.

Figure 6.5  Calculated added internal energies, $\Delta E_{\text{int}}$, versus reaction time for negative holomyoglobin ions formed from ferromyoglobin solution; -4 (●); -5 (○); -6 (▲); -7 (▼).

Figure 6.6  Model potentials for heme binding in neutral and +7 gas phase myoglobin, using Coulomb and Morse potentials.
List of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Symbol or Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Peaks of apomyoglobin</td>
</tr>
<tr>
<td>a</td>
<td>Sum of radii of the collision partners</td>
</tr>
<tr>
<td>aMb</td>
<td>ApoMyoglobin</td>
</tr>
<tr>
<td>$a_x, a_y, a_z$</td>
<td>Mathieu parameter</td>
</tr>
<tr>
<td>A</td>
<td>Projection area</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionization</td>
</tr>
<tr>
<td>b</td>
<td>Distance from the cell entrance</td>
</tr>
<tr>
<td>BIRD</td>
<td>Blackbody Infrared Radiation Dissociation</td>
</tr>
<tr>
<td>c</td>
<td>Concentration</td>
</tr>
<tr>
<td>$C_D$</td>
<td>Drag coefficient</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<td>CEM</td>
<td>Channel Electron Multiplier</td>
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<tr>
<td>CI</td>
<td>Chemical Ionization</td>
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<tr>
<td>CID</td>
<td>Collisionally Induced Dissociation</td>
</tr>
<tr>
<td>CM</td>
<td>Center of mass</td>
</tr>
<tr>
<td>CRM</td>
<td>Charged Residue Model</td>
</tr>
<tr>
<td>d</td>
<td>Diameter</td>
</tr>
<tr>
<td>D</td>
<td>Cross-sectional diameter</td>
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</table>
\[ D_c \] Well depth (binding energy)

DC Direct Current

DMAPA 3-(dimethylamino) propylamine

e Elemental charge

\[ E \] Kinetic energy of an ion at the collision cell exit

\[ E^0 \] Kinetic energy of an ion at the collision cell entrance

\[ E_{1/10} \] Stopping energy at one tenth of initial intensity

\[ E_{el} \] Electric field

\[ E_{int} \] Internal energies

EI Electron Ionization

ESI Electrospray Ionization

EXIT Aperture plate

ferriMb Ferrimyoglobin

ferroMb Ferromyoglobin

\[ F_d \] Drag force

FAB Fast Atom Bombardment

FT Fourier Transform

GdmCl Guandinium chloride

Gly Glycine

\[ h \] Peaks of holomyoglobin

hMb Holomyoglobin

H/D Hydrogen/Deuterium
<table>
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</tr>
</thead>
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<td>His</td>
<td>Histidin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performan Liquid Chromatography</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>$I_{aMh}$</td>
<td>Relative intensity of the fragment ion</td>
</tr>
<tr>
<td>$I_{hMh}$</td>
<td>Relative intensity of the precursor ion</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>ICR</td>
<td>Ion Cyclotron Resonance</td>
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<tr>
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<td>Ion Evaporation Model</td>
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<td>IMS</td>
<td>Ion Mobility Spectrometry</td>
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<td>$k_B$</td>
<td>Boltzmann’s constant</td>
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<td>K</td>
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</tr>
<tr>
<td>$K_0$</td>
<td>Reduced ion mobility</td>
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<tr>
<td>$K(c)$</td>
<td>Equilibrium constant</td>
</tr>
<tr>
<td>$K_n$</td>
<td>Knudsen number</td>
</tr>
<tr>
<td>l</td>
<td>Length traveled in the collision cell</td>
</tr>
<tr>
<td>$l_d$</td>
<td>Length of a drift tube</td>
</tr>
<tr>
<td>$l_r$</td>
<td>Length of the collision cell over which reaction occurs</td>
</tr>
<tr>
<td>Leu</td>
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</tr>
<tr>
<td>Lys</td>
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<td>m</td>
<td>Constant</td>
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<td>$m_1, m_2, m_3$</td>
<td>Masses of ions</td>
</tr>
<tr>
<td>$m_i$</td>
<td>Mass of an object</td>
</tr>
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<td>Symbol</td>
<td>Description</td>
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<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$m_2$</td>
<td>Mass of the collision gas</td>
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<tr>
<td>$m_i$</td>
<td>Mass of an ion</td>
</tr>
<tr>
<td>$m_b$</td>
<td>Mass of a buffer gas</td>
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<tr>
<td>$m/z$</td>
<td>Mass to charge ratio</td>
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<tr>
<td>$M$</td>
<td>$M = m_1 + m_2$</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionization</td>
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<tr>
<td>Mb</td>
<td>Myoglobin</td>
</tr>
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<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>MTBD</td>
<td>1,3,4,6,7,8-hexahydro-1-methyl-2H-pyrimido-[1,2-a] pyridine</td>
</tr>
<tr>
<td>$n$</td>
<td>Number gas density</td>
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<tr>
<td>$N$</td>
<td>Number of Collisions</td>
</tr>
<tr>
<td>$P$</td>
<td>Pressure</td>
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<tr>
<td>$q_1, q_2$</td>
<td>Electrostatic charges</td>
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<td>$q_u, q_x, q_y$</td>
<td>Mathieu parameter</td>
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<td>$Q$</td>
<td>Quadrupole</td>
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<td>$Q0$</td>
<td>Radio frequency only quadrupole</td>
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<td>$Q1$</td>
<td>First mass analyzer</td>
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<tr>
<td>$Q2$</td>
<td>Radio frequency only quadrupole/ collision cell</td>
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<td>$Q3$</td>
<td>Second mass analyzer</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>Q0/Q1, Q1/Q2, Q2/Q3</td>
<td>Ion lenses</td>
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<tr>
<td>r</td>
<td>Distance between heme and protein</td>
</tr>
<tr>
<td>r₀</td>
<td>Distance from the center of a quadrupole to the rods</td>
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<tr>
<td>rₑ</td>
<td>Distance where the Morse potential has a minimum</td>
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<td>r₉</td>
<td>Distance between charges</td>
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<td>Rₑ</td>
<td>Reynolds number</td>
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<tr>
<td>Rₑₜₜₑₜₑ</td>
<td>Reaction time</td>
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<td>RF</td>
<td>Radio frequency</td>
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<td>s</td>
<td>Speed ratio</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SIMS</td>
<td>Secondary Ion Mass Spectrometry</td>
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<tr>
<td>SR0</td>
<td>Short rods</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>tₑₜₑₜₑ</td>
<td>Drift time</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
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<td>TI</td>
<td>Thermal Ionization</td>
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<td>TOF</td>
<td>Time-of-Flight</td>
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<td>x or y</td>
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$v_0$</td>
<td>Velocity of an object relative to the gas</td>
</tr>
<tr>
<td>$v_e$</td>
<td>Speed of ions entering the collision cell</td>
</tr>
<tr>
<td>$v_l$</td>
<td>Speed of ions leaving the collision cell</td>
</tr>
<tr>
<td>$V$</td>
<td>RF voltage</td>
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<td>$V_d$</td>
<td>Drift velocity</td>
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<td>$V(r)$</td>
<td>Potential</td>
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<td>$z$</td>
<td>Number of charges on the ion</td>
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<tr>
<td>$\beta$</td>
<td>Constant</td>
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<tr>
<td>$\Delta b$</td>
<td>Change in distance</td>
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<tr>
<td>$\Delta E_a$</td>
<td>Difference in activation energy</td>
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<tr>
<td>$\Delta E_{int}$</td>
<td>Added internal energy</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>Change in free energy of binding</td>
</tr>
<tr>
<td>$\Delta V_{OS}$</td>
<td>Orifice-skimmer voltage difference</td>
</tr>
<tr>
<td>$\varepsilon_0$</td>
<td>Permittivity of vacuum</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Average fraction of centre of mass kinetic energy transferred to internal energy in a single collision</td>
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<tr>
<td>$\Phi_0$</td>
<td>Potential applied between the rods</td>
</tr>
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<td>$\Phi(x, y)$</td>
<td>Potential at point $(x, y)$</td>
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<tr>
<td>$\eta$</td>
<td>Gas viscosity</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Mean free path</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Reduced mass</td>
</tr>
</tbody>
</table>
\( \rho \)  
Gas density

\( \sigma \)  
Collision cross section

\( \omega \)  
Angular frequency

\( \Omega \)  
Cross section integral for ion mobility calculations

\( \xi \)  
\[ \xi = \frac{\omega t}{2} \]
Acknowledgements

I would like to thank my supervisor Don Douglas, whose teachings about work and science I could never have received from any textbook. Many thanks to my labmates Annie, Xian Zhen, Professor Chuanfan, Peter John, Ori, Winnie, Jim Zhang, Anthony, Aaron, Dunmin, Samir and Wei. I have enjoyed our many “scientific” discussions these past five years.

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I would like to thank my parents for believing in me no matter what decisions I have made throughout my life. This accomplishment is as much yours as it is mine. To my kid sister, for taking all the butt end of my jokes (a right an older brother has) throughout the years.

And to my brothers Allan, Isaac and Geoff- guys who I would go to battle with anytime.
Chapter 1

Introduction

Mass spectrometry (MS) is a method of identifying the chemical constitution of substances by means of the separation of gaseous ions, according to their difference in mass to charge (m/z) ratios. The first apparatus measuring m/z ratios was constructed by Thomson in the early twentieth century. It was stated by Thomson: “I feel sure that there are many problems in chemistry which could be solved with far greater ease by this than by any other method. The method is surprisingly sensitive, requires an infinitesimal amount of material and does not require this to be specially purified” [1]. Over the course of the last century, with the development of new technologies, all components of mass spectrometers have undergone major advances. Their performance has improved to such an extent that many different types of samples can now be analyzed.

The main components of a mass spectrometer are: sample inlet, ionization source, mass analyzer, and ion detector. Ionization methods available to a mass spectrometrist today are numerous: chemical ionization (CI) [2, 3], atmospheric pressure chemical ionization (APCI) [4], thermal ionization (Ti) [5], electron ionization (EI) [6], fast atom bombardment (FAB) [7, 8], secondary ion mass spectrometry (SIMS) [9], electrospray ionization (ESI) [10, 11], matrix assisted laser desorption ionization (MALDI) [12, 13],
and inductively coupled plasma (ICP) [14, 15]. There are many different types of mass analyzers available: magnetic sector [16], quadrupole [17], time of flight (TOF) [18-22], ion cyclotron resonance (ICR) [23], and ion trap [24, 25]. The choice of the ionization source and mass analyzer depends on the nature of the sample and the type of analysis.

In this thesis, the use of an ESI-triple quadrupole mass spectrometer to study conformation, binding, and the influence of Coulomb effects on binding of the heme prosthetic group to the globin in gas phase myoglobin ions is described.

Chapter 1 of this thesis reviews the principles of ESI, and its application to study non-covalent complexes of biomolecules, particularly myoglobin (Mb), in solution and in the gas phase.

1.1 Electrospray Ionization Mass Spectrometry

“Soft” ionization techniques are techniques that do not cause extensive fragmentation of ions. The most commonly used soft ionization techniques in modern mass spectrometers are ESI [10, 11, 26] and MALDI [12, 27]. The application of these two ionization techniques to study biological macromolecules led to the award of one half of the Nobel Prize in chemistry 2002 to Koichi Tanaka for MALDI and to John Fenn for ESI. The second half of the prize was awarded to Kurt Wüthrich for his work in determining three-dimensional biological macromolecular structures in solution by nuclear magnetic resonance (NMR).
The first experiments to employ ESI, as a source for the formation of gas phase ions, were carried out by Dole and coworkers [10, 11] in 1968. A dilute solution of polystyrene molecules was sprayed into an evaporation chamber that contained a flowing nitrogen bath gas at atmospheric pressure. A molecular beam containing ions was then passed through a nozzle-skimmer region to a Faraday cage for ion detection. Two decades later, Fenn and coworkers observed the mass spectra of multiply charged protein ions [26] with an ESI-quadrupole mass spectrometer.

Figure 1.1 Schematic of ESI and a mass spectrometer interface region.
The ESI process involves three steps: the formation of charged droplets, the shrinkage of droplets, and the formation of gas phase ions at atmospheric pressure. This process is shown schematically in Figure 1.1. A solution is passed through a metal capillary (typical flow rates on the order of μL/min [28]), where the solution is electrically charged to a high voltage (±2-5 kV). Ions accumulate at the surface of the liquid at the metal capillary tip. At the tip, the charge build-up forms an elongated meniscus (Taylor cone) that splits into individual highly charged droplets. As the charged droplets are drawn to a counter electrode of the interface region by the potential difference between the electrospray and counter electrode, solvent molecules evaporate and the droplets become smaller. The charge density on the droplets increases, and eventually the charged droplets reach a critical state (Rayleigh limit), where they become unstable. After a series of Coulombic explosions, ions are formed. Ions then pass through the counter electrode aperture followed by the orifice-skimmer region of the mass spectrometer.

Two models for the formation of gas phase ions from solution have been proposed. The first, the ion evaporation model (IEM), was introduced by Thomson and Iribarne [29, 30]. In this model, consecutive Coulomb fission and solvent evaporation reduce the size of the charged droplets (radius ~ 10 nm) to the point where the electric field on the surface of the droplet is sufficiently high for an ion to leave the droplet. The second model, proposed by Dole and coworkers, and later improved by Rollgen and coworkers [31], is a charged residue model (CRM) [10]. According to this model, when the concentration of the analyte is low, repeated solvent evaporation and Coulomb fission produce a droplet so small that it contains a single ion.
Many ESI improvements and variations have been made, including pneumatically assisted nebulization [32], and the addition of heat [33]. More recently, the development of nano-ESI [34, 35] as an ion source, by Wilm and Mann, has allowed for sample sizes injected into the source to be significantly reduced. In principle, the nanospray process is similar to electrospray with less sample being injected (1-10 μL) into the capillary at a lower flow rate (typically ~20 nL/min), which conserves the sample. Also, the voltage applied to the sprayer tip is lower (< 2000 V) than in ESI.

ESI has been adopted as a useful method to investigate gas phase binding of non-covalent complexes of biomolecules. In addition, it has been used to study the conformations of non-covalent protein complexes in solution and the gas phase. The production of high charge state ions allows for proteins of high molecular weight to be observed with mass spectrometers that have moderate mass ranges (typically ≤ m/z 4000).

1.2 Studies of Non-Covalent Interactions in Myoglobin

Non-covalent interactions include hydrogen bonding, electrostatic, hydrophobic and van der Waals interactions. Many complexes of biomolecules are held together by non-covalent interactions: protein-protein, protein-peptide, protein-nucleic acid, protein-saccharide, protein small-molecule, and protein-metal ion. They play an important role in many cellular functions. It is of great importance to study these complexes in order to gain better insight into the mechanisms of their action.
1.2.1. Non-Covalent Interactions in Myoglobin

Among the numerous protein small-molecule complexes, heme-protein complexes are the most extensively studied [36]. Heme consists of a porphyrin molecule with a central iron atom, which is attached to the porphyrin by four bonds to the central nitrogen atoms. Iron binds to the protein through the nitrogen atom of a histidine residue that is situated below the plane containing the porphyrin. The sixth coordination site of iron is used for ligand binding [37]. The structure of heme is shown in Figure 1.2.

Figure 1.2 Structure of heme [38].
Heme-protein complexes are involved in many biological processes, such as inhalation and detoxification in mammals. One of the most frequently studied heme-protein complexes is myoglobin. In myoglobin, heme is non-covalently attached to the globin through:

i) hydrophobic interactions between the π electrons from the tetrapyrrole ring and hydrophobic amino acid side chains of the globin,

ii) coordination of the nitrogen from a histidine side chain with the iron heme,

iii) hydrogen bonding between heme propionates and charged amino acid side chains on the globin surface [37, 39, 40].

When the heme is attached to the globin, the protein is referred to as holomyoglobin (hMb), and when the globin has no heme attached, the protein is called apomyoglobin (aMb). The polypeptide chain, which consists of 153 amino acid residues, folds into a single domain of eight α-helices [37]. Myoglobin is found in the muscle of most mammals and its primary role is to reversibly bind molecular oxygen. The ability to bind and release oxygen is facilitated by the iron atom in the heme.

1.2.2 Application of Various Analytical Techniques to Study Myoglobin

Due to its importance, myoglobin has been studied extensively by various analytical techniques. In 1958, Kendrew et al. [41] determined the structure of myoglobin, including the location of the heme iron by x-ray crystallography. The dimensions of native myoglobin have been reported to be 45 x 35 x 25 Å [42]. Later, x-ray
crystallography was used to determine the three-dimensional structures of the met (oxidized form of heme) \[43\], deoxy (no oxygen bound to heme) \[44\], and oxy (oxygen bound to heme) \[45\] forms of myoglobin. UV/VIS spectroscopy can be used to detect the oxidation state of heme iron \[46\]. Ferrous (Fe\(^{+2}\)) and ferric (Fe\(^{+3}\)) myoglobin show two distinct absorption spectra: ferriMb (myoglobin containing Fe\(^{+3}\)) has a maximum absorbance at 555 nm, while ferroMb (myoglobin containing Fe\(^{+2}\)) has maxima at 545 and 580 nm.

Conformations of myoglobin have been investigated by various techniques such as fluorescence, circular dichroism (CD), NMR and calorimetry. Denaturation of myoglobin can be monitored by tryptophan fluorescence \[47\]. Under denaturing conditions, a shift in a fluorescence emission peak to longer wavelengths and an increase in fluorescence intensity occur. Different types of secondary structures (helices, sheets, turns and coils) give rise to different CD spectra. Therefore, a CD spectrum of myoglobin which consists solely of alpha helices is specific, and any changes in conformation can be easily detected by observing the changes in a CD spectrum \[48, 49\]. NMR has been used to determine the hydrogen/deuterium (H/D) exchange levels in oxymyoglobin and deoxymyoglobin by comparing NMR spectra obtained in water and deuterium-oxide \[50\]. This can give direct insight into differences in conformation between these two forms of myoglobin, since more open protein conformations exchange more hydrogens than more folded conformations. In addition, NMR has been used to monitor changes in myoglobin conformation upon oxygenation \[51\]. Calorimetry has been used to examine the dynamics of enthalpy and volume changes upon
photodissociation of carbon monoxide from carboxymyoglobin [52]. Unfolding of apomyoglobin and the influence of the hydrophobic interactions on the unfolding enthalpy were studied by isothermal titration calorimetry [53].

These and many other studies of myoglobin were performed with solution myoglobin, where the solvent can have a significant influence on binding and conformation. It is of interest to investigate the behavior of myoglobin in the gas phase, where a solvent-free complex can reveal the nature of the interactions between components, without solvent interferences.

1.2.3 Application of ESI-MS to Study Myoglobin

The first detection and identification of non-covalent complexes by ESI-MS was reported by Ganem et al. in 1991 [54, 55]. Since then, among the many protein small-molecule complexes that have been studied by ESI-MS, myoglobin has been the most common [39, 40, 56-63]. Katta et al. were the first to identify holomyoglobin ions and to observe different charge state distributions of ions with a change in solution pH [61]. Since then, ESI-MS has been used to study myoglobin behavior in both solution and the gas phase.

In solution, conformations of different forms of myoglobin in different environments have been extensively studied by ESI-MS. Monitoring of the charge state distributions of Mb ions in mass spectra has been used in folding/unfolding studies [64-66]. It is well known that folded proteins have fewer sites available for protonation in positive MS ion
mode than unfolded proteins. This results in a more compact conformations and narrower charge state distributions of folded proteins, with lower charge states. In addition, H/D exchange has been used to study conformations of Mb in solution [65, 67]. Native proteins exchange fewer hydrogens than denatured proteins, since many hydrogens are buried within the tightly folded structures. These measurements showed that in solution, aMb is more unfolded than hMb [67, 68].

ESI-MS has been used to study myoglobin in the gas phase. Conformations of gas phase myoglobin ions were studied by collision cross section measurements [59, 69-71] and gas phase H/D exchange [62]. Collision cross sections give an estimate of the ion size and therefore can be used to study the change in conformations upon binding heme. H/D exchange has been done with trapped gas phase myoglobin ions. Vapor of deuterium oxide (D$_2$O) or methanol-d$_4$ (CD$_3$OD) was introduced directly into the trap. Collisions with myoglobin ions gave H/D exchange. Gas phase conformations were determined since, as in solution, folded proteins have fewer hydrogens available for exchange than unfolded proteins [62]. The application of mass spectrometry to study the conformation of myoglobin ions, in both the gas phase and solution, is discussed in greater detail in section 1.3.

In addition, ESI-MS has been applied to study binding of heme in gas phase myoglobin. Tandem mass spectrometry was used to measure the relative internal energies transferred to hMb ions to induce dissociation [72]. Blackbody infrared radiative dissociation (BIRD) was used to measure activation energies and Arrhenius parameters for the
removal of heme from gas phase myoglobin ions [60]. Another type of dissociation experiment performed on myoglobin gas phase ions, involved the dissociation of hMb in the orifice-skimmer region of a mass spectrometer, as shown in Figure 1.1 [39, 73]. The potential difference between the orifice and the skimmer was adjusted to cause the dissociation of holomyoglobin. In the orifice-skimmer region, energetic collisions transfer translational energy into internal energy. This additional internal energy causes dissociation of gas phase non-covalent complexes such as holomyoglobin.

In addition to studies with positive ions, MS studies with negative myoglobin ions have been reported. Time-resolved MS was used to study conformational changes of negative Mb ions under denaturing conditions [74], while tandem mass spectrometry was used to examine the gas phase stability of negative ions of the heme-globin complex compared to positive ions [75].

1.3 Studies of Myoglobin Conformations by Mass Spectrometry

1.3.1 Myoglobin Conformations in Solution

The transition from a folded protein to an unfolded protein in solution can be observed in ESI mass spectra [61, 76-79]. In positive ion MS, where a more folded conformation has fewer basic amino acids accessible for protonation, less charging of ions and higher m/z ratios of ions are seen in the protein mass spectrum [76]. Denaturing conditions cause unfolding of proteins, an increase in surface area, and exposure of buried basic amino
acids making them more accessible for protonation [77, 80]. In negative ion MS, more compact protein conformations have fewer acidic amino acids accessible for deprotonation, which results in less charging of ions. This gives a lower charge state distribution at higher m/z ratios in the mass spectrum. In some cases, denaturing conditions cause protein unfolding in solution, which results in an increase in surface area. This causes more deprotonation sites to be exposed, yielding more charging and a high charge state distribution at lower m/z, similar to positive ion MS [81-83]. This is not the case for all proteins. With some proteins, the ion charge state distribution in negative ion mode was not affected by protein unfolding [76].

ESI-MS can be used to monitor changes of conformation of myoglobin in solution. Several methods to induce denaturation of myoglobin have been implemented in combination with MS studies, such as altering the solvent conditions by adding acids, bases and organic solvents [84, 85]. Increasing the temperature of a solution has also been used to cause conformational changes [86].

A detailed solution conformational study of myoglobin unfolding induced by methanol [65] was reported. At MeOH concentrations ranging from 0-30%, the mass spectrum comprised of mostly lower charge states of hMb, from +9 hMb to +13 hMb. An increase in the methanol concentration to 40% produced a bimodal distribution of low charge states, from +9 to +13, and high charge states, from +14 to +23, of hMb, along with high charge states (+12 to +26) of aMb. This indicated that two different myoglobin conformations, folded hMb and unfolded aMb, existed in equilibrium in solution. The
methanol content was further raised to 70%, and the major peaks in the mass spectrum were those of aMb ions in high charge states.

Protein folding and unfolding can also be studied by systematic changes in solution pH. So far, myoglobin conformational changes due to pH changes have been studied with positive ions. Time-resolved ESI-MS [87], where two syringes are simultaneously pumped and the solutions are mixed at a tee prior to being electrosprayed, was used to monitor the unfolding of myoglobin [64]. Recording of mass spectra from 0.7 to 15.1 seconds after mixing showed the following unfolding mechanism of solution Mb:

\[
\text{native hMb} \rightarrow \text{unfolded hMb} \rightarrow \text{heme} + \text{unfolded aMb}
\]

This was confirmed by investigating the change in charge state distributions of hMb and aMb ions with the change in pH [66]. The gradual shift in charge states, with a change in pH from 8.5 to 2.5, indicated a non-cooperative myoglobin unfolding process which included multiple protein conformations.

Conformations of myoglobin ions in solution have been studied by H/D exchange. Rates of exchange of backbone amide hydrogens were measured for both aMb and hMb ions [68]. It was found that the percentage of deuteration for aMb was four times higher than the percentage of deuteration for hMb, indicating a more compact conformation of hMb. Neutral and acidic pH values were used to investigate aMb conformations, by monitoring different charge state distributions and online H/D exchange [67].
In the work presented in this thesis, conformational changes of aMb and hMb in solution are investigated with negative ion MS, through observations of differences in charge state distributions of ions produced from solutions at different pH values.

1.3.2 Myoglobin Conformations in the Gas Phase

Determination of the size of protein ions in the gas phase may lead to better understanding of gas phase protein ion conformations. Initial experiments to evaluate the size of chemical compounds were performed by Mack [88]. Average cross section areas were determined for iodine, benzene, naphthalene, anthracene, toluene, aniline and benzidine by measuring their rates of evaporation.

Measurement of protein cross sections is one mass spectrometric method to probe gas phase protein conformations. Cross sections have been measured by ion mobility and ion energy loss. Ion mobility spectrometry (IMS) was first applied as an analytical method to separate ions by Cohen and coworkers in 1970 [89]. In this technique, gas phase ions move through a drift tube under a constant electric field, while an inert buffer gas flows opposite to the drift direction of the ions of interest. The ion drift time can be recorded by a plasma chromatograph [89], or an ion detector following the mass analyzer [90]. Several mass analyzers have been used in combination with the drift tubes such as magnetic sectors [91], FT-ICR [92], ion traps [93], quadrupoles [94] and TOF instruments [95]. In general, ions with more folded structures have smaller cross
sectional areas, undergo fewer collisions with the buffer gas and therefore have larger ion mobilities. This is a unique feature, since ions with identical masses can be separated based on their sizes.

The average drift velocity of an ion, $V_d$, is determined by the number of collisions with the buffer gas in the drift tube. The drift velocity is proportional to the electric field $E_{el}$:

\[ V_d = KE_{el} \]  

(1.1)

where $K$ is the ion mobility constant. As ions pass through the drift tube with length $l_d$, $K$ can be calculated from

\[ K = \frac{l_d}{t_d E_{el}} \]  

(1.2)

where $t_d$ is the drift time. To account for different temperatures and pressures, a reduced mobility $K_0$ is calculated from:

\[ K_0 = K \frac{P}{760} \frac{273.15}{T} \]  

(1.3)
where \( P \) is the gas pressure in torr and \( T \) is the gas temperature in degrees Kelvin.

Substitution of equation 1.2 into equation 1.3 gives the following expression for the reduced ion mobility constant:

\[
K_0 = \frac{l_d}{t_d E_{el}} \frac{P}{760} \frac{273.15}{T}
\]  

(1.4)

To obtain information about conformation, mobility can be converted to a collision integral:

\[
K = \frac{3}{16} \frac{z e}{n} \left( \frac{2\pi}{\mu k_B T} \right)^{\frac{1}{2}} \left( \frac{1}{\Omega} \right)
\]  

(1.5)

where \( n \) is the number gas density, \( e \) is the elemental charge, \( \mu = \frac{m_i m_b}{m_i + m_b} \) is the reduced mass, \( m_i \) is the ion mass, \( m_b \) is the buffer gas mass, \( T \) is the temperature, \( k_B \) is Boltzmann’s constant, and \( \Omega \) is the averaged collision integral [96, 97]. In the case of diffuse (inelastic) scattering of large ions like proteins, the collision integral is given by:

\[
\Omega = 1.22 \pi a^2 = 1.22 A
\]  

(1.6)

where \( a \) is the sum of the radii of the collision partners, and \( A \) is the projection area [98].
Ion mobility measurements were used to distinguish different conformations of aMb ions in the gas phase [69]. The aMb charge states ranged from +4 to +22. The collision cross sections for the charges +8 to +22 of aMb ions were from 2800 to 3800 Å². High proton affinity bases (DMAPA and MTBD) were added in the interface region to "strip" protons from high charge states, to produce low charge state aMb ions. The collision cross sections for +4 aMb to +7 aMb ranged from 1500 to 1700 Å². It was concluded that "proton stripping" of higher charge state unfolded ions causes a spontaneous collapse into a partially folded conformation in the gas phase.

The second method used to determine protein cross sections by MS is a measurement of the kinetic energy losses of ions [70, 71]. This technique has lower resolution than ion mobility [99]. Unlike IMS, energy loss experiments can be used to study weakly bound non-covalent complexes [59, 100]. Covey and Douglas [71] first reported the determination of collision cross sections by energy loss experiments with an ESI-triple quadrupole system. They found that an increase in charges on the ion leads to an increase in collision cross sections. The collision cross sections of proteins interpreted by ion mobility and by energy loss have been compared [70]. Douglas included an aerodynamic drag coefficient [101] that takes into account the thermal motion of the collision gas. Average center-of-mass scattering angle of 90° is assumed [71]. The drag coefficient model has been applied to calculate collision cross sections of gas phase ions of hMb and aMb [59, 72], cytochrome c [70], cytochrome c-cytochrome b₅ complexes [100], DNA [99], and enzyme-inhibitor complexes [102].
Collision cross sections of hMb and aMb ions have been measured at several orifice-skimmer voltage differences (ΔVOS) [59], with an ESI-triple quadrupole system. By increasing the ΔVOS from 30 V to 180 V, the intensity of aMb ions in the mass spectrum increased, accompanied by a decrease in hMb ion intensity. Cross sections were measured for the +8 to +14 charge states of both aMb and hMb. For a given charge state, the increase in ΔVOS caused both aMb and hMb ions to unfold to give larger cross sections.

1.4 Tandem Mass Spectrometry of Myoglobin

Tandem mass spectrometry is a process of multiple steps of mass analysis separated by ion fragmentation. It is used to provide structural information, by fragmenting an analyte ion and identifying the resulting fragments. Tandem mass spectrometry has been grouped into two methods, tandem in-time and tandem in-space [103]. In-time refers to mass analysers which can store, select, and fragment precursor ions such as quadrupole ion traps [104] and ion cyclotron resonance (ICR) traps [105]. In-space refers to at least two consecutive mass analyzers such as magnetic sectors [106], quadrupoles [107] and TOF [108]. In an ESI triple-quadrupole MS/MS system, an ion is selected in a first mass analyzing quadrupole. The ion then undergoes collisions with a neutral gas in a collision cell where it fragments. The product ions from the fragmentation are scanned by a second mass analyzing quadrupole.
One of the first tandem mass spectrometry investigations of myoglobin was done with an ESI-triple quadrupole system in 1994 [58]. Solutions of both ferri and ferromyoglobin were prepared for a comparative study. It was shown that fragmentation of positive ferriMb ions gives mostly charged heme loss, whereas fragmentation of positive ferroMb ions gives mostly neutral heme loss. Fragmentation of the +10 hMb ions, formed from a solution mixture of ferrimyoglobin and ferromyoglobin yielded +10 aMb and +9 aMb, as well as singly charged heme ions. Also in 1994, McLuckey and Ramsey used an ion trap to perform collisional activation of the +8 hMb ions in MS/MS experiments [57]. They observed a charged heme loss for positive ferriMb ions. Since then numerous MS/MS studies on myoglobin have been performed to investigate different aspects of its dissociation [60, 72, 75, 109].

1.5 Coulomb Energy and the Dissociation of Heme From Myoglobin

The Coulomb energy is the electrostatic potential energy between two charged bodies (for example heme and globin) and is given by:

\[
U_{el-stat} = \frac{1}{4\pi\varepsilon_0} \frac{q_1 q_2}{r_q}
\]  

(1.7)

where \( \varepsilon_0 \) is the permittivity of vacuum, \( q_1 \) and \( q_2 \) are the electrostatic charges and \( r_q \) is the distance between them. The Coulomb energy is positive for like charges, where the
force between the charges is repulsive. With opposite charges, the force is attractive and the Coulomb energy is negative.

Coulomb effects can influence the dissociation of heme from gas phase myoglobin ions, because heme can leave the globin with an overall charge of -1, 0 or +1 [58, 109]. Simplistic arguments suggest that with ferrimyoglobin, the heme group will have an overall charge of +1, and this has led some authors to speculate on the effects of this on the gas phase binding of heme to the multiply charged gas phase protein ions. Dissociation of +10 holomyoglobin ions, formed from a mixture of ferrimyoglobin and ferromyoglobin in the collision cell of a triple quadrupole system, was performed by Konishi and Feng [58]. The ratio of neutral to charged heme loss was independent of energy or collision gas target thickness, and it was concluded that ferrimyoglobin and ferromyoglobin had the same gas phase stability. McLuckey and Ramsey [57] speculated that loss of "Coulombic strain" would favour loss of charged heme from positive ferrimyoglobin ions particularly in higher charge states, and this was consistent with their observation of charged heme loss from +8 holomyoglobin ions in a trap MS/MS experiment. Schmidt and Karas [75] observed the preservation of negative holomyoglobin ions under "harsh" conditions (capillary 200°C, 20 V skimmer–octopole CID), which led to loss of heme from positive holomyoglobin ions. They concluded that negative ions were more stable because of Coulombic attraction between the positive heme and negative globin.
The overall charge on the heme group in solution is complicated by the presence of two propionic acid groups on the heme [37]. When iron is bound to a neutral free porphyrin, the porphyrin loses two protons and has a net charge of -2. If Fe$^{+2}$ is bound, the overall heme group will be neutral, and if Fe$^{+3}$ is bound, the heme will have an overall charge of +1 [110]. The propionic acid groups can also be charged, depending on solution pH. The $pK_a$ of these acid groups when heme is bound in the protein will differ from the $pK_a$ of free heme ($pK_a^{solution} = 4.4$) [111]. In cytochrome $c$ the effects of binding the heme has been investigated. Wright et al. reported that the $pK_a$ values remain less than 6.5 for three cytochrome $c$ variants [112]. Electrostatic calculations show the propionate groups have $pK_a$ values from about 4.0 to 6.5 in a variety of cytochromes [113]. The $pK_a$ values for the propionate groups of the heme in myoglobin have not been reported. Nevertheless, the groups are usually considered ionized at neutral pH [37]. The propionates are often described as participating in hydrogen bonds with the protein; one propionate shares a hydrogen with Lys45, and the second with Ser92 and Leu89 [39]. Thus, in solution depending on the description of the hydrogen bonding, the degree of ionization of the propionate groups and the Fe oxidation state, the heme can be considered to have an overall charge from -2 to +1.

Measurement of the energies needed to dissociate the heme-globin complexes can give insight into Coulomb interactions within the complexes. Chen et al. [72] have introduced a collision model (described in detail in Chapter 6), to calculate the relative energies transferred to complexes to cause their dissociation in tandem mass spectrometry experiments. They applied this model to hMb and measured the relative energies
necessary to dissociate heme from myoglobin, for the charge states from +8 to +21. It was found that for lower and higher charge states, the calculated internal energies are similar. Another approach to investigate the Coulomb effect, in the process of heme loss from myoglobin, is the measurement of activation energies for heme loss by BIRD experiments. Gross and coworkers [60] studied the decomposition of hMb ions (charge states +9 to +12) in an ICR cell. They reported similar activation energies for the heme loss from the +9 to +12 hMb ions. These two types of experiments showed only small differences in heme binding energies between the low charge states, which therefore have similar Coulomb barriers for dissociation.

1.6 Goals of This Work

Studies of non-covalent protein complexes by negative or positive ion ESI-MS can give insight into solution and gas phase protein conformations, their folding and unfolding mechanisms, energies required to dissociate these complexes, as well as the Coulomb effects on their dissociation. As discussed above, of the many non-covalent complexes, myoglobin has been extensively studied by various mass spectrometric techniques. In this study, collision cross sections and tandem mass spectrometry in positive and negative ion mode ESI-MS are used to investigate Coulomb effects on the dissociation of heme from myoglobin.

Previously, acid and methanol-induced protein unfolding were studied by positive ion MS for lysozyme, cytochrome c, myoglobin and ubiquitin ions [64-66]. In addition,
comparison of unfolding for several proteins was investigated in positive and negative ion MS [76]. In this study, different Mb conformations in solutions at low and high pH were investigated, by negative ion MS. The solution pH was changed, and different charge state distributions which indicate different protein conformations were observed.

Here, conformations of gas phase negative myoglobin ions are probed by collision cross section measurements. Previously, Douglas and Collings [59] found that positive myoglobin ions can be activated in the orifice-skimmer region of an ESI triple-quadrupole mass spectrometer system. They dissociated hMb in the orifice-skimmer region to produce aMb ions and measured cross sections of the ions at three ΔVOS values. At 30 V, only hMb was observed. At 110 V, both aMb and hMb were observed having similar cross sections. At 180 V, only aMb ions were observed, having cross sections similar to cross sections of aMb and hMb ions at 110 V. It was found that cross sections of hMb at 30 V were lower than cross sections of aMb and hMb at 110 V and 180 V, indicating more compact conformations of the protein with the heme bound at lower ΔVOS. Part of the work of this thesis describes a comparison of the collision cross sections of aMb ions, formed from a myoglobin in solution and measured at ΔVOS of 30 V, with the collision cross sections of hMb ions measured at the same ΔVOS. At ΔVOS of 30 V, it is not possible to form aMb ions from holomyoglobin ions in the orifice-skimmer region. It is of interest to measure the cross sections of aMb at ΔVOS of 30 V, in order to compare them directly to those of hMb ions at that same ΔVOS. In this way, aMb ions do not gain excess energy that can cause them to unfold. The observed result contributes to a better understanding of the unfolding mechanism of myoglobin.
during heme dissociation, and the role of heme binding in the stabilization of the myoglobin complex.

Gas phase non-covalent complexes are free of the influence of solvent, and therefore it has been suggested that their dissociation is greatly influenced by Coulomb interactions between the charges on the gas phase ions [114]. Coulomb effects on the dissociation of non-covalent gas phase complexes have been reported for many protein multimers [115-121]. In many cases, it was found that the dissociation results in an asymmetric distribution of charges and masses between the fragments. These include homodimers of ecotin [115], cytochrome c and α-lactalbumin [117], tetramers of streptavidin [116], avidin, concavalin A and human hemoglobin [118, 120], pentamer of Shiga-like toxin I [119], and a 24-mer of heat-shock protein HSP16.5 [121]. The asymmetry of charge distributions in the fragment ions of all these complexes was found to depend on the precursor charge state, internal energy and conformation. It is of interest to investigate Coulomb effects in the dissociation of other types of gas phase non-covalent complexes, like protein small-molecule complexes. Myoglobin is particularly suited as a model system for this study, because the charge can be at least partially controlled by oxidizing or reducing the iron heme, which can therefore provide a more detailed study. Coulomb effects could in principle, either increase or decrease the barrier for heme loss, depending on the position of charges in the heme-protein complex and in the transition state for heme loss. The influence of Coulomb effects on the energies added to the ions to cause dissociation is discussed in the last chapter of this thesis. In principle, similar experiments could be performed with other protein small-molecule complexes, if the
charge on the small molecule can be controlled in a systematic way. Measured collision cross sections, along with tandem MS experiments, are used to determine the energies necessary to dissociate positive and negative hMb ions. A simple model potential is proposed, to take into account the differences in Coulomb energies between neutral and charged heme loss from myoglobin. The MS/MS results for different oxidation states of the heme iron are of interest, since tandem mass spectrometry experiments on ferro and ferriMb ions show different heme loss pathways in positive ion mode MS [58, 109]. The possible differences in binding between heme Fe$^{2+}$ and Fe$^{3+}$ and the globin complicate the interpretation of the MS/MS experiments, but the simplest conclusion is that Coulomb repulsion lowers the barrier for heme loss.

1.7 Outline of This Work

Chapter 2 details the basics of quadrupole theory, the ESI triple-quadrupole apparatus and other experimental details.

Chapter 3 shows positive and negative ion mass spectra of ferri and ferromyoglobin in solution, at pH 7.1. A discussion is given of the conformation of ferrimyoglobin in solution, during acid and base-induced equilibrium unfolding, as monitored by charge states in negative ion ESI-MS.
Chapter 4 shows the results of collision cross section measurements for positive and negative hMb ions, produced from ferri and ferroMb in solution. The positive and negative ion cross sections are compared.

Chapter 5 contains the results of collision cross section measurements of positive aMb ions formed upon removal of heme from hMb in solution, prior to ESI-MS. Cross sections of these ions, measured at different ΔVOS which can induce unfolding of ions, are compared with the cross sections of positive aMb ions formed by dissociating ferriMb ions in the orifice-skimmer region of the mass spectrometer. Removal of the heme prior to ESI-MS provides the possibility for a direct comparison of cross sections for aMb and hMb at a low ΔVOS, when aMb is not unfolded.

Chapter 6 describes measurements of the added energies necessary to dissociate heme from positive and negative ions of hMb in the gas phase. Calculation of the internal energies for heme dissociation can be used to examine the effects of a neutral and charged heme loss, and whether any heme loss pathways are influenced by Coulomb effects. The collision cross section data from Chapter 4 are used, along with dissociation voltages obtained from tandem mass spectrometry experiments, to calculate these energies. The different internal energies needed to cause neutral and charged heme loss from myoglobin are rationalized by differences in Coulomb energy. A model for heme binding is described using Coulomb and Morse potentials.
Chapter 2

Experimental Methods

This chapter includes an overview of the electrospray ionization triple quadrupole mass spectrometer operation. It also describes the materials and methods used to prepare myoglobin solutions.

2.1 Electrospray Ionization Mass Spectrometer

Figure 2.1 Diagram of the ESI-MS system. ESI is the electrospray ionization source, Q0 is the RF only quadrupole, SR0 are short rods, Q0/Q1, Q1/Q2, and Q2/Q3 are ion lenses, Q1 is the first mass analyzer, Q2 is an RF only quadrupole, Q3 is the second mass analyzer, EXIT is an aperture plate and CEM is the detector.
In the ESI-MS system used here [70], gas phase ions formed at atmospheric pressure by ESI pass through an orifice in a curtain plate, a \( \text{N}_2 \) curtain gas, and a sampling orifice (0.25 mm diameter), followed by a skimmer (0.75 mm diameter orifice). Ions then enter an RF (radio frequency)-only quadrupole (Q0), a first quadrupole mass analyzer (Q1), an RF-only quadrupole or collision cell (Q2), a second mass analyzer (Q3), followed by a channel electron multiplier (CEM) for ion detection. The low pressures of the system are achieved by a three stage differential pumping system. A mechanical pump is used for the interface region and one turbo molecular pump is used for each of the Q0 chamber and the main chamber containing the quadrupoles. Table 2.1 gives the background pressures of the different regions in the MS system.

<table>
<thead>
<tr>
<th>Region</th>
<th>Pressure (torr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curtain-Orifice</td>
<td>760</td>
</tr>
<tr>
<td>Orifice-Skimmer</td>
<td>1.3</td>
</tr>
<tr>
<td>Q0 Chamber</td>
<td>( 4 \times 10^{-3} )</td>
</tr>
<tr>
<td>Main Chamber</td>
<td>( 8 \times 10^{-6} )</td>
</tr>
</tbody>
</table>

**Table 2.1** Pressures in the different regions of the mass spectrometer.
2.2 Electrospray Ionization

Figure 2.2 Electrospray source used in Mb experiments.

Figure 2.2 shows a schematic of the homemade electrospray source. The sprayer consists of a 3 cm long fused-silica capillary (Polymicro Technologies, Phoenix, AZ), with an inner diameter (i. d.) of 74 μm, and an outer diameter (o. d.) of 145 μm, connected to a 5 cm long stainless steel tube (Small Parts Inc., Miami Lakes, FL), with an i. d. of 0.02 cm, and an o. d. of 0.04 cm. A syringe pump (model 22, Harvard Apparatus, South Natick, MA) infuses solutions into the source through a capillary, at a flow rate of 1 μL/min. High voltages (-2.0 to 4.5 kV) are applied to the stainless steel tube.

2.3 Interface Region

As shown in Figure 1.1, charged droplets formed at atmospheric pressure travel towards the curtain plate inlet. Nitrogen (curtain gas) is passed through the region between the
curtain plate and the orifice. The curtain gas helps desolvate ions and prevents solvent from entering the first vacuum pumping stage. The flow of the curtain gas is important. Too high a flow may lead to a loss of analyte ions, while too low a flow could cause formation of analyte-solvent adducts.

After passing through the orifice, ions are accelerated by a potential difference between the orifice and the skimmer. The curtain plate is held at 1000 V, the orifice at 290-320 V, and the skimmer at 120-150 V. Ions and gas pass through the skimmer and into the Q0 chamber. Operation of the quadrupoles is described below.

### 2.4 Quadrupole Potentials

![Figure 2.3 Schematic of the quadrupole rods. The distance from the center to the rods is $r_0$.](image)

Figure 2.3 Schematic of the quadrupole rods. The distance from the center to the rods is $r_0$. 

30
The ideal quadrupole mass filter consists of a set of four parallel hyperbolic rods with DC (direct current) and RF (radio frequency) potentials applied to the electrodes [122], as shown in Figure 2.3. Each pair of opposite rods is electrically connected, providing them with the same polarity. One pair of rods has an applied potential:

\[ \Phi_0 = U - V \cos(\omega t) \]  (2.1)

where \( U \) is the DC voltage rod to ground, \( V \) is the zero to peak amplitude of the RF voltage between each rod and ground, \( \omega \) is the angular frequency of the RF voltage, \( t \) is time, and \( r_0 \) is the minimum distance from the center of the quadrupole to any of the rods. The other pair of electrodes has the opposite potential:

\[ -\Phi_0 = U - V \cos(\omega t) \]  (2.2)

In two dimensions, the potential at any point \((x, y)\) within the quadrupole is given by:

\[ \Phi(x, y) = \left( \frac{x^2 - y^2}{r_0^2} \right) \Phi_0 \]  (2.3)

At the center of the quadrupole, \( \Phi(x, y) = 0 \).
2.5 Ion Motion in a Quadrupole

The motion of ions in the $x$-direction of a quadrupole field is determined by:

$$\frac{d^2x}{dt^2} + \frac{2e}{m_i r_o^2} (U - V \cos(\omega t))x = 0$$

(2.4)

where $m_i$ is the mass of an ion, and $e$ is the elemental charge. Similarly in the $y$-direction:

$$\frac{d^2y}{dt^2} - \frac{2e}{m_i r_o^2} (U - V \cos(\omega t))y = 0$$

(2.5)

The motion of ions in the $x$ and $y$ directions are independent. Let:

$$a_x = -a_y = \frac{8eU}{m_i \omega^2 r_o^2}$$

(2.6)

$$q_x = -q_y = \frac{4eV}{m_i \omega^2 r_o^2}$$

(2.7)

$$\xi = \frac{\omega t}{2}$$

(2.8)
From equations (2.4) to (2.8), the equation of motion can be written as:

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

where \( u = x \) or \( y \). Equation 2.9 is known as the Mathieu equation. The solutions of the Mathieu equation give the trajectories of the ions which can be “stable” or “unstable”. Stable motion refers to the ion displacement in the \( x \) and \( y \) directions being less than \( r_0 \), for all values of \( t \). In this case, the oscillation of the ion through the quadrupole will be confined within the rods, and the ion will be transmitted. Conversely, unstable ion motion results in the ion striking the rods and not being transmitted through the quadrupole. For the set of different values of the Mathieu parameters, \( a_u \) and \( q_u \), a stability diagram like one presented in Figure 2.4, can be constructed [122, 123].

![Figure 2.4](image)

**Figure 2.4** First stability region of ion motion in a quadrupole field based on the Mathieu equation. Different masses (\( m_1 \), \( m_2 \), and \( m_3 \)) lie on the operating lines labeled A and B.
Dividing equation 2.6 by equation 2.7 gives \( \frac{2U}{V} = \frac{a}{q} \). Since this ratio is independent of m/z, masses of all the ions are located on the same line, called the "operating line" or "mass scan line". The \( \frac{a}{q} \) value is the slope of the operating line. A series of operating lines can be created by changing the ratio \( \frac{2U}{V} \). One operating line corresponds to one resolution setting. In Figure 2.4 three ions: \( m_1 \), \( m_2 \), and \( m_3 \) with different m/z ratios, lie on the operating line with \( m_1 < m_2 < m_3 \). In the case of the operating line A, \( m_2 \) is stable in both the x and y directions, and therefore the ion \( m_2 \) is transmitted through the quadrupole. In the case of the operating line B, all three ions lie within the stability region and are transmitted. The operating line with the larger slope (line A) results in higher resolution, because a shorter length of the operating line lies within the stability region. As a result, ions of mass \( m_2 \) are separated from ions with masses \( m_1 \) and \( m_3 \), as both \( m_1 \) and \( m_3 \) lie in regions of instability. In the case of the operating line B, all three ions are transmitted with lower resolution.

2.6 Instrument Operation

The triple quadrupole mass analyzer consists of four quadrupoles. After ions pass through the skimmer, they enter the Q0 quadrupole. Only RF voltage is applied between the rods of Q0. Ions are transported to the first mass analyzer Q1, where ions with selected m/z ratios are transmitted. By scanning the voltages on Q1, ions with different
m/z are transmitted through Q2 and Q3 to the detector to produce a Q1 mass spectrum. While Q1 is scanning, both Q2 and Q3 are in RF-only mode. Similarly, a Q3 mass spectrum can be generated by scanning the RF and DC voltages on Q3, to transmit different m/z ratios. In this case, Q1 is operated in RF-only mode.

In collision cross section experiments, Q1 was operated as an ion guide in RF-only mode. Kinetic energies of ions leaving Q2 were determined by increasing the rod offset voltage of Q3, until the ion signal was decreased by several orders of magnitude for each pressure of argon.

In MS/MS experiments with myoglobin, a product ion scan mode was used. Holomyoglobin ions that were mass selected in Q1, collided with argon in Q2 to cause dissociation. Fragment ions were mass analyzed in Q3. Collision energies of the hMb ions were determined by the difference between the Q0 and Q2 rod offset voltages. The Q3 rod offset was kept the same as the Q2 rod offset. The voltages used for tandem mass spectrometry and collision cross section experiments are listed in Table 2.2. When changing from positive to negative ion mode, all polarities were reversed.

### 2.7 Ion Detection

A channel electron multiplier (CEM) was used as the ion detector. It is capable of detecting positive and negative ions. The CEM consists of a continuous dynode. When an ion strikes the surface of the dynode, secondary electrons are released. This process
continues as secondary electrons strike the dynode to further release more electrons, which create an electron cascade to produce a current. The total number of ions with different m/z ratios was recorded and presented as a mass spectrum. When changing from positive to negative ion detection, only the polarity was changed on the detector.

| Table 2.2 Voltages (V) used in positive ion mode MS/MS and collision cross section measurements. For negative ions, the polarity of these voltages was reversed. |
2.8 Myoglobin Solutions and Reagents

Horse heart myoglobin was purchased from Sigma (St. Louis, MO, USA).

Ferrimyoglobin (Fe$^{3+}$) was reduced to ferromyoglobin (Fe$^{2+}$) by mixing equal volumes of $1.6 \times 10^{-3}$ M sodium L-ascorbate from Sigma (St. Louis, MO, USA) and $1 \times 10^{-5}$ M myoglobin solutions. Reaction was allowed to proceed for 30 minutes [109]. The reduction of iron was verified by recording UV spectra (Beckmann DU 800).

Apomyoglobin from equine skeletal muscle was purchased from Sigma (St. Louis, MO, USA). The aMb was $1.2 \times 10^{-5}$ M in 10% methanol. HPLC-grade methanol, acetic acid and ammonium hydroxide were purchased from Fisher Scientific (Nepean, ON, Canada). Argon (99.9999%, manufacturer’s stated purity) and nitrogen (99.999%, manufacturer’s stated purity, UHP grade) were from Praxair (Mississauga, ON, Canada). Solution pH was measured with an Accumet pH meter (Fisher Scientific, model 15, Arvada, CO).
Chapter 3

Myoglobin Spectra

The first part of this chapter shows positive and negative ion mass spectra of myoglobin ions from ferri and ferromyoglobin solutions at pH 7.1. The second part focuses on negative myoglobin ions from ferrimyoglobin solutions with different pH values, to induce unfolding. The ion charge states which can give an indication of protein conformations are monitored by ESI-MS.

3.1 Negative and Positive Ion Mass Spectra

Mass spectra of negative Mb ions, formed from ferri and ferromyoglobin solutions, are shown in Figures 3.1 a) and 3.1 b), respectively. The solutions contained 50% MeOH and the pH was measured to be 7.1. The orifice-skimmer voltage difference was 170 V. Under these experimental conditions, low charge states of holomyoglobin dominate the spectra.

In the mass spectrum of ferriMb, shown in Figure 3.1 a), holomyoglobin ions have peaks at the following m/z ratios: 4393 (-4h), 3514 (-5h), 2928 (-6h), and 2510 (-7h). Corresponding to the hMb peaks, are lower intensity aMb peaks, at m/z ratios: 3389
Figure 3.1 Negative ion ESI-MS spectra of a) $1 \times 10^{-5}$ M ferrimyoglobin, and b) $5 \times 10^{-6}$ M ferromyoglobin, in a solution of 50% methanol, at pH 7.1. The orifice-skimmer voltage difference was 170 V. Notation: -5h is -5 holomyoglobin (hMb) and -5a is -5 apomyoglobin (aMb).
(-5a), 2824 (-6a) and 2420 (-7a). The peak at m/z 3905 has the same nominal mass as the -9 (hMb)_2 dimer ions. Dimer ions with even charges have the same m/z ratios as monomer ions (e.g. the -12 dimer ion has the same m/z ratio as the -6 hMb monomer). Full scan MS/MS on the m/z 3905 hMb dimer ion, at a 1.7 mtorr argon pressure in the collision cell, and for a range of different collision energies from 400 to 730 eV, shows no significant fragment ions in the mass spectrum. The mass spectrum of negative ferroMb ions, shown in Figure 3.1 b), is similar to the mass spectrum of ferriMb. The hMb ions with charge states -4 to -7 show the highest intensities. The aMb peaks are not well resolved, possibly because of the addition of 8 x 10^{-4} M sodium L-ascorbate which causes the formation of clusters between the protein and sodium and ascorbate ions. A possible dimer peak of the -9 ion appears at approximately m/z 3900.

Figures 3.2 a) and b) show the mass spectra of positive myoglobin ions from solutions of ferri and ferroMb at pH 7.1, respectively. In the mass spectrum of ferriMb, charge states range from +5 to +9, with m/z ratios: 3516 (+5h), 2930 (+6h), 2511 (+7h), 2197 (+8h) and 1953 (+9h). Apomyoglobin peaks appear at m/z ratios: 2826 (+6a), 2423 (+7a), 2120 (+8a) and 1884 (+9a). At m/z 2703, there is a minor peak which is assigned to the +13 charge state of a hMb dimer. The mass spectrum of positive ferroMb ions shows the charge states +4 to +8. The mass spectrum in Figure 3.2 b) has a higher noise level than the mass spectrum in Figure 3.2 a), due to the addition of 8 x 10^{-4} M sodium L-ascorbate, and a lower concentration of myoglobin (5 x 10^{-6} M).
Figure 3.2 Positive ion mass spectra of a) $1 \times 10^{-5}$ M ferriMb, and b) $5 \times 10^{-6}$ M ferroMb, in a solution of 50% MeOH, at pH 7.1. The $\Delta$VOS was 170 V.
The shift in charge states in the mass spectra, seen in the Figure 3.2 (+5 to +9 for ferriMb to +4 to +8 for ferroMb), could be attributed to the different oxidation states of iron in the heme. FerroMb contains iron in the oxidation state +2, while ferriMb contains an iron in the oxidation state +3. This gives ferroMb ions overall one less charge compared to ferriMb. The charge state distributions of negative ferri and ferroMb ions are identical. It is likely that Fe$^{+3}$ is reduced to Fe$^{+2}$ in the negative electrospray process [124]. This is further discussed in Chapter 6.

### 3.2 Myoglobin Conformations in Solution Monitored in Negative Ion Mode

Conformations of negative myoglobin ions, formed from a solution of ferriMb, are investigated at different solution pH values. Figure 3.3 shows mass spectra of negative myoglobin ions formed from a ferrimyoglobin solution containing 50% MeOH and having low and high pH values, respectively. Figure 3.3 a) shows that upon addition of acid (pH 3.2) to the solution of ferrimyoglobin, higher charge states appear in the spectrum. This indicates more open conformations, when heme is lost from hMb ions. The dominant peaks with m/z ratios of 4238 (-4a), 3390 (-5a), 2825 (-6a), 2422 (-7a), 2119 (-8a), 1883 (-9a), 1695 (-10a), 1541 (-11a), 1413 (-12a) all correspond to aMb. The free heme is observed at m/z 614. Among the peaks of aMb, there appears to be a bimodal distribution centered on the -9 and -6 charge states, which may indicate the existence of two protein conformations in solution. In addition to the peaks of the
Figure 3.3 Negative ion mass spectra of ferrimyoglobin ions formed from a solution containing 50% methanol, at pH: a) 3.2, and b) 10.1. ΔVOS was 170V.
denatured apoprotein, peaks of holomyoglobin ions with minor intensity appear in the spectrum. The mass spectrum in Figure 3.3 a), recorded using a solution at pH 3.2, shows a wide charge state distribution of ferriMb, which corresponds to an unfolded conformation in solution. In comparison, the mass spectrum of native myoglobin ions formed from the ferriMb solution containing 50% MeOH at a neutral pH, 7.1, is shown in Figure 3.1 a). The charge state distribution in Figure 3.1 a) is narrow, with low charge states from -4h to -7h. The highest observed charge state is at m/z 2510.

Figure 3.3 b) shows that with a solution at pH 10.1, two bell-shaped distributions of aMb charge appear in the mass spectrum, with one having a maximum at -11 and the other at -7. The dominant peaks at m/z ratios: 4238 (-4a), 3390 (-5a), 2825 (-6a), 2422 (-7a), 2119 (-8a), 1883 (-9a), 1695 (-10a), 1541 (-11a), 1413 (-12a), 1303 (-13a), 1211 (-14a), 1130 (-15a), 1059 (-16a), 997 (-17a) all correspond to aMb. The free heme is observed at m/z 614. One of the first negative ion ESI-MS spectra of myoglobin was obtained by Loo and coworkers [125]. In their study, a basic Mb solution contained 1% ammonium hydroxide (pH 11.3), and the mass spectrum showed predominantly peaks of aMb, with charge states ranging from -10 to -19, and hMb peaks having lower intensity. Comparison of their results obtained for solutions at pH 11.3 with results shown here for solutions at pH 10.1, shows that even a small change in solution pH of 1.2 causes a change in protein conformation that can be detected in the mass spectrum.
The change in the solution pH from 7.1 to 10.1, appears to lead to a transition of native, folded negative hMb to a denatured, unfolded aMb. At pH 10.1, some low charge state hMb peaks remain in the mass spectrum, indicating that not all native hMb is lost. Conversely, a decrease in the pH to 3.2 shows mainly peaks of aMb, up to -12a. Both acids and bases cause unfolding of myoglobin in solution. As a result, peaks of higher charge state aMb ions dominate the mass spectrum. The different charge state distributions of aMb ions formed from the solutions at pH 3.2 and 10.1 suggest that these two processes result in different myoglobin conformations. This was previously observed in time-resolved experiments by Sogbein and coworkers. They monitored the acid and base induced unfolding of Mb [74], and showed that hMb acid-induced unfolding to aMb recorded in positive ion mode occurs through an intermediate unfolded conformation of hMb. During base-induced unfolding recorded in negative ion mode, an intermediate state is not observed, and native hMb directly unfolds to aMb.

In Figures 3.3 a) and b), besides the peaks of hMb and aMb, for each charge state a third peak appears, corresponding to aMb with two heme groups attached. Two heme groups attached to aMb were previously observed in positive myoglobin reconstitution studies [126]. Karas and Schmidt have speculated [75] that with negative ions the heme group, even when removed from the binding site in myoglobin, may still be involved in nonspecific binding, due to Coulombic attraction in the gas phase.

Acid induced Mb unfolding has not been previously studied in negative ion ESI-MS. The results obtained for solutions at pH 3.2 and presented here show mostly peaks of
aMb, with charge states from -4a to -12a, and free heme appearing at m/z 614. Positive ion mode ESI-MS experiments to study conformational changes in Mb have been done by Babu and Douglas [65]. A solution of myoglobin containing 50% MeOH at pH 4, showed a positive charge state distribution of aMb, with charges from +12 to +28. In the spectra of ions formed from a solution of myoglobin containing 50% MeOH at pH 3.2, presented here, negative ion charge states are low, from -5a to -12a.

Konermann and Douglas have studied the equilibrium unfolding transitions of lysozyme, cytochrome c and ubiquitin in solution by positive and negative ion ESI-MS [76]. To induce unfolding of these proteins, the pH of the solutions was adjusted with hydrochloric acid and ammonium hydroxide. In the case of cytochrome c and ubiquitin, the unfolding showed minimal shifts in charge state distributions of ions in negative ion mode, whereas in positive ion mode, large shifts in charge state distributions were observed. For ubiquitin, both acid (pH 2) and base-induced (pH 11.7) unfolding in solution were studied. At pH 7.2, positive ion MS for ubiquitin showed the +5 and +6 charge states as the peaks of the highest intensity. A decrease in pH caused ubiquitin to unfold in solution and as a result, higher charge state distributions were seen at pH 2 (charge states +5 to +12) and pH 11.7 (charge states +5 to +11). In negative ion mode, changes in the solution pH did not cause a major shift in charge state distributions in all three mass spectra, at pH 2, 7.2 and 11.7, which showed only one charge state (-5) with high intensity. Circular dichroism (CD) spectra of ubiquitin at these three pH values showed three different curves, indicating three different ubiquitin conformations. A possible rationale for these results was that positive and negative charge state
distributions monitor different structural features in the protein, and that the protein conformation in solution determines the charge state distribution in positive ion mode.

Results shown here indicate that changes of solution pH do have an influence on the charge state distributions of negative Mb ions. Changes of pH in solutions of Mb cause different charge distributions of negative ions in the mass spectra. Monitoring the charge state distributions in the mass spectra of negative Mb ions, at pH 3.2, 7.1 and 10.1, shows a shift to higher charge states for both acid (pH 3.2) and base-induced (pH 10.1) unfolding, from a low charge state native hMb conformation at pH 7.1.

3.3 Summary

Mass spectra are obtained for positive and negative myoglobin ions, formed from a ferri and ferroMb solutions containing 50% MeOH, at pH 7.1. It is known that under physiological conditions, at pH ~7, myoglobin is in its native state [37]. ESI mass spectra show that in its native state, myoglobin produces low charge states. To date, studies of equilibrium folding/unfolding of myoglobin in solution by negative ion MS have not been reported. Base-induced solution unfolding (pH 10.1) of ferriMb to produce peaks of high charge state ions of aMb in the mass spectrum, studied by negative ion MS, is described. The charge state distribution is found to be slightly different from the charge state distribution of negative myoglobin ions presented by Loo and coworkers [125]. A solution of ferriMb, containing 50% MeOH, is adjusted to pH 3.2 by addition of acetic acid, and studied in negative ion MS. Predominantly ions of aMb are observed. In
comparison to positive ion mode, with a solution containing 50% MeOH at pH 4 [65], the 
charge state distribution of negative myoglobin ions at pH 3.2 is narrower, with low 
charges (-5a to -12a versus +12a to +27a). In the pH range from 3 to 4, mass spectra of 
positive and negative ions show different charge state distributions, implying different 
conformations.
Chapter 4

Collision Cross Sections of Holomyoglobin Ions

This chapter describes the measurement of collision cross sections of protein ions with an ESI-triple quadrupole system. The drag coefficient model used to calculate the cross sections is described in detail. This method is then applied to determine the cross sections of ferri and ferroMb ions.

4.1 Drag Model

Collision cross sections can give insight into structural information of gas phase protein ions. When a protein ion travels through a collision cell containing a neutral gas, the number of collisions the protein ion undergoes is governed by the protein's conformation.

Protein ions traveling through a collision gas experience an average aerodynamic drag force from the collision gas. Factors that influence the aerodynamic force on the ion are the size, shape, surface characteristics and velocity of the ion relative to the gas, as well as the thermal speed of the collision gas [70, 101]. The aerodynamic drag force on an object, $F_d$, traveling through a collision gas with speed $v$, and having a cross sectional area, $\sigma$, is [101]:

$$F_d = \frac{1}{2} \rho C_d \frac{1}{2} \sigma v^2$$
where $m_1$ is the mass of the object, $m_2$ is the mass of the collision gas, $n$ is the number density, and $C_D$ is the drag coefficient. The drag coefficient varies with:

1) The gas density, viscosity and the relative velocity of the object, through the Reynolds number. The Reynolds number, is defined as:

$$R_e = \frac{\rho v_o D}{\eta}$$  \hspace{1cm} (4.2)

where $\rho$ is the gas density, $\eta$ is the gas viscosity, $v_o$ is the velocity of the object relative to the gas and $D$ is the cross-sectional diameter of the object [127].

2) The ratio of the mean free path of the collision gas to the size of the object defined by the Knudsen number, $K_n$:

$$K_n = \frac{\lambda}{d}$$  \hspace{1cm} (4.3)

where $\lambda$ is the mean free path and $d$ is the diameter of the object.
3) The ratio, $s$, of the object's speed, $v$, to the thermal speed of the gas, given by:

$$s = \frac{v}{\sqrt{\frac{2k_BT}{m_2}}}$$  \hspace{1cm} (4.4)

where $k_B$ is the Boltzmann's constant and $T$ is the gas temperature. For the following collision cross section calculations with myoglobin, the Reynolds number is nearly zero and the Knudsen number is very high.

Scattering of molecules from the surface of an object has been modeled in two categories: "specular" and "diffuse". Specular scattering involves the molecules leaving the surface in the direction determined by the angle of incidence. All memory of the incident momentum is conserved, where the component of momentum parallel to the surface is unchanged, and the normal component of velocity is reversed. Diffuse scattering involves the molecules leaving the surface in a cosine distribution about the normal to the surface, and all memory of the incident velocity is lost [128].

The drag coefficient can be calculated by taking into account the momentum transfer from the incident gas striking the surface, and from the scattered gas leaving the surface, integrated over all scattering angles. For the case of all speed ratios, $s$, Stadler and Zurich [128] calculated the drag coefficient for a sphere for specular and diffuse scattering.
A summary of all theoretical and experimental data for drag coefficients up to 1976 has been reported by Henderson [129]. As mentioned, Douglas has developed a drag model, by incorporating an aerodynamic drag to the energy loss measurements [101]. The model is described below. Equation 4.1 can be rewritten as:

\[
\frac{1}{v} \frac{dv}{dx} = -C_D \frac{\sigma m_2}{2m_1} dx
\]

(4.5)

Integration of equation 4.5 gives:

\[
\int_{v_e}^{v} \frac{1}{v} dv = - \int_0^l \frac{C_D \sigma m_2}{2m_1} dx
\]

(4.6)

\[
\ln \left( \frac{v}{v_e} \right)^2 = - \frac{C_D \sigma m_2 l}{m_1}
\]

(4.7)

where \(v_e\) is the speed of the ions entering the collision cell, \(v_l\) is the speed of the ions leaving the cell, and \(l\) is the length of the collision cell. Equation 4.5 can be rewritten as:

\[
\left( \frac{v_l}{v_e} \right)^2 = \exp \left( - \frac{C_D \sigma m_2 l}{m_1} \right)
\]

(4.8)

The kinetic energy, \(E\), of the particle is proportional to the square of the velocity of the particle, thus the energy of the ions in the collision cell is:
\[ \frac{E}{E_0} = \exp\left( -\frac{C_D \sigma m \omega^2}{m_1} \right) \]  \hspace{1cm} (4.9)

where \( E_0 \) is the kinetic energy at the entrance of the collision cell. Experimental drag coefficients for diffuse scattering include the effects of inelastic collisions, the thermal speed of the target and scattering through a range of angles from a rough surface. It was shown by Chen et al. [70] that the drag coefficient model with diffuse scattering provides a good description of the collisions between protein ions and gases.

### 4.2 Energy Loss Measurements

The experimental setup for cross section measurements is shown in Figure 2.1. Gas phase protein ions are injected into the RF only quadrupole (Q0), pass through the first mass analyzer (Q1), and enter the collision cell (Q2). In these experiments, the rod offset difference between Q0 and Q2 is 10 volts, and therefore the initial kinetic energy given to protein ions entering Q2 is 102 eV, where \( z \) is the number of charges on the ion. In the cell, ions lose kinetic energy through collisions with the collision gas (Ar). The kinetic energy of the ions leaving Q2 is measured by scanning the second mass analyzer Q3 rod offset. Quadrupole Q3 is set to mass analyze ions. The rod offset voltage is increased in increments of 1 volt until the signal intensity drops two to three orders of magnitude. Stopping curves are acquired at several different collision cell pressures from 0.0 to 1.5 mtorr. The collision cell length is 20.6 cm and the collision gas is argon.
4.3 Interpreting the Data From Energy Loss Experiments

The stopping energy is taken as the charge multiplied by the stopping voltage. The stopping voltage for each collision cell pressure is determined as the Q3-Q2 value at which the ion intensity is decreased to one-tenth of the initial value. Stopping curves are plots of the ion intensity versus the potential difference between Q2 and Q3. Figure 4.1 is an example of stopping curves for the +6 charge state of ferriMb ions. Stopping curves are obtained for five different pressures.

Figure 4.1 The stopping curves of +6 ferriMb ions at different cell pressures of Ar. The stopping curves from right to left correspond to added argon pressures of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mtorr.
The following gives a sample calculation of the collision cross section of the +6 ferriMb ions from measurements at Ar pressures of 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mtorr. Values of the stopping energies at one tenth of the initial intensity ($E_{1/10}$), are determined at each of these pressures. In this case, the stopping energy with no Ar gas in the collision cell, $E^0$, is 132 eV, while stopping energies for different Ar pressures in the collision cell, $E_{1/10}$, are 120, 108, 96, 87 and 76 eV. The ratios $E/E^0$ can be calculated accordingly. For the +6 hMb ion, the $C_D$ value is 2.5. Drag coefficients are determined from the graph of $C_D$.
versus $s$ in [128]. The collision cross section of 1167 Å² is determined from the slope of the plot $-\ln \frac{E}{E_0}$ versus $\frac{C_0 l m_n n}{m_i}$, shown in Figure 4.2 ($R^2 = 0.9995$, y intercept is $+0.0046$).

4.4 Collision Cross Sections of Holomyoglobin Ions from Ferri and Ferromyoglobin Solutions

Collision cross sections measured for holomyoglobin ions from solutions of ferri and ferromyoglobin are shown in Table 4.1. The cross sections are averages of three experiments performed on separate days. Errors are standard deviations.

<table>
<thead>
<tr>
<th>Charge</th>
<th>Ferrimyoglobin</th>
<th>Ferromyoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>1252 ± 96</td>
<td>1271 ± 102</td>
</tr>
<tr>
<td>-5</td>
<td>1261 ± 74</td>
<td>1270 ± 97</td>
</tr>
<tr>
<td>-6</td>
<td>1271 ± 21</td>
<td>1259 ± 110</td>
</tr>
<tr>
<td>-7</td>
<td>1467 ± 54</td>
<td>1416 ± 75</td>
</tr>
</tbody>
</table>

Table 4.1 a) Collision cross sections (Å²) of negative hMb ions produced from solutions of ferri and ferromyoglobin.
Table 4.1 b) Collision cross sections (Å²) of positive ions produced from solutions of ferri and ferromyoglobin.

<table>
<thead>
<tr>
<th>Charge state</th>
<th>Ferrimyoglobin</th>
<th>Ferromyoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>+5</td>
<td>1278 ± 149</td>
<td>1265 ± 57</td>
</tr>
<tr>
<td>+6</td>
<td>1235 ± 98</td>
<td>1264 ± 81</td>
</tr>
<tr>
<td>+7</td>
<td>1594 ± 54</td>
<td>1515 ± 101</td>
</tr>
<tr>
<td>+8</td>
<td>1777 ± 79</td>
<td>1791 ± 32</td>
</tr>
<tr>
<td>+9</td>
<td>1812 ± 20</td>
<td></td>
</tr>
</tbody>
</table>

Typical uncertainties for the collision cross sections of hMb are about ± 5% with the largest (± 11%) for the +5 ferriMb ion. In general, the data show that an increase in the charge state of hMb ions leads to an increase in collision cross section. The increase in cross sections can be attributed to an increase in Coulomb repulsion as the number of charges on the ion increases. This trend is consistent with the previous findings for myoglobin and other proteins [59, 71]. A comparison between the cross sections from Table 4.1 and [59, 71] could not be done, as the charge states and ΔVOS used to measure Mb cross sections were different from each other.

These results show that for a given charge state, positive ions from solutions of ferri and ferroMb have similar cross sections. Also, for a given charge state, negative ions from
solutions of ferri and ferroMb have similar cross sections. These results indicate that these low charge state myoglobin ions have nearly identical sizes. It appears that cross sections of myoglobin do not depend greatly on the oxidation state of iron in heme and the ion polarity. As discussed later in detail, similar cross sections for ions formed from ferro and ferriMb solutions are a part of the evidence that, Fe$^{+3}$ is reduced to Fe$^{+2}$ in the negative electrospray process.

Cross sections of positive and negative ions of cytochrome c, with charges ±4 to ±11, measured by ion mobility, have been compared by Hoaglund-Hyzer and coworkers [130]. It was found that for the low charge states (±4 to ±6), cross sections of positive and negative ions are similar. In addition, for higher charge states (±9 to ±11), cross sections are similar. However, the intermediate charge states, ±7 and ±8, differ by approximately 200 Å$^2$. It was reasoned that the difference was attributed to different gas phase stabilities of positive and negative intermediate charge states of cytochrome c.

The collision cross sections from Tables 4.1 a) and b) are used later in this thesis (Chapter 6), in the calculation of the internal energies needed to dissociate holomyoglobin.

4.5 Summary

Energy loss experiments are performed with positive and negative myoglobin ions from ferri and ferroMb solutions. A drag coefficient model is used to calculate the cross
sections. The results show cross sections ranging from about 1250 Å$^2$ to 1460 Å$^2$ for the charge states -4 to -7 hMb. For the +5 to +9 hMb ions, the cross sections gradually increase from about 1260 Å$^2$ to 1810 Å$^2$. For a given charge state, positive and negative ferri and ferroMb ions have comparable cross sections.
Chapter 5

Collision Cross Sections of Apomyoglobin Ions

Collings and Douglas have studied dissociation of hMb in the orifice-skimmer region, to produce gas phase aMb ions [59]. It was reported that gas phase hMb ions have relatively compact structures that partially unfold when heme is dissociated from the globin. It was also shown that collisions in the orifice-skimmer region can significantly unfold hMb. They proposed the following unfolding mechanism:

\[
\text{hMb} \rightarrow \text{unfolded hMb} \rightarrow \text{aMb}
\]

At AVOS of 30 V, only peaks of hMb ions were present in the mass spectrum. At high AVOS, only ions of aMb could be produced. A direct comparison of cross sections between hMb and aMb ions at the same low AVOS is needed, since at low AVOS aMb ions are still folded. This comparison is given in this chapter. Results of collision cross section measurements of aMb ions are given. These ions are formed from aMb in solution. Heme is removed from solution prior to ESI-MS. The cross sections are compared to collision cross sections of aMb formed in the gas phase by dissociating hMb ions in the interface region, by increasing the AVOS.
5.1 Mass Spectra of Apomyoglobin

Figure 5.1 Mass spectra of apomyoglobin ions, formed from a solution containing 10% MeOH. The concentration of aMb was $1.2 \times 10^{-5}$ M. The ΔVOS was a) 30 V, and b) 110 V. Solution pH was 6.9.
**Figure 5.2** Mass spectra of apomyoglobin ions, formed from a solution containing 10% MeOH. The concentration of aMb was $1.2 \times 10^{-5}$ M. The AVOS was 180 V. Solution pH was 6.9.

Mass spectra of ions formed from a solution of apomyoglobin at different AVOS are shown in Figures 5.1 and 5.2. It can be seen that, at a AVOS of 30 V, the charge state distribution of apomyoglobin is wide, with charge states from +6 to +19. When the AVOS is increased to 110 V, the charge state distribution narrows to charge states from +6a to +15a. The intensities of the higher charge states (+12a and greater) decrease relative to the peak with the highest intensity (+10a). It can be seen that the noise level is higher in Figures 5.1 b) and 5.2, compared to Figure 5.1 a). It is possible that the energy given to the higher charge state ions causes them to fragment, which in turn increases the noise level. This is observed in the mass spectrum at AVOS of 110 V, as the noise level
increases between m/z 800 and 1800. An increase of ΔVOS to 180 V further narrows the charge state distribution, with charge states from +6 to +14. Apomyoglobin ions further fragment at this ΔVOS, and the noise level increases.

Mass spectra of apomyoglobin, in which heme is removed in solution, prior to ESI-MS, have been reported [67, 131]. In a solution containing 50% MeOH at pH 6.5, DePauw and coworkers [131] produced high charge states of apomyoglobin, up to +19a. They observed a decrease in ion intensity for higher charge states at high ΔVOS, similar to the results shown above. It was suggested that higher charge states preferentially fragment. Ion intensity versus cone voltage was plotted for the charge states +9, +11, +14, +17 and +19. It was shown that, for the +14a ion, decrease in the cone voltage from 160 V to 60 V (analogous to an increase in ΔVOS), caused the ion intensity of the +14a ion to increase from $10^7$ up to a maximum of $10^9$ (arbitrary units). A further decrease in cone voltage to 20 V, resulted in an ion intensity drop from $10^9$ to $10^8$. This trend was observed for the charge states +9, +11, +14, +17 and +19.

5.2 Collision Cross Sections of Apomyoglobin Ions

Collision cross sections are measured for the +6a to the +14a ions, at ΔVOS of 30 V, 110 V and 180 V, and for the +15a and +16a ions, at ΔVOS of 30 V, and the results are shown in Figure 5.3. Typical standard deviations for these measurements are ± 5%. The results show that for a given charge state, collision cross sections of apomyoglobin ions formed from solution increase with an increase in ΔVOS. This indicates that when aMb
ions undergo more energetic collisions in the orifice-skimmer region at higher ΔVOS, the aMb ions unfold. Table 5.1 shows the collision cross sections of aMb ions formed from aMb in solution.

![Graph](image)

**Figure 5.3** Collision cross sections versus charge state of positive apomyoglobin ions formed in solution at ΔVOS (●) 30 V, (○) 110 V, and (▼) 180 V.

In addition, collision cross sections were measured for holomyoglobin ions from a 1 x 10⁻⁵ M solution, containing 10% MeOH (pH 6.9), at ΔVOS values of 30 V, 110 V, and 180 V. In this case, an increase in ΔVOS causes the formation of gas phase aMb ions from hMb ions by CID. Table 5.2 shows the cross sections of hMb and aMb ions
obtained at different ΔVOS values. Collings and Douglas have previously reported similar spectra and collision cross sections [59].

<table>
<thead>
<tr>
<th>Charge</th>
<th>ΔVOS = 30 V</th>
<th>ΔVOS = 110 V</th>
<th>ΔVOS = 180 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>+6</td>
<td>1875 ± 103</td>
<td>1889 ± 112</td>
<td>2068 ± 121</td>
</tr>
<tr>
<td>+7</td>
<td>2005 ± 73</td>
<td>2030 ± 105</td>
<td>2102 ± 114</td>
</tr>
<tr>
<td>+8</td>
<td>2171 ± 55</td>
<td>2304 ± 107</td>
<td>2405 ± 90</td>
</tr>
<tr>
<td>+9</td>
<td>2275 ± 63</td>
<td>2497 ± 55</td>
<td>2655 ± 93</td>
</tr>
<tr>
<td>+10</td>
<td>2521 ± 70</td>
<td>2487 ± 45</td>
<td>2702 ± 80</td>
</tr>
<tr>
<td>+11</td>
<td>2568 ± 87</td>
<td>2583 ± 80</td>
<td>2951 ± 105</td>
</tr>
<tr>
<td>+12</td>
<td>2569 ± 86</td>
<td>2743 ± 105</td>
<td>3002 ± 122</td>
</tr>
<tr>
<td>+13</td>
<td>2742 ± 109</td>
<td>2860 ± 50</td>
<td>3012 ± 69</td>
</tr>
<tr>
<td>+14</td>
<td>2740 ± 110</td>
<td>2895 ± 120</td>
<td>3019 ± 92</td>
</tr>
<tr>
<td>+15</td>
<td>2825 ± 69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+16</td>
<td>2833 ± 93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.1** Collision cross sections (Å²) of aMb ions formed in solution at ΔVOS of 30, 110 and 180 V.
Table 5.2 Collision cross sections (Å²) of hMb ions at ΔVOS of 30 V and 110 V, and aMb ions formed in the gas phase by dissociating hMb ions, at ΔVOS of 110 and 180 V.

A direct comparison between aMb ions (Table 5.1) formed from solution aMb, and hMb ions (Table 5.2), at the same ΔVOS of 30 V, shows that for all charge states, aMb ions have greater collision cross sections than hMb, by an average of about 400 Å². This indicates that for the same ΔVOS, gas phase hMb ions have a more folded structures than aMb ions. Heme binding to the globin helps maintain the compact structures of myoglobin in the gas phase. Figure 5.4 shows a direct comparison of collision cross sections of hMb and aMb at ΔVOS of 30 V.
Figure 5.4 Collision cross sections vs. charge state for hMb and aMb ions at ΔVOS of 30 V.

NMR has been used to characterize the structure of apomyoglobin in solution [132]. These studies show aMb as being an unstable native, globule-like protein with some disorder at the heme binding site. The conformations and stability of aMb formed in solution at neutral pH, by ESI-MS and CD [67, 68]. It was found that aMb exchanges more hydrogen atoms in H/D exchange experiments than hMb, indicating a more open conformation of aMb. Small-angle x-ray scattering [133] and size exclusion chromatography [134] experiments showed that the cross section of Mb in solution increases about 1.3 times after heme loss, and another 2-4 times after complete denaturation. The data presented here show that at ΔVOS of 30 V, hMb ions have cross
sections about 10-30% lower than aMb ions, for both low and high charge states, indicating more compact gas phase hMb ions.

5.3 Summary

Collision cross sections are measured for hMb and aMb ions from hMb and aMb solutions, respectively, and for aMb ions formed by dissociation of gas phase hMb ions in the orifice-skimmer region of the mass spectrometer, causing native hMb to unfold, followed by heme loss.

Mass spectra of aMb ions formed from a solution of aMb, are recorded, and collision cross sections are measured at ΔVOS of 30 V, 110 V and 180 V. The cross section data show that an increase in the ΔVOS causes the aMb ions to unfold moderately. Comparison of the cross sections of hMb and aMb ions at ΔVOS of 30 V provides evidence that heme, when attached to the globin, helps maintain a more compact myoglobin structure. This goes further than the findings by Collings and Douglas [59], since conformations of aMb and hMb are compared at a low ΔVOS, where both these forms of myoglobin are folded, as well as at high ΔVOS, at which both are unfolded. Thus, the conformations can be compared in all stages of the unfolding process. The charge state distribution and collision cross sections of hMb at ΔVOS of 30 V are found to be similar to those found by Collings and Douglas. With the ΔVOS voltage that gives similar amounts of aMb and hMb in the spectra (110 V), cross sections are found to be similar to those of Collings and Douglas [59].
Chapter 6

Coulomb Effects in Binding of Heme to Myoglobin

This chapter describes tandem mass spectrometry experiments with hMb ions formed from solutions of ferri and ferroMb. A collision model is applied to calculate the added energy needed to induce dissociation of heme from myoglobin ions. A simple model potential (Coulomb and Morse) is used to account for the differences in the energies needed to induce dissociation of heme for different charge states of hMb. Most of the results in this chapter are taken from Mark and Douglas [124].

6.1 Tandem Mass Spectrometry of Holomyoglobin Ions

Holomyoglobin ions were formed from a solution containing 50% MeOH, at pH 7.1. Holomyoglobin ions were mass selected in Q1 and fragmented in Q2, with argon as a collision gas. Fragment ions were mass analyzed in Q3. For MS/MS of hMb ions, the Q3 rod offset was set equal to Q2. Holomyoglobin dissociates into aMb and heme.
Figure 6.1 MS/MS spectra of -7 holomyoglobin ions from a solution of ferrimyoglobin at a collision gas pressure of 1.7 mtorr. The collision energy $E^0$ was a) 840 eV, b) 1085 eV, c) 1190 eV, and d) 1260 eV.

Figure 6.1 is a set of MS/MS spectra for dissociation of the -7 ferriMb ions, at a collision cell pressure of 1.7 mtorr. It can be seen that the major dissociation pathway of the -7 hMb ion is the loss of neutral heme, to form the -7 aMb ion. There is a small loss (10% of fragments) of negative heme to form -6 aMb. The major dissociation pathway, neutral heme loss, is observed for all charge states, from -4 to -7. Neutral heme loss as a major
dissociation pathway is also observed for negative and positive hMb ions, formed from a ferroMb solution. On the other hand, for positive ferriMb ions, the major dissociation pathway is the loss of a positively charged heme with a minor dissociation channel being neutral heme loss. Tandem mass spectra of +7 ferriMb ions are shown in Figure 6.2.

![Tandem mass spectra of +7 holomyoglobin ions](image)

Figure 6.2 Tandem mass spectra of +7 holomyoglobin ions formed from a solution of ferrimyoglobin, at a collision gas pressure of 1.7 mtorr. The collision energy $E^0$ is a) 490 eV, b) 630 eV, c) 770 eV, and d) 875 eV.
Figure 6.3 Precursor and fragment ion intensities versus Q0-Q2 voltage difference for the -7 holomyoglobin ions formed from a solution of ferrimyoglobin.

In MS/MS experiments, lab kinetic energies are systematically increased, to cause loss of precursor ions, and formation of fragment ions. A plot of fragment yields versus the rod offset difference between Q0 and Q2 is constructed to show the change in intensity of precursor and fragment ions during the tandem MS experiment. The precursor (hMb) and fragment (aMb) relative intensities are calculated as follows:

$$I_{hMb} (%) = \frac{I_{hMb}}{I_{hMb} + I_{aMb}} \cdot 100\%$$  \hspace{1cm} (6.1)
\[ I_{aMb} (\%) = \frac{I_{aMb}}{I_{hMb} + I_{aMb}} \cdot 100\% \] (6.2)

where \( I_{hMb} \) is the relative intensity of the precursor ion and \( I_{aMb} \) is the relative intensity of the fragment ion. Figure 6.3 shows that, for the -7 hMb ion, an increase in Q0-Q2 causes a decrease in the intensity of the -7 hMb ion, and an increase in the intensity of the -7 aMb ion. The intensity of -6 aMb, corresponding to a charged heme loss, remains less than 5% of the initial precursor intensity, and is always less than 12% of the neutral loss in all experiments with negative ions formed from ferrimyoglobin in solution. The sum of the intensities of precursor and fragment ions is normalized to 100%, at each of the collision energies used. The collision energy that creates a 50% loss of precursor ions is taken as the nominal energy, \( E^0 \), required to induce dissociation. The Q0-Q2 rod offset voltage difference determining this energy is referred to as the dissociation voltage.

Figure 6.4 shows dissociation voltages of negative ions formed from ferromyoglobin at five different collision cell pressures. At higher pressures there are more collisions, and the required energy per collision is lower. Therefore, at higher pressures, ions require lower initial kinetic energies to induce dissociation.
Figure 6.4 Dissociation voltage vs. pressure for negative ferroMb ions, for charges -4h to -7h.

6.2 Collision Model

The maximum possible energy an ion can acquire in the collision cell is the center of mass energy of each collision, summed over all collisions. As shown, higher dissociation energies are needed at lower collision cell pressures. Also, different charge states of hMb have different collision cross sections, and therefore at a given cell pressure have a different number of collisions. A collision model has been developed [72, 100], which takes into consideration the effects of different collision cross sections, and different
kinetic energy losses as the ions move through the cell. An ion at a distance $b$ from the cell entrance has a lab kinetic energy given by:

$$E = E^0 e^{\frac{-C_{\mu}m_2\Phi}{m_i}}$$

(6.3)

The mean free path, $\lambda$, of the ion is given by:

$$\lambda = \frac{1}{n\sigma}$$

(6.4)

In traveling a distance $\Delta b$ through the collision cell, the number of collisions the ion experiences, $N$, is:

$$N = \frac{\Delta b}{\lambda} = n\sigma\Delta b$$

(6.5)

Let $\Phi$ be the average fraction of energy transferred to internal energy in a collision. This is taken as $\Phi = 1.0$ [135]. The increase in internal energy of an ion, $\Delta E_{\text{int}}$, while traveling the distance $\Delta b$ is:

$$\Delta E_{\text{int}} = \Phi \frac{m_i}{M} E^0 e^{\frac{-C_{\mu}m_2\Phi}{m_i}} n\sigma\Delta b$$

(6.6)
where \( M = m_1 + m_2 \). Writing equation 6.6 in differential form and integrating over the length traveled, \( l \), leads to:

\[
\int_0^{E_{\text{int}}} dE_{\text{int}} = \int_0^l \Phi \frac{m_2}{M} E^0 e^{-\frac{-C_D m_2 e b}{m_1 n \sigma l b}} \quad (6.7)
\]

\[
\Delta E_{\text{int}} = \phi \frac{m_2}{M} E^0 \frac{m_1}{m_2} C_D \left[ 1 - e^{-\frac{-C_D m_2 e b}{m_1}} \right] \quad (6.8)
\]

For these experiments, \( C_D \) is typically between 2.1 and 2.3. Table 6.1 a) shows the dissociation voltages and collision cell pressures used for MS/MS experiments, collision cross sections (Chapter 4), and the calculated added internal energies required to dissociate negative ions formed from ferriMb in solution. The calculated internal energy represents the change in internal energy from one state (hMb) to another (aMb). The absolute internal energy of hMb entering the collision cell is not known. Uncertainties in the cross sections and dissociation voltages are the standard deviations of three experiments. Uncertainties in \( E_{\text{int}} \) values are calculated from the combined uncertainties of the cross sections and dissociation voltages.

The same experiments were repeated with negative ions, formed from ferromyoglobin in solution. Tandem mass spectra show predominantly loss of neutral heme, with less than 12% loss of charged heme. Dissociation voltages, collision cell pressures, cross sections and \( \Delta E_{\text{int}} \) values are shown in Table 6.1 b). Tables 6.1 c) and d) list dissociation
voltages, collision cell pressures, cross sections, and $\Delta E_{\text{int}}$ values for positive ions formed from solutions of ferri and ferroMb.

### a)

<table>
<thead>
<tr>
<th>Charge</th>
<th>Dissociation voltage (V)</th>
<th>P (mtorr)</th>
<th>Cross section ($\text{Å}^2$)</th>
<th>$\Delta E_{\text{int}}$ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>269 ± 2</td>
<td>2.15</td>
<td>1252 ± 96</td>
<td>273 ± 21</td>
</tr>
<tr>
<td>-5</td>
<td>279 ± 10</td>
<td>1.70</td>
<td>1261 ± 74</td>
<td>302 ± 20</td>
</tr>
<tr>
<td>-6</td>
<td>222 ± 3</td>
<td>1.70</td>
<td>1271 ± 21</td>
<td>290 ± 6</td>
</tr>
<tr>
<td>-7</td>
<td>165 ± 8</td>
<td>1.70</td>
<td>1467 ± 54</td>
<td>278 ± 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>average 285 ± 16</td>
</tr>
</tbody>
</table>

### b)

<table>
<thead>
<tr>
<th>Charge</th>
<th>Dissociation voltage (V)</th>
<th>P (mtorr)</th>
<th>Cross section ($\text{Å}^2$)</th>
<th>$\Delta E_{\text{int}}$ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>257 ± 11</td>
<td>2.15</td>
<td>1271 ± 102</td>
<td>263 ± 24</td>
</tr>
<tr>
<td>-5</td>
<td>248 ± 11</td>
<td>1.7</td>
<td>1270 ± 97</td>
<td>270 ± 24</td>
</tr>
<tr>
<td>-6</td>
<td>205 ± 7</td>
<td>1.7</td>
<td>1259 ± 110</td>
<td>266 ± 25</td>
</tr>
<tr>
<td>-7</td>
<td>160 ± 7</td>
<td>1.7</td>
<td>1416 ± 75</td>
<td>263 ± 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>average 265 ± 23</td>
</tr>
</tbody>
</table>
Table 6.1 Dissociation voltages, pressures, collision cross sections and $\Delta E_{\text{int}}$ values for different charge states of holomyoglobin: a) negative ions from a ferriMb solution, b) negative ions from a ferroMb solution, c) positive ions from a ferriMb solution, and d) positive ions from a ferroMb solution.
For comparisons between the experiments, slight corrections are applied to these
\( \Delta E_{\text{int}} \) values since in experiments at different pressures, and for ions with different
collision cross sections, ions have slightly different times to react [100]. It was found by
simulations, that under the conditions where there is a 50% loss of precursor and 50%
formation of fragment ions, that most of the reaction occurs in the last 4 cm of the
collision cell [100]. The ion speed in this region is calculated from equation 4.8 and the
time to traverse this region is taken as the time available for reaction \( R_{\text{time}} \):

\[
R_{\text{time}} = \frac{l_r}{\sqrt{\frac{2E^0}{m_1} \exp\left(-\frac{C_D n m_2 l \sigma}{m_1}\right)}}
\]

(6.9)

where \( l_r = 4 \text{ cm} \) is the length over which the reaction occurs.

The calculated energies required for reaction, \( \Delta E_{\text{int}} \), added to the negative hMb ions,
formed from ferromyoglobin solution for different reaction times are shown in Figure
6.5. The best comparison of these energies is done by using a constant reaction time of
say 18 \( \mu \text{s} \). The internal energies required to react in this time can be determined from
this plot. This procedure is repeated for positive and negative ions from ferri and
ferromyoglobin. The added internal energies required to induce reaction in 18 \( \mu \text{s} \) are
shown in Table 6.2.
Figure 6.5 Calculated added internal energies, $\Delta E_{\text{int}}$, versus reaction time for negative holomyoglobin ions formed from ferromyoglobin solution; -4 (●); -5 (○); -6 (▼); -7 (▽).

<table>
<thead>
<tr>
<th>Charge</th>
<th>$\Delta E_{\text{int}}$ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>293</td>
</tr>
<tr>
<td>-5</td>
<td>293</td>
</tr>
<tr>
<td>-6</td>
<td>282</td>
</tr>
<tr>
<td>-7</td>
<td>280</td>
</tr>
</tbody>
</table>

average $287 \pm 7$
### Table 6.2 $\Delta E_{int}$ values for a constant reaction time of 18 µs for a) negative ions from ferriMb solution, b) negative ions from ferroMb solution, c) positive ions from ferriMb solution, and d) positive ions from ferroMb solution.

<table>
<thead>
<tr>
<th>Charge</th>
<th>$\Delta E_{int}$ (eV)</th>
<th>Average $\Delta E_{int}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4</td>
<td>268</td>
<td></td>
</tr>
<tr>
<td>-5</td>
<td>257</td>
<td></td>
</tr>
<tr>
<td>-6</td>
<td>248</td>
<td></td>
</tr>
<tr>
<td>-7</td>
<td>247</td>
<td>255 ± 10</td>
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<table>
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<th>Charge</th>
<th>$\Delta E_{int}$ (eV)</th>
<th>Average $\Delta E_{int}$</th>
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<tr>
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</tr>
<tr>
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<td>174</td>
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<tr>
<td>+7</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>+8</td>
<td>205</td>
<td>183 ± 15</td>
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<table>
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<th>Charge</th>
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<th>Average $\Delta E_{int}$</th>
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<tbody>
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<tr>
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<td>295</td>
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<tr>
<td>+7</td>
<td>291</td>
<td></td>
</tr>
<tr>
<td>+8</td>
<td>269</td>
<td>290 ± 15</td>
</tr>
</tbody>
</table>
6.3 Neutral versus Charged Heme Loss

Loss of neutral heme is seen for negative ions from both ferromyoglobin and ferrimyoglobin in solution. Loss of positive heme from a negatively charged protein is not expected, because of the Coulomb attraction between the heme and protein. For example, from the cross section of 1271 Å² for the -6 ion, a nominal ion "radius" of about 20 Å can be calculated. If the -6 ion is comprised of -7 protein and +1 heme ions, the Coulomb binding energy at 20 Å is 5.0 eV, considerably greater than the binding energy of heme in solution (1.1 eV) [39], or the activation energy for loss of positive ions from the positive protein (0.9 eV) [60]. Thus neutral heme loss might be expected even if the heme is positively charged in the protein. The energies required to cause neutral heme loss from negative ions, formed from ferrimyoglobin and ferromyoglobin solutions, are similar (Table 6.1 a), and b)). This suggests the same reaction mechanism, loss of a neutral heme with neutral propionate groups and Fe⁺². The small loss of a negative heme likely arises from heme with Fe⁺² and one ionized propionic acid group.

For positive ions, charged heme loss dominates with ions formed from ferrimyoglobin, and neutral loss with ions formed from ferromyoglobin solution. The simplest interpretation is that heme in gas phase holomyoglobin ions has an overall charge +1 when formed from ferrimyoglobin, and is neutral when formed from ferromyoglobin. If the propionate groups are ionized in solution, they are likely re-protonated in the ESI process. The energy required to induce loss of neutral heme from positive ions is similar
to the energy required to induce neutral loss from the negative ions. It appears that the heme is overall neutral in these three cases.

6.4 Comparison of $\Delta E_{\text{int}}$ Values

The values of $\Delta E_{\text{int}}$ in Tables 6.1 and 6.2 are very similar, because the ions have similar cross sections and similar binding energies. Corrections for different reaction times, to $\Delta E_{\text{int}}$ calculated from equation 6.8, are quite small in this particular case. The values of $\Delta E_{\text{int}}$ do not vary much over the limited range of charge states formed in these experiments. Tables 6.1 and 6.2 also show the $\Delta E_{\text{int}}$ values averaged over charge states, and the average uncertainties. With positive or negative ions, the average energies required to induce loss of neutral heme are all about 280 eV. In contrast to this, the energy required to induce loss of charged heme from positive ions is substantially less, about 184 eV. Thus, it appears that Coulomb repulsion between the heme and positively charged globin lowers the barrier for heme loss.

In the case of Fe$^{+2}$ and Fe$^{+3}$, it is possible that the binding between heme and the protein in the gas phase complex changes. This could contribute to the difference in binding energy of the heme. In solution myoglobin, when Fe is in the oxidation state +2, the heme is slightly more strongly bound to the proximal histidine of the protein, than when Fe is in oxidation state +3, and this increases the overall binding energy of heme to the protein [136]. This difference in solution binding energy can be estimated in four different ways, as follows:
1) When CN\textsuperscript{-} is bound to myoglobin, the Fe-His bond becomes stronger, analogous to Fe\textsuperscript{+2} myoglobin. Bunn and Jandl measured rates of heme exchange between different hemoglobins [137]. They found that ferrihemoglobin (37 °C, pH 7.18) exchanged 76% of its hemes in 5 hours whereas cyanohemoglobin showed only 3.1-3.3% exchange. If this difference in rates (a factor of about 76/3 ~ 25) is attributed to differences in activation energies for heme loss (ΔE\textsubscript{a}), a difference of ΔE\textsubscript{a} = 0.086 eV is calculated.

2) The equilibrium constant for heme association with wild type myoglobin is 8×10\textsuperscript{13} M\textsuperscript{-1} and for His93Gly myoglobin, where binding between the heme and proximal histidine is completely removed, 6×10\textsuperscript{9} M\textsuperscript{-1} [138]. These equilibrium constants give ΔG values for binding of 0.807 and 0.567 eV respectively, and a difference of 0.24 eV or 22% of the activation energy for heme loss.

3) Hargrove and coworkers [139] compared rates of heme loss from wild type and many different mutant myoglobins. For the His93Gly mutant of sperm whale myoglobin (37 °C, pH 5.0) the rate increases from 1.0 ± 0.5 hr\textsuperscript{-1} to 660 hr\textsuperscript{-1}. If this change in rate is attributed to differences in activation energy for heme loss, a difference of ΔE\textsubscript{a} = 0.16 eV is calculated. This is about 15% of the activation energy for heme loss (1.1 eV). Both these experiments with His93Gly overestimate the difference between binding of Fe\textsuperscript{+2} and Fe\textsuperscript{+3} heme.
4) Hargrove and coworkers [140] compared unfolding of aquometmyoglobin with cyanometmyoglobin by guanidinium chloride (GdmCl). The unfolding was modeled as an equilibrium between denatured and folded myoglobin with an equilibrium constant given by $K(c) = K \exp(mc)$ where $c$ is the GdmCl concentration and $m = 4.1 \text{ M}^{-1}$. For aquoferrimyoglobin $K = 140 \times 10^{-6}$ and for cyanoferrimyoglobin $K = 6 \times 10^{-6}$. This gives a difference in $\Delta G$ for binding heme of 0.0787 eV, small in comparison with the total binding energy of 1.1 eV.

These four estimates, while approximate, show that the difference in heme binding between Fe$^{2+}$ and Fe$^{3+}$ is relatively small in comparison to the total binding energy, likely about 10% or less different. If these differences in binding energy apply to gas phase myoglobin ions, they seem too small to explain the ca. 30% difference in added energy, required for dissociation between neutral and charged heme loss. The simplest interpretation of these experiments is that Coulomb repulsion between the positive heme and positive protein lowers the barrier for heme loss.

6.5 Model Potential for Myoglobin

The change in energy for charged and neutral heme loss is determined by the difference in Coulomb energy when heme is bound, and when heme is at the transition state for heme loss. The differences in energies required to dissociate charged heme from different charge states of holomyoglobin are small (Tables 6.1, 6.2), as are the differences in activation energies for loss of heme measured in BIRD experiments [60]. The differences
in Coulomb energy when heme is bound and when heme is at the transition state, must be small. This implies that the difference in spacing between the charged heme and charges on the protein, when the heme is bound and at the transition state, must also be relatively small. A simple-model potential for the heme binding that accounts for this and other properties of gas phase myoglobin is shown in Figure 6.6. The potential is given by:

\[
V(r) = \left[D_e \left(1 - \exp(-\beta(r - r_e))^2\right) - D_E\right] + \left[\frac{q_1 q_2}{4\pi \varepsilon_0 r}\right]
\]  

(6.10)

where the first term is a Morse potential with well depth \(D_e\), modified so \(V(r) \to 0\) as \(r \to \infty\), and the second term describes the Coulomb energy between the heme and protein. The contributions of these two terms and their sum are shown in Figure 6.6. In equation 6.10, \(r\) is the distance between the heme and the protein, \(r_e\) is the distance between the heme and neutral protein when the neutral heme is bound, \(\beta\) is a constant that determines the range of the Morse potential, \(q_1\) is the heme charge, \(q_2\) is the protein charge and \(\varepsilon_0\) is the permittivity of vacuum. Figure 6.6 shows this potential calculated with \(D_e = 1.1 \text{ eV}\), \(r_e = 22 \text{ Å}\), \(\beta = 2.4 \text{ Å}^{-1}\), a charge of +6 on the protein and +1 on the heme (overall ion charge +7). The parameters of this potential are chosen as follows: (1) The value \(r_e = 22 \text{ Å}\) gives a cross section \(\pi r_e^2\) of 1520 Å², like that of holomyoglobin +7 (Table 6.1); (2) The binding energy \(D_e = 1.1 \text{ eV}\) of heme in the absence of Coulomb repulsion is the activation energy to remove heme from solution myoglobin; (3) The value of \(\beta = 2.4 \text{ Å}^{-1}\) was chosen because this gives a difference in activation energy for
heme loss from holomyoglobin +9 and holomyoglobin +12 ions of 0.1 eV, as found approximately in BIRD experiments [60].

Figure 6.6 shows that binding has a long range Coulomb repulsion, followed by a very short range attraction between the heme and protein. This behavior has been described by Rockwood and coworkers [141], for simple models of proteins as charges on a string, although it is now known that gas phase proteins [142], including myoglobin [72], in low charge states, contain considerable folded structure and are not "strings".

Figure 6.6 Model potentials for heme binding in neutral and +7 gas phase myoglobin, using Coulomb and Morse potentials.
6.6 Summary

Myoglobin is particularly suited for studies of the Coulomb effects on binding because the charge can be at least partially controlled by oxidizing or reducing the heme. In principle, similar experiments could be performed with other protein small-molecule complexes, if the charge on the small molecule could be controlled in a systematic way. With gas phase myoglobin ions, the possible different bonding between heme Fe$^{+2}$ and Fe$^{+3}$ and the protein complicate the interpretation of the MS/MS experiments, but the simplest conclusion is that Coulomb repulsion lowers the barrier for heme loss. This is consistent with a model potential that has long range Coulomb repulsion and short range binding.
Chapter 7

Conclusions and Future Work

7.1 Conclusions

This thesis describes a study of the non-covalent heme-globin complex myoglobin. It examines the influence of Coulomb effects on the energy needed to dissociate the heme. This work is also an investigation of myoglobin conformations in solution and the gas phase, by monitoring charge state distributions and measuring collision cross sections with an electrospray ionization triple quadrupole system.

A collision model is used to calculate the energy needed to dissociate the heme from myoglobin in tandem mass spectrometry experiments. The MS/MS results show that negative hMb ions from ferri and ferroMb solutions, and positive hMb ions from a ferroMb solution, give neutral heme loss as a major dissociation pathway and charged heme loss, as a minor dissociation pathway. For positive hMb ions formed from a solution of ferriMb, the charged heme loss is the major dissociation channel.

Tandem mass spectrometry for a given charge state shows that a lower dissociation voltage is needed at higher Ar collision gas pressures. Calculated internal energies show
that about 1.5 times more energy is needed to induce neutral heme loss, than to induce charged heme loss. The differences in energy can be attributed to Coulomb effects. Coulomb repulsion between the charged protein and charged heme lowers the barrier for heme loss. A model potential shows a short range attraction between heme and globin, and a long range Coulomb repulsion.

The solution conformation change of negative myoglobin ions, formed from a ferriMb solution, is monitored by ESI-MS. At pH 7.1, low charge states (-4h to -7h) of holomyoglobin are observed in the ESI mass spectra. At pH 3.2, a narrow charge state distribution of aMb ions, with low charge states, is formed. In comparison, in positive ion mode MS of myoglobin at a pH 4 [65], a broad charge state distribution of aMb ions with high charge states is observed.

Collision cross sections of hMb and aMb ions, formed from a solution of hMb, are measured at different ΔVOS, and the results are similar to those reported by Collings and Douglas [59]. They found that the increase in ΔVOS activates hMb ions and causes them to unfold, followed by a loss of heme, to give aMb ions in the gas phase. In this study, apomyoglobin ions are formed from a solution of aMb. Apomyoglobin ions formed this way, have cross sections greater than cross sections of hMb ions at low ΔVOS. This implies that the heme helps maintain a more compact conformation of the gas phase myoglobin complex ions at low ΔVOS.
7.2 Future Work

Protein conformations in solution have been monitored by ESI-MS, mainly focusing on positive protein ions. For a given protein, differences in charge state distributions between positive and negative protein ions have been observed [76]. For a more complete comparison between negative and positive Mb ions, different Mb charge state distributions at different solution pH values should be evaluated by ESI-MS. Furthermore, collision cross section measurements can be performed for ions formed from solutions at different pH values. As well, upon acid and base-induced denaturation protein unfolding in solution could be probed by measuring H/D exchange levels of negative Mb ions. This may lead to better understanding of the differences between these two types of unfolding mechanisms at different pH values.

The collision cross section results for aMb ions formed from aMb solution, could be supplemented by gas phase H/D exchange measurements. This might give an insight into the conformation differences between aMb and hMb ions when they are not unfolded. Measuring H/D exchange levels at different ΔVOS may give different exchange rates for gas phase aMb and hMb ions, which could be compared to the collision cross section data obtained in this study.

It would be of interest to use the collision model to measure the energy needed for the dissociation of heme from complexes in which heme is bound covalently, like
cytochrome c, as well as from non-covalently bound protein-protein complexes, like hemoglobin, and to compare it to the energies required to dissociate myoglobin. Another way to explore the binding of heme would be to allow free heme to react with aMb in solution, prior to ESI-MS. Measuring the energy needed to dissociate heme from myoglobin in solution formed this way, could indicate the binding strength of the heme-globin complex as the heme attaches itself to aMb. Comparison to the binding in the gas phase can reveal if differences exist. If they do, they could be attributed to the influence of solvent on heme binding.
References


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