STUDY OF SELECTED PROTEINS AND
NONCOVALENT PROTEIN-PROTEIN COMPLEXES
BY NANO- AND ELECTROSPRAY IONIZATION
TANDEM MASS SPECTROMETRY

By
Victor Joseph Nesati
M.Eng (Biotechnology) Lomonosov Institute of Fine Chemical Technology, Moscow, 1992
M.Sc. (Chemistry) Weizmann Institute of Science, Rehovot, 1996

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(Department of Chemistry)
We accept this thesis as conforming
to the required standard

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FEBRUARY 2000
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Department of Chemistry
The University of British Columbia
Vancouver, Canada

Date 28 April 2000
Abstract

This thesis summarizes the results of the investigation of conformational changes of individual proteins under denaturing/unfolding conditions by electrospray ionization mass spectrometry and an assessment of the stability of gas phase noncovalent protein-protein complexes by tandem mass spectrometry.

The experiments studying changes in protein conformations utilized two relatively small, very stable proteins with a well characterized native structure, bovine pancreatic trypsin inhibitor (BPTI) and ubiquitin. Unfolding experiments were performed using changes in pH, and the addition of organic solvents. The results of the experiments confirmed the known resistance of these two proteins to strongly denaturing conditions.

Proteins that are stabilized by multiple disulphide bonds can be unfolded by simply reducing the disulfides. BPTI contains three disulphide bonds, which can be removed by reduction with DTT. In addition, mutants with one or two native or non-native disulphide bonds can be prepared by different techniques. The contribution of the disulphide bonds to the folding of gas-phase BPTI was assessed using cross sections measured by an ion energy loss method. The charge states produced in ESI show that reduced BPTI is partially unfolded under the solution conditions used here. Collision cross sections show that removing the disulphide bonds causes BPTI to unfold in the gas phase but the increase in size is rather small (~17% in area). The results show that the disulphide bonds of BPTI contribute to the folding of the gas phase ion but that even in the absence of disulphide bonds, the protein maintains a compact structure.

The experiments on evaluation of the stability of noncovalent interactions in the gas-phase utilized complexes between BPTI and tryptic proteins (trypsin, chymotrypsin, and trypsinogen). Trypsin, chymotrypsin and trypsinogen have similar
molecular weights but different binding energies for BPTI in solution thus forming a system of natural mutants which become an attractive model for studying protein-protein interactions. Experiments were performed using electro- and nanospray ionization sources. The results of tandem mass spectrometry experiments were analyzed using a recently proposed collision model. It was found that the relative binding energies of the protein-protein complexes in the gas-phase do not correlate with their stability in solution.
Table of Contents

ABSTRACT .................................................................................................................. II

TABLE OF CONTENTS ............................................................................................... IV

LIST OF TABLES .......................................................................................................... VII

LIST OF FIGURES ....................................................................................................... VIII

LIST OF SYMBOLS ..................................................................................................... XII

LIST OF ABBREVIATIONS .......................................................................................... XIV

1. INTRODUCTION .................................................................................................... 1

2. OVERVIEW ............................................................................................................. 5

  2.1. ELECTROSPRAY IONIZATION .......................................................................... 5
      2.1.1. Atmosphere-Vacuum Interface ................................................................. 7
      2.1.2. ESI-MS of Proteins and M, Determination ............................................... 8

  2.2. NANOSPRAY IONIZATION .............................................................................. 11

  2.3. QUADRUPOLE MASS ANALYZERS ............................................................... 14

  2.4. MS/MS ............................................................................................................ 20
      2.4.1. General concepts ....................................................................................... 21
      2.4.2. Ion Activation ........................................................................................... 21
      2.4.3. Scan modes .............................................................................................. 22
      2.4.4. Triple Quadrupole Mass Spectrometers .................................................... 22

  2.5. COLLISION INDUCED DISSOCIATION ......................................................... 24
      2.5.1. General concept ....................................................................................... 24
      2.5.2. Collision dynamics .................................................................................... 26
      2.5.3. Collision energy: High vs. Low ................................................................. 26
      2.5.4. Instrumentation ......................................................................................... 28
      2.5.5. Related applications .................................................................................. 29

3. MATERIALS AND METHODS .............................................................................. 35
3.1. MATERIALS ........................................................................................................... 35
3.2. REDUCTION OF DISULPHIDE BONDS ................................................................. 35
3.3. PURIFICATION OF α-AMYLASE ............................................................................. 36
3.4. FORMATION OF NONCOVALENT COMPLEXES ................................................... 37
3.5. ELECTROSpray IONIZATION-MASS SPECTROMETRY ......................................... 38
3.6. TANDEM MASS SPECTROMETRY ...................................................................... 39
3.7. NANO-ESI-MS .................................................................................................. 40
3.8. CROSS SECTION MEASUREMENTS ................................................................... 41
3.9. ANALYSIS OF MS/MS EXPERIMENTS AND CALCULATIONS ......................... 43

4. PROBING PROTEIN CONFORMATIONS BY ESI-MS .............................................. 45

4.1. ESI-MS AS A TOOL TO STUDY PROTEIN CONFORMATION ................................. 46
4.2. SELECTED PROTEINS USED IN THIS STUDY ..................................................... 51
   4.2.1. BPTI ........................................................................................................... 51
   4.2.2. Bovine ubiquitin ...................................................................................... 52
4.3. EFFECT OF THE pH ON CHARGE STATE DISTRIBUTIONS ................................. 54
4.4. EFFECT OF ORGANIC SOLVENTS ON CHARGE STATE DISTRIBUTIONS .......... 56
4.5. REDUCTION OF THE DISULPHIDE BONDS ....................................................... 57
4.6. CROSS SECTION MEASUREMENTS .................................................................. 59
   4.6.1. Typical example of collision cross section calculations ............................... 59
   4.6.2. Collision cross sections for BPTI samples ................................................ 61
4.7. DISCUSSION ..................................................................................................... 63
4.8. SUMMARY ......................................................................................................... 70

5. NONCOVALENT INTERACTIONS STUDIED BY NANO- & ESI-MS ......................... 72

5.1. PROTEIN - LIGAND NONCOVALENT INTERACTIONS ....................................... 73
5.2. DETECTION OF NONCOVALENT COMPLEXES BY ESI-MS .............................. 76
5.3. BRIEF SURVEY OF THE LITERATURE ............................................................... 78
5.4. SELECTED NONCOVALENT COMPLEXES USED IN THIS STUDY .................... 83
   5.4.1. Noncovalent interactions between BPTI and its target enzymes ............... 83
   5.4.2. The α-amylase - tendamistat system ....................................................... 85
5.5. DETECTION OF NONCOVALENT PROTEIN-PROTEIN COMPLEXES .............. 88
   5.5.1. α-Amylase - tendamistat noncovalent complexes ..................................... 88
   5.5.2. BPTI-tryptic proteins noncovalent complexes ......................................... 91
List of Tables

Table 1. Voltages used in MS/MS experiments ................................................. 40
Table 2. Voltages used in energy loss experiments .......................................... 42
Table 3. Values of speed ratios, drag coefficients, and collision cross sections for 4 charge states of BPTI gas phase ions ................................................................. 60
Table 4. Cross sections for BPTI samples .......................................................... 61
Table 5. Comparison of cross sections for native BPTI measured by Energy loss and Ion Mobility methods .............................................................. 66
Table 6. Comparison of $r_g$ and cross sections ................................................. 67
Table 7. Relative areas calculated from gas phase cross sections and hydrodynamic radii in solution ................................................................................. 69
Table 8. Collision cross-sections for protein complexes ....................................... 108
Table 9. Internal energy required to cause dissociation calculated for noncovalent protein-protein complexes at different pressures of collision gas ................................................. 109
Table 10. Internal energy required to cause dissociation calculated for noncovalent protein-protein complexes ............................................................ 111
Table 11. Collision cross-sections for protein-protein complexes formed by Nanospray Ionization MS ......................................................................... 114
Table 12. Change of internal energy required to cause dissociation calculated for the +13 charge state of noncovalent complexes using different pressure of Kr ................................................. 115
Table 13. Change of internal energy required to cause dissociation calculated for the +13 charge state of the noncovalent protein-protein complexes ................................................................. 115
Table 14. Comparison of collision cross sections for noncovalent complexes using ESI and NanoESI ......................................................................... 117
Table 15. Comparison of the changes in the internal energies using ESI and NanoESI sources for noncovalent complexes ............................................................ 118
Table 16. Comparison of halfway dissociation voltages for Chymotrypsin-BPTI complex using ESI and NanoESI sources ............................................................ 119
Table 17. Comparison of halfway dissociation voltages for +13 charge state of Trypsinogen-BPTI complex using ESI and NanoESI sources ............................................................ 119
Table 18. Examples of positive and negative correlations based on comparison in affinities for noncovalent complexes in solution and in the gas-phase ................................................. 126
List of Figures

Fig. 1. Electrospray Ionization Mass Spectrometry: Schematic representation of the process. 5
Fig. 2. ESI mass spectra of bovine ubiquitin. Peaks from 952 through 1713 are m/z values for these multiply protonated (5+ through 9+) ions. 9
Fig. 3. Schematic of a Nanospray Ionization source. 12
Fig. 4. Schematic of the ideal quadrupole geometry. X and Y are distances from the center of the field and \( 2r_0 \) is the separation of the rods. 15
Fig. 5. First region quadrupole stability diagram illustrating conditions for stable and unstable oscillations. \( m_3 > m_2 > m_1 \) 17
Fig. 6. Collision model for an ion activation. \( E_0 \) is the initial kinetic energy of an ion at the cell entrance, \( m_i \) is the ion mass, \( m_2 \) is the collision gas mass, \( Z \) is the distance travelled by the ion from the cell entrance, \( n \) is the gas number density, and \( l \) the collision cell length. 34
Fig. 7. Schematic of the triple quadrupole mass spectrometer. \( Q_0 \), rf only quadrupole; \( PF \), prefilter; \( Q_1 \), mass analyzing quadrupole; \( Q_2 \), rf only quadrupole; \( Q_3 \), mass analyzing quadrupole. 38
Fig. 8. Bovine pancreatic trypsin inhibitor. Dashed lines represent disulphide bonds. 52
Fig. 9. Bovine ubiquitin. 53
Fig. 10. ESI mass spectra of 10 µM BPTI recorded at different pH. The pH was adjusted by addition of hydrochloric acid. Numbers denote the charge states of protein ions. 54
Fig. 11. ESI mass spectra of bovine ubiquitin recorded at different pH. Samples of ubiquitin were dissolved to 10 µM in the aqueous solutions adjusted to different pH values with hydrochloric acid. Numbers denote the charge states of protein ions. 55
Fig. 12. ESI mass spectra of 10 µM BPTI recorded in water and methanol. Numbers denote the charge states of protein ions. 56
Fig. 13. ESI mass spectra of BPTI in different concentrations of urea. Numbers denote the charge states of protein ions. 56
Fig. 14. ESI mass spectra of bovine ubiquitin recorded in different methanol/water solutions. Numbers denote charge states of protein ions. Samples of ubiquitin were dissolved to 10 µM. 57
Fig. 15. Electrospray ionization mass spectra of BPTI samples. Native BPTI(a), recombinant wild type(b), recombinant mutant [5-55]Ala BPTI (c), and reduced native BPTI(d). Numbers
indicate the charge states of the BPTI ions. Samples [10 μM] were dissolved in aqueous solution at pH 3 adjusted by hydrochloric acid.

Fig. 16. Stopping curves for native BPTI + 4 ions (m/z 1627). Pressures were 0, 0.25, 0.5, 0.75, 1 mtorr of Neon.

Fig. 17. Natural logarithm of ratio of ion energy (E) to ion energy with no collision gas added \(E_0\) versus target thickness for four charge states of BPTI ions. Solid lines are linear fits to the data.

Fig. 18. Collision cross sections vs. charge state for BPTI samples. Native BPTI (•), mutant [5-55]_{Ala}(O), reduced BPTI (○).

Fig. 19. ESI-MS of noncovalent protein complexes. The process of solvent evaporation during transfer of a noncovalent complex to the gas-phase.

Fig. 20. Noncovalent complexes between BPTI and its target enzymes. The drawings are made from coordinates in the Brookhaven Protein Data Bank files 1TPA, 1CBW and 2TGP respectively.

Fig. 21. α–Amylase-Tendamistat noncovalent complex. The drawings is made from coordinates in the Brookhaven Protein Data Bank file 1 BVN.

Fig. 22. ESI mass spectra of α-amylase and tendamistat. Proteins were dissolved in aqueous solution at pH 7 to a final concentrations of 10 μM. Numbers denote the charge states of the protein ions.

Fig. 23. ESI mass spectra of noncovalent complex between α-amylase and tendamistat. Tendamistat and α-amylase were mixed to final concentrations of 50 μM and 10 μM respectively at pH 7 and allowed to incubate overnight at room temperature to form a complex. Tendamistat- (T), α-amylase- (A), complex-(*).

Fig. 24. Deconvoluted mass spectra of the noncovalent complex between α-amylase and tendamistat.

Fig. 25. ESI mass spectra of BPTI, trypsin, trypsinogen and chymotrypsin samples. Proteins were dissolved in aqueous solution to a 10 μM concentration. Numbers indicate the charge states of the protein ions.

Fig. 26. ESI mass spectra of protein-protein complexes. Trypsin/BPTI (a), Chymotrypsin/BPTI (b), and Trypsinogen-BPTI (c), at pH 7. T-trypsin, C-chymotrypsin, Tg-trypsinogen, *-complex, numbers indicate the charge state.
Fig. 27. Nanospray ionization mass spectra of protein-protein complexes. (a) Trypsin/BPTI, (b) Chymotrypsin/BPTI, and (c) Trypsinogen/BPTI. T-trypsin, C-chymotrypsin, Tg-trypsinogen, *-complex, numbers indicate the charge state. Proteins were dissolved in water with 5/1 inhibitor excess and incubated overnight at 4 °C.

Fig. 28. Mass spectrum of the +12 charge states (*,+12) for protein-protein complexes. (a) Trypsin/BPTI, (b) Chymotrypsin/BPTI, and (c) Trypsinogen/BPTI. B-BPTI, T-trypsin, C-chymotrypsin, Tg-trypsinogen, *-noncovalent complex. Numbers indicate the charge states of fragment/precursor ions. The collision gas was 1 mtorr of argon and Q0-Q2 was 120 volts.

Fig. 29. Mass spectrum of the +13 charge states (*,+13) for protein-protein complexes. (a) Trypsin/BPTI, (b) Chymotrypsin/BPTI, and (c) Trypsinogen/BPTI. B-BPTI, T-trypsin, C-chymotrypsin, Tg-trypsinogen, *-noncovalent complex. Numbers indicate the charge states of fragment/precursor ions. 1 mtorr of argon at the value of Q0-Q2 equal 90 volts.

Fig. 30. Effect of Or/Sk potential difference on D50% of Trypsin/BPTI complex. 0.5 mtorr of Kr, +12 c.s. Legends indicate differences in volts.

Fig. 31. Effect of Or/Sk potential difference on D50% of Trypsinogen/BPTI complex. 0.5 mtorr of Kr, +12 c.s. Legends indicate differences in volts.

Fig. 32. Effect of charge state on D50% of BPTI/Chymotrypsin complex. P=1 mtorr of Ar.

Fig. 33. Effect of charge state on D50% of Trypsin/BPTI complex. P=1.4 mtorr of Ne.

Fig. 34. Effect of collision gas on D50% of ChT/BPTI complex. P = 0.8 mtorr, +12 charge state.

Fig. 35. Effect of collision gas on D50% of Tgen/BPTI complex. P = 1.2 mtorr, +13 charge state.

Fig. 36. Effect of the collision gas pressure on the dissociation of Trypsin-BPTI complex. +12 c.s., gas Krypton. Legends indicate pressure in mtorr.

Fig. 37. Effect of the collision gas pressure on the dissociation of ChT/BPTI complex. +12 c.s., gas Argon. Legends indicate pressure in mtorr.

Fig. 38. Dissociation curves of the noncovalent complexes between BPTI and its target enzymes. P=0.5 mtorr of Kr, +13 charge state.

Fig. 39. Dissociation curves of the noncovalent complexes between BPTI and its target enzymes. P=1 mtorr of Ar, +12 charge state.

Fig. 40. Mass spectrum of the +13 charge states for protein-protein complexes using nanospray ionization. (a) Trypsin/BPTI, (b) Chymotrypsin/BPTI and (c) Trypsinogen/BPTI. B-BPTI,
T-trypsin, C-chymotrypsin, Tg-Trypsinogen. *-complex. The charge states of ions are indicated by the numbers. 0.3 mtorr of krypton at the value of Q0-Q2 equal 80 volts. __112

Fig. 41. Effect of pressure on dissociation of ChT-BPTI complex. +13 charge state, collision gas Krypton. Legends indicate pressure in mtorr. ____________________________113

Fig. 42. Effect of pressure on dissociation of Trypsin-BPTI complex. +13 charge state, collision gas Krypton. Legends indicate pressure in mtorr. ____________________________113

Fig. 43. Dissociation curves of noncovalent complexes between BPTI and its target enzymes.
P=0.3 mtorr of Kr, +13 charge state. ____________________________114

Fig. 44. Dissociation curves of noncovalent complexes between BPTI and its target enzymes.
P=0.4 mtorr of Kr, +13 charge state. ____________________________114
# List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>( \theta_{cm} )</td>
<td>Center-of-mass scattering angle</td>
</tr>
<tr>
<td>( a )</td>
<td>Parameter in the Mathieu equation depending on direct current potential</td>
</tr>
<tr>
<td>( a )</td>
<td>Acceleration</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>Ratio of energies before and after a collision</td>
</tr>
<tr>
<td>( A )</td>
<td>Particle projected area</td>
</tr>
<tr>
<td>( C_D )</td>
<td>Drag coefficient</td>
</tr>
<tr>
<td>( C_{Dd} )</td>
<td>Drag coefficient using a diffuse scattering model</td>
</tr>
<tr>
<td>( \Delta G )</td>
<td>Change in free energy</td>
</tr>
<tr>
<td>( E )</td>
<td>Energy</td>
</tr>
<tr>
<td>( E_0 )</td>
<td>Initial collision energy</td>
</tr>
<tr>
<td>( E_{1/10} )</td>
<td>Energy at which the ion intensity is decreased to 1/10 of its original value</td>
</tr>
<tr>
<td>( E_{cm} )</td>
<td>Center-of-mass kinetic energy</td>
</tr>
<tr>
<td>( E_{int} )</td>
<td>Relative internal energy</td>
</tr>
<tr>
<td>( E_{lab} )</td>
<td>Collision energy in the lab frame</td>
</tr>
<tr>
<td>( E_z )</td>
<td>Ion injection energy</td>
</tr>
<tr>
<td>( \phi )</td>
<td>Average fraction of center-of-mass energy transferred to ion internal energy in a collision</td>
</tr>
<tr>
<td>( f )</td>
<td>Applied frequency</td>
</tr>
<tr>
<td>( F_x )</td>
<td>Force in the ( x ) direction</td>
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<tr>
<td>( K_a )</td>
<td>Association constant</td>
</tr>
<tr>
<td>( k_B )</td>
<td>Boltzmann's constant</td>
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<tr>
<td>( K_D )</td>
<td>Dissociation constant</td>
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<td>( \lambda )</td>
<td>Mean free path</td>
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<td>( m )</td>
<td>Mass</td>
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<td>( m_i )</td>
<td>Mass of an ion</td>
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<tr>
<td>( m_2 )</td>
<td>Mass of a neutral collision gas</td>
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<tr>
<td>( N )</td>
<td>Number of collisions</td>
</tr>
<tr>
<td>( n )</td>
<td>The gas number density</td>
</tr>
<tr>
<td>( n_c )</td>
<td>Number of cycles experienced by an ion in rf field</td>
</tr>
<tr>
<td>( p )</td>
<td>Number of degrees of freedom</td>
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<td>( q )</td>
<td>Parameter in the Mathieu equation depending on applied rf voltage</td>
</tr>
<tr>
<td>( Q )</td>
<td>Quadrupole</td>
</tr>
<tr>
<td>( Q1 )</td>
<td>The first quadrupole</td>
</tr>
<tr>
<td>( Q2 )</td>
<td>The second quadrupole (collision cell)</td>
</tr>
<tr>
<td>( Q3 )</td>
<td>The third quadrupole</td>
</tr>
<tr>
<td>( R )</td>
<td>Gas constant</td>
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<tr>
<td>( r_o )</td>
<td>One-half of the distance between opposite rods of a quadrupole</td>
</tr>
<tr>
<td>( RE )</td>
<td>Resolution</td>
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<tr>
<td>( r_g )</td>
<td>Radius of gyration</td>
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<td>( r_h )</td>
<td>Hydrodynamic radius</td>
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<td>( S )</td>
<td>Target thickness</td>
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<td>( \sigma )</td>
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<tr>
<td>( s )</td>
<td>Speed ratio</td>
</tr>
<tr>
<td>( T )</td>
<td>Temperature</td>
</tr>
<tr>
<td>( U )</td>
<td>Direct current (dc) potential on quadrupole rods</td>
</tr>
<tr>
<td>( v )</td>
<td>Object speed</td>
</tr>
<tr>
<td>( V_o )</td>
<td>Applied rf voltage</td>
</tr>
<tr>
<td>( \omega )</td>
<td>Angular frequency of the rf field</td>
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<tr>
<td>( x,y )</td>
<td>Spatial coordinates</td>
</tr>
<tr>
<td>( z )</td>
<td>Total charge on an ion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>4OT</td>
<td>4-oxalocrotonate tautomerase</td>
</tr>
<tr>
<td>Abu</td>
<td>α-amino-n-butyric acid</td>
</tr>
<tr>
<td>amu</td>
<td>atomic mass units</td>
</tr>
<tr>
<td>BIRD</td>
<td>blackbody infrared radiative dissociation</td>
</tr>
<tr>
<td>BPTI</td>
<td>Bovine pancreatic trypsin inhibitor</td>
</tr>
<tr>
<td>CA</td>
<td>Collisional activation</td>
</tr>
<tr>
<td>c.s.</td>
<td>charge state</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CID/CAD</td>
<td>Collisionally induced/activated dissociation</td>
</tr>
<tr>
<td>CM</td>
<td>Centre-of-Mass</td>
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<tr>
<td>cyt b₅</td>
<td>cytochrome b₅</td>
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<tr>
<td>cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>H/D</td>
<td>Hydrogen/Deuterium</td>
</tr>
<tr>
<td>ICR</td>
<td>ion cyclotron resonance</td>
</tr>
<tr>
<td>IRMPD</td>
<td>infrared multiphoton dissociation</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>Mb</td>
<td>Myoglobin</td>
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<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<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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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>nanoESI</td>
<td>Nanoelectrospray ionization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>----------------------------------------------</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Or</td>
<td>Orifice</td>
</tr>
<tr>
<td>PPA</td>
<td>Porcine pancreatic α-amylase</td>
</tr>
<tr>
<td>QET</td>
<td>Quasi equilibrium theory</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soybean pancreatic trypsin inhibitor</td>
</tr>
<tr>
<td>Sk</td>
<td>Skimmer</td>
</tr>
<tr>
<td>SORI</td>
<td>Sustained off resonance irradiation</td>
</tr>
<tr>
<td>SRM</td>
<td>Single reaction monitoring</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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1. Introduction

Mass Spectrometry (MS) began with studies on electrical discharges in gases by Sir J. J. Thomson of the Cavendish Laboratory of the University of Cambridge which resulted in the discovery of the electron in 1897. The first mass spectrometer, called a parabola spectrograph, was constructed by Thomson in the first decade of the 20th century.\(^1\) It was used to determine mass-to-charge ratio of the ions. The 1906 Nobel Prize in Physics was awarded to Thomson "in recognition of the great merits of his theoretical and experimental investigations on the conduction of electricity by gases."

An improvement of MS resolving power by an order of magnitude over the resolution that Thomson had been able to achieve, was accomplished by his protege Francis W. Aston of the University of Cambridge. He designed a mass spectrometer in which ions were dispersed by mass and focused by velocity.\(^2\) Aston received the 1922 Nobel Prize in Chemistry "for his discovery, by means of his mass spectrograph, of isotopes, in a large number of non-radioactive elements, and for his enunciation of the whole-number rule."

A magnetic deflection instrument with direction focusing was developed by A. J. Dempster\(^3\) of the University of Chicago around 1920. This format was later adopted commercially and is still in use today. The first electron impact source was also developed by him. Volatile molecules were ionized with a beam of electrons from a hot wire filament. Modern mass spectrometers still widely employ electron impact ion sources.

The quadrupole mass filter was first reported in the mid-1950s by the group of Wolfgang Paul of the University of Bonn.\(^4\) In this instrument a quadrupolar electrical field comprising radiofrequency (rf) and direct-current (dc) components is used to
separate ions. However it was Paul's work on ion trapping which was cited for the 1989 Nobel Prize in Physics. Quadrupole instruments have become very popular as stand-alone spectrometers. Today the number of quadrupoles sold and in use far exceeds the total of all other types of mass spectrometers.

Triple quadrupole mass spectrometers which were invented by Tim Morrison in Australia and developed at Michigan State University by Richard A. Yost and Christie G. Enke, have become one of the most popular types of instruments used for tandem MS (MS/MS). Generally this technique requires two or more mass analyzers in series to mass analyze the precursor and product ions. Structural information can be deduced by establishing relationships between precursor ions and their fragmentation products. Fragmentation of mass-selected precursor ions by "collision-induced/activated dissociation" (CID/CAD) is followed by mass analysis of the resulting product ions. Collision-induced dissociation was introduced in 1968 by Keith R. Jennings and Fred McLafferty.

It was not until the 1980s that mass spectrometry was applied to the field of protein chemistry. Prior to this period, mass spectrometry was largely limited to the analysis of small molecules with molecular weights (MW) below 500. Considerable experimental skills were required to obtain reliable and reproducible results. The situation changed dramatically when two new ionization techniques - matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) were introduced in the mid to late 1980s.

ESI was investigated by Malcolm Dole in the 1960s, but it was not until early the 1980s that it was put into practice for mass spectrometry by molecular beam researcher John B. Fenn. During the ESI process, ions formed by evaporation of highly charged droplets dispersed from a capillary in an electric field, are drawn into an MS inlet.
MALDI was simultaneously developed by a Japanese team led by Koichi Tanaka at Shimadzu Corporation and by German scientists Franz Hillenkamp and Michael Karas in 1985. Laser desorption of sample molecules from a solid or liquid matrix containing a highly UV-absorbing substance is the essence of the MALDI ionization technique.

These two techniques had a major impact on the use of MS for the study of large biomolecules and made MS increasingly useful for biomedical analysis. The ability of ESI and MALDI to create intact gas-phase biomolecules merged mass spectrometry and protein chemistry into a new interdisciplinary field and redefined scientific boundaries. High mass proteins were observed in ESI as multiply charged ions which allowed their MW determination on limited mass-to-charge range mass spectrometers such as quadrupoles. Coupling of ESI sources to quadrupole mass analyzers and introduction of tandem mass spectrometry to extract structural information once more changed the face of modern mass spectrometry. DNA sequencing, the analysis of intact viruses and immunological molecules, the sequencing and analysis of peptides and proteins, are among routine applications of modern MS.

Following success in analysis and detection of individual proteins, attempts were made to expand the application of ESI-MS to the analysis of noncovalent complexes between biomolecules. Numerous reports have been published on this matter. One critical question remains to be answered. Do the interactions between biomolecules in the gas-phase reflect those found in solution? The goals of the work in this thesis are as follows:

- Detection of noncovalent protein complexes in the gas phase by mass spectrometry
- Determination of the relative dissociation energies for these complexes in the gas-phase by tandem mass spectrometry
• Comparison of the relative binding energies of protein complexes in the gas-phase with their solution binding energies

The following methods were applied in order to achieve these goals.

1. Formation of gas phase ions for protein complexes by nano- and electrospray ionization sources and their subsequent detection by MS

2. Determination of the collision cross-sections of the gas-phase ions by an energy loss method

3. Evaluation of relative energies required to dissociate protein complexes by tandem mass spectrometry using a collision model for ion activation

An overview of the ESI technique, principles of operation and techniques employed, new theories and their application to the study of individual biomolecules and noncovalent complexes are the focus of the second chapter of the thesis.

The Materials and Methods section, Chapter 3, is followed by results and their discussion. Chapter 4 presents and discusses the study of conformational changes of proteins such as bovine pancreatic trypsin inhibitor (BPTI) and ubiquitin in solution. The detection of noncovalent complexes by electro- and nanospray ionization mass spectrometry is the subject of the 5th Chapter. A recently proposed collision model was used in the 6th Chapter to calculate binding energies of noncovalent complexes between BPTI and its target enzymes studied by tandem MS.

Finally the last chapter provides a summary of the thesis, outlines of future work and discusses the possibility of using MS to study complex interactions between biomolecules.
2. Overview

After a brief introduction into the history of mass spectrometry (MS) this chapter introduces the principles of operation of electro- and nanospray ionization sources and briefly describes the theory of quadrupole mass analyzers. The fundamentals of tandem mass spectrometry, collision induced dissociation processes and related applications can be found in the remaining sections of this chapter.

2.1. Electrospray Ionization

Electrospray ionization occurs during the nebulization of a solution of analyte ions in a high electrostatic field. Highly charged droplets are formed in a dry bath gas, at near atmospheric pressure. Figure 1 depicts the three principal steps of electrospray ionization mass spectrometry.

![Electrospray Ionization Mass Spectrometry: Schematic representation of the process.](image)

Small highly charged droplets are formed by electrostatic dispersion of analyte solution under the influence of a high electric field. Gas phase ions are produced by desorption of analyte ions from the droplets. This process is assisted by a countercurrent flow of nitrogen (curtain) gas. There are three major steps involved in the production of gas-phase ions from solution by electrospray ionization.
The first step consists of production of charged droplets at the tip of the electrospray capillary. The second step involves shrinkage of these charged droplets due to solvent evaporation and repeated droplet decomposition resulting in very small highly charged droplets. The third step is the conversion of these highly charged droplets to gas-phase ions. Currently there are two rival theories to explain the formation of gas phase ions. The first, proposed by Dole, assumes the formation of extremely small droplets containing only one ion followed by solvent evaporation leading to a gas-phase ion. Later this theory was modified by Röllgen who suggested that charged droplets shrink as neutral solvent evaporates until the charge repulsion overcomes the cohesive droplet forces leading to a "Coulombic explosion" ultimately leading to a droplets containing only one ion per droplet.

An alternative theory, published by Iribarne and Thompson, predicts possible direct ion emission from the droplet after a significant decrease in the size of that droplet. This process, which was called "ion evaporation", becomes dominant for droplets with radii less than about 10 nm. An equation predicting the detailed rate of ion emission from charged droplets was derived. The formation of gas-phase ions can be explained with either of these two theories. Both hypotheses agree that ions that interact weakly with the solvent due to low solvation energy or high surface activity, will be the first to appear in the gas-phase. The real difference in these theories exists only in the range from 1 to 10 nm. Both theories agree that droplets with radii more than 10 nm release growing electrostatic repulsion of the charges on the surface by a Coulomb explosion. However for droplets with radii between 1 and 10 nm the Iribarne theory predicts that direct ion emission is dominant over the Coulomb fission, described by the Dole-Röllgen theory. For droplets with radii less than 1 nm, it is virtually impossible to distinguish between these two mechanisms. In the positive ion mode ESI produces multiply charged ions from neutral polar molecules by attachments.
of such cations as protons, alkali cations, or ammonium ions. In the negative ion mode ESI produces multiply charged ions from neutral polar molecules by cation abstraction. Ionic species bearing permanent charges in solution also can be the source for the production of gas-phase ions. Typically ESI employs a capillary with internal diameter from 10 to 100 μm allowing flow rates typically ranging from 0.1 to 10 μL/min.

ESI was originally described by Dole et al. in studies of the intact ions formed from synthetic and natural polymers of molecular mass in excess of 100,000 Da, based on measurements of the ion mobility in the gas-phase. These experiments were expanded by Yamashita and Fenn at Yale University in 1984 using atmospheric pressure sampling of ions and analysis with a quadrupole mass spectrometer, and essentially simultaneously by researchers from the Soviet Union using a magnetic sector instrument. Fundamental aspects of ESI, demonstration of its utility for the analysis of biomolecules and the possibility of interfacing with liquid chromatography was outlined in later work by Fenn and co-workers. Multiple protonation of basic residues during the ESI process laid the ground for the structural analysis and determination of molecular weights of oligopeptides and proteins. Proteins with MW up to 40,000 Da were analyzed on a quadrupole mass spectrometer with a maximum mass/charge ratio of 1600.

2.1.1. Atmosphere-Vacuum Interface

Efficient sampling and transport of ions into a mass spectrometer operating in vacuum is the critical feature of an ion source operated at atmospheric pressure. The oldest method which was used in some commercial instruments utilized a single laser-drilled pinhole orifice between the atmospheric pressure source and high-vacuum region of the mass spectrometer. This arrangement required a cryopump capable of very high pumping speed. More recently, differential pumping technology has been
used in commercial instruments. Some systems employ a capillary-inlet skimmer system as described by Yamashita and Fenn. In this arrangement the electrospray needle is maintained at a potential of a few kilovolts with respect to the surrounding electrode. The sprayed solution encounters a countercurrent flow of bath gas which flushes uncharged material and solvent vapor away from the mass spectrometer inlet. As droplets drift forward, ions are formed and some are captured in the flow of gas which emerges as a free jet expansion in the first stage of differential pumping. Ions are then transmitted through a skimmer into the inlet optics of the mass analyzer. The operation of the ESI source in combination with the atmospheric vacuum interface requires the empirical adjustment of a number of parameters according to the details of the particular apparatus, the species being analyzed, and the object of the experiment. If the counter flow of desolvating gas is too high, analyte ions can be swept away from the entrance of the interface with a drastic decrease in sensitivity. If this flow is too low analyte solvation may be excessive due to a reduced evaporation rate, resulting in broadened peaks and decreased signal. These effects can be explained by an excessive amount of solvent entering the mass spectrometer, leading to formation of solvent adducts with analyte ions. Similarly, the voltages on the nozzle and skimmer elements vary the ion collision energy in this region, and this affects the extent of analyte solvation and ion focusing.

2.1.2. ESI-MS of Proteins and M<sub>r</sub> Determination.

Examination of the mass spectra of large oligopeptides and small proteins by ESI-MS typically shows a distinct bell shaped distribution of multiply charged molecular ions arising by either proton or alkali ion attachment. No evidence of fragmentation is detected unless high energy collisions are imposed upon the analyte ions during their transport into the mass spectrometer vacuum system.
The determination of $M_r$ of proteins from their positive ion ESI mass spectra, is straightforward given two assumptions:
- adjacent peaks of a series differ by only one charge
- charging is due to cation attachment (usually a proton)

A series of simple linear simultaneous equations with unknown values for $M_r$, charge state and the mass of the charging agent can be used to describe the observed $m/z$ ratios for each charge state in the recorded charge state distribution. Any two peaks (assuming the mass of the charging agent is known) are sufficient to uniquely determine $M_r$ and charge as is indicated by the relations shown for the adjacent peaks at $m/z$ 1427 and 1224 in the Figure 2. However if the mass of the charging agent is unknown it is necessary to use at least three consecutive peaks in the spectrum in order to determine $M_r$ unequivocally.

$$1427 = \frac{(M_r + n + 1)}{n}$$
$$n = 6$$

**Fig. 2. ESI mass spectra of bovine ubiquitin.** Peaks from 952 through 1713 are $m/z$ values for these multiply protonated (5+ through 9+) ions.
The centroid of the peak was used to extract observed $m/z$ values which is consistent with the fact that isotopic contributions for these large multiply charged ions are not resolved and the average mass is defined by $M_r$. Methods based on this fundamental principle for extracting $M_r$ from these data with high precision and accuracy have been developed. Suppose $k_i$ is the mass-to-charge ratio of a multiply charged ion bearing $n_i$ charges. Then relationship between multiply charged ion at $m/z$ value $k_i$ with charge $n_i$ and the $M_r$ is given by

$$k_i \cdot n_i = M_r + M_a \cdot n_i = M_r + 1.0079 \cdot n_i$$  \hspace{1cm} (1)$$

where $M_a$ is the MW of the charge carrying species. In equation 1 the assumption is that they are protons.

The molecular weight of a second multiply protonated ion at $m/z = k_2$ (where $k_2 > k_1$) that is one peak away from $k_1$ is given by

$$k_2 \cdot (n_i - 1) = M_r + 1.0079 \cdot (n_i - 1)$$  \hspace{1cm} (2)$$

These two equations can be solved for the charge of $k_i$:

$$n_i = (k_2 - 1.0079) / (k_2 - k_1)$$  \hspace{1cm} (3)$$

The molecular weight is obtained by taking $k_i$ as the nearest integer value, and thus the charge of each peak in the charge state distribution can be assigned.

This approach can easily be generalized to determine the mass of other charged adduct species $M_a$ (e.g. $H^+$, $Na^+$, $K^+$) $$k_i = (M_r/n_i) + M_a$$  \hspace{1cm} (4)$$

There are three unknowns in this equation. Consequently three equations are needed to determine $M_a$, $M_r$, and the corresponding charge state. Calculation of $M_r$ for each of the observed $m/z$ values provides enhanced precision. Thus for the data shown
in Figure 2 a relative standard deviation of 1.87 Da is observed for 5 m/z measurements from one spectrum. In this example, the uncertainty in the determination, i.e.,

\[
100\% \times \frac{M_r(\text{theoretical}) - M_r(\text{measured})}{M_r(\text{theoretical})},
\]

where the theoretical \( M_r \) was calculated from the sequence is

\[(8558.9 - 8556/8556) \times 100\%, \text{ or } 0.034 \%.\]

Molecular weights have now been demonstrated to be measured to better than 0.005% with quadrupole mass spectrometers. The relative "inaccuracy" of this particular measurement can be explained by a combination of such factors as lower resolution, insufficient number of scans, and a relatively "big" scanning step (1 m/z). However a precision of 0.03% is sufficient in many biochemical applications for unambiguous MW determination.

2.2. Nanospray Ionization

The overall efficiency in electrospray mass spectrometry can be defined as the number of analyte ions recorded at the detector divided by the number of analyte molecules sprayed and this is determined by a combination of the desolvation, ionization, and transfer efficiencies into the vacuum system as well as the transmission of the mass analyzer and detector efficiency. The nanoelectrospray ion source (nanoESI) which has been developed and described recently\(^\text{20}\) improves desolvation, ionization, and transfer efficiencies in comparison to conventional ESI sources.

Figure 3 shows a nanospray ionization source. A spraying orifice with internal diameter 1-2 \( \mu \text{m} \) is achieved by pulling the spraying capillary to a fine tip. Smaller droplets are generated at a very low flow rate of \(~20 \text{ nL/min} \). The flow is dictated by the electrospray process itself with slight assistance from a pneumatic pump.
It has been reported that only 1 molecule out of 280,000 sprayed was detected using a conventional ESI source. A review of other numbers published in the literature shows similar values. Smith et al. estimated that that only one ion out of 100,000 leaving the needle is detected, while Covey assessed it as 1 in 20,000. However these values do not take into account the ionization efficiency of the ESI process itself. In contrast a nanospray ionization source allowed the detection of one molecule out of 390 with a 510 times improvement in overall efficiency. Using the above mentioned numbers as a upper limits to the overall efficiency of ESI resulted in two orders of magnitude difference in favor of NanoESI, which is approximately the same magnitude of difference (~2 orders) in flow-rate between the two sources.

Highly efficient conversion of analyte molecules into gas-phase ions by nanospray can be attributed to several factors. Separation of analyte molecules into different droplets prevents them from clustering. The small size of the droplets and their monodispersity contribute to the increase in desolvation efficiency. In addition the overall charge-to-volume ratio in the NanoESI source is much higher then that for
ESI resulting in better ionization efficiency due to a greater number of charges within reach of binding sites on the analyte molecule.\textsuperscript{20}

A large percentage of ions generated by NanoESI can be transferred into the inlet of the mass spectrometer because of the geometrical setup. The distance between the tip of the spraying capillary and the orifice is about 2-4 mm. Additionally the tip of the capillary is arranged in line with the quadrupole axis. Thus the short distance between the tip of the capillary and the front orifice of the vacuum system, the reduced flow (roughly by 50\%) of the counter current (curtain) gas and the small size of the droplets, which are below 200 nm, contribute to more efficient transfer of ions generated by the NanoESI source. However the onset voltage and flow rate are not exactly reproducible due to inconsistencies in the exact placement of the spray tip, the density of the metal coating and the length of the tip itself.

The differences from other conventional and miniaturized electrospray sources are as follows. The calculated initial droplet diameter is less than 200 nm.\textsuperscript{20} The droplet volume is therefore about 100-1000 times smaller than the volume of the 1-2 \( \mu \)m droplets generated by conventional electrospray sources. Small droplets have a high surface-to-volume ratio which makes a large proportion of analyte ions available for desorption. The benefit of the low flow consists of providing a longer measurement time at unchanged signal level for a given sample volume.

At least two other micro-electrospray ion sources have been developed. Gale and Smith coupled capillary electrophoresis to electrospray without a liquid sheath flow.\textsuperscript{22} In 1992 they described the use of small-diameter capillaries (down to 5 \( \mu \)m inner diameter) terminating in sharp tips etched by hydrogen fluoride. However this interface has not been widely adapted in practical applications. Emmett and Caprioli integrated a chromatographic packing into the tip of an electrospray needle.\textsuperscript{23} A total of 1 fmol of a peptide was injected as 10 \( \mu \)l of a 100-amol/\( \mu \)l solution. Peptides absorbed
on this material were step eluted at a flow rate of about 0.3-0.8 μL/min. Detection limits in the attomole to subattomole range have been reported with this source.

The nanoESI is stable at a flow rate that seems to be lower than that possible or practical in a pumped system, probably because of the very small diameter of the spraying capillary. The typical mode of operation of nanoESI is to load a 0.2-10 μL volume of analyte solution directly into the needle rather than loading the sample into an injector loop or syringe.

The advantages of NanoESI source can be best put in words of Matthias Mann, one of the inventors of this source. The NanoES ion source has been shown to possess a number of unique analytical features. These include freedom to choose the solvent in a wide range of composition and pH and an unprecedented tolerance to salt contamination. Advantages of nanoES in protein analysis include the possibility of long signal averaging to obtain accurate mass measurements in protein mixtures and the possibility to desolvate, for example, glycoproteins better. The analysis of unseparated peptide mixtures shows the possibility for rapid and sensitive analytical strategies.

2.3. Quadrupole Mass Analyzers

The popularity of the quadrupole mass analyzer, which was originally developed by Paul and coworkers, is based on the following. It requires no magnet and thus is very compact, the source operates at a potential very close to ground potential, the quadrupole may be scanned very rapidly, and finally it requires no slits and hence, in principle, has high transmission.

Quadrupoles are now the most widely used mass spectrometers. Application of electric potentials to four parallel rods creates a quadrupole electric field. Such fields are widely used in mass spectrometry not only to filter ions according to their m/z ratio but also to trap ions and focus ion beams. The ideal quadrupole field is generated by a set
of four hyperbolic rods with adjacent oppositely charged electrodes (Fig. 4). However for practical reasons such as decreased complexity in manufacture, round rods closely approximating the rods with hyperbolic cross-sections are generally used. This change in rod geometry introduces some faults in the field and can impose certain limitations like slight loss in resolution.²⁴

![Diagram of the ideal quadrupole geometry](image)

Fig. 4. Schematic of the ideal quadrupole geometry. X and Y are distances from the center of the field and 2r₀ is the separation of the rods.

Rods of 20 to 30 cm. length can be used to achieve mass selectivity for ions with energies typically below about 10 eV. Electrical connection of the opposite pairs of rods and subsequent application of a dc voltage U and rf voltages V₀ cos (ωt) to the rods makes the mass filter act as a path stability device. Only ions having a small range of m/z values have stable paths through the filter for particular fields and all other ions are not transmitted. Mathieu equations can be used to calculate the stability and paths of...
singly charged ions within an ideal quadrupole mass filter. The motion is described mathematically as the solution of a second-order linear differential equation. It is applied to the movement of an ion of mass-to-charge ratio \( m/z \) along the \( x \) and \( y \) axes perpendicular to the direction of motion of the ions in the axial direction. The equation of ion motion along the \( x \) axis derives from Newton's equation \( F_x = ma \), where \( F_x \) is the force in \( x \) direction, \( m \) is the mass and \( a \) is the acceleration of the ion in the \( x \) direction.

The acceleration of the ion can be expressed as the second derivative of distance with respect to the time and force as a first derivative of potential with respect to distance. A series of substitutions and differentiations results in the following set of equations.

\[
\frac{d^2 x}{d(\omega t/2)^2} + (a + 2q \cos 2(\omega t/2)) x = 0 \tag{5}
\]

\[
\frac{d^2 y}{d(\omega t/2)^2} - (a + 2q \cos 2(\omega t/2)) y = 0 \tag{6}
\]

where

\[
a = \frac{8zU}{mr_0^2 \omega^2} \quad \text{and} \quad q = \frac{4zV_0}{mr_0^2 \omega^2} \tag{7}
\]

In these expressions, \( x \) and \( y \) are distances from the center of the field,

\( U \) is the direct current (dc) potential, \( V_0 \) is the applied rf voltage, \( \omega \) is the angular frequency of the rf field, \( r_0 \) is one-half of the distance between opposite rods and \( z \) is charge.

Solutions to the Mathieu equation with combinations of values \( a \) and \( q \) defining stable motion of the ion in a quadrupole field lead to a "stability diagram". The stability of an ion trajectory within the quadrupole field can be defined as experimental conditions which determine whether the ion remains within the device and is
successfully transmitted through the analyzer or not. Regions with stable solutions can be plotted using $a$ and $q$ as coordinate axes. The overall stability diagram of a quadrupole mass filter can be constructed by superimposing two single stability diagrams. Ions can be transmitted by the quadrupole mass filter only if they are stable in both the $x$ and $y$ directions simultaneously.

![First region quadrupole stability diagram illustrating conditions for stable and unstable oscillations.](image)

The stability region closest to the origin, called the "first stability region" is depicted in Fig. 5. Certain values of $a$ and $q$ lead to stable oscillations in the $x$ and $y$ directions for ions of a given mass. The oscillations are unstable for other ions, in either the $x$ or $y$ direction so that their trajectories tend toward infinite displacement from the center resulting in the loss of the ions which strike the rods.

In general, the $a$ and $q$ diagram shows regions of stability which may be satisfied by many combinations of $U$, $V$ and $m/z$ assuming constant $r_0$ and $\omega$. 

$m_3 > m_2 > m_1$
Examination of the equations (7) reveals that:

\[ a/q = 2U/V \]  
\[ (8) \]

The \( a/q \) ratio does not depend on \( m/z \). Keeping the ratio of \( U \) over \( V \) constant will maintain a fixed value of \( a/q \) thus generating a scan or operating line passing through the origin of the stability diagram. Thus the mass scan line consists of the \( U \), \( V \) and \( m/z \) values having a constant \( a/q \) ratio. Changes in the values of \( U \) and \( V \) will result in the changes of masses stable within the device. Transmission of an ion occurs when \( U \) and \( V \) are such that the ion has values of \( a \) and \( q \) lying within the stable region.

Increasing the slope of the line results in its passing closer to the peak of the stability region thus increasing the resolution and decreasing the sensitivity. Intersection of the scan line with the tip of the stability region at \( U/V_\theta = 0.167 \) produces the highest resolution. In principle the resolution can be limitless. In practice, the resolution of a quadrupole is limited by the time spent by the ions in the quadrupole field which in turn depends on the finite length of the quadrupole rods (\( l \)), the rf frequency and ion injection energy (\( E_0 \)). Imperfections in the quadrupole field also impose some limitation on mass resolution. The number of cycles experienced by the ions in the \( rf \) field is a major factor determining the resolution limit. The following expression representing this relation was proposed.\(^{24}\)

\[ RE = \frac{n_c^2}{20} \]  
\[ (9) \]

where \( n_c \) is the number of cycles experienced by the ion in the \( rf \) field, and \( RE \) is the resolution.

The number of cycles \( n_c \) can be defined as:
Thus equation (9) can be re-written as follows:

$$RE = \frac{0.05m f^2 l^2}{2zE_0} = \frac{0.05 f^2 l^2}{v^2} \quad (11)$$

Extension of the length of the rods, decrease in the ions' injection energy and increase in the frequency may theoretically lead to increases in the resolution. An increase of the resolving power of the mass filter with mass occurs because higher mass ions have lower velocities and so spend a longer time in the analyzer.

The maximum mass range of the quadrupole mass filter is determined by the radius of the rods, the maximum rf supply voltage to the rods and the rf frequency. The maximum mass is given by

$$M_{\text{max}} = \frac{7 \times 10^6 \times V_0}{r_0^2 f^2} \quad (12)$$

where $M_{\text{max}}$ is the maximum mass measured in atomic mass units (amu), $f = \omega / 2\pi$ (MHz), $r_0$ is the inscribed radius of the rods in meters, and $V_0 cos(\omega t)$ is the rf voltage applied between adjacent rods.

In principle, the mass range can be extended by increasing the maximum values of $U$ and $V_0$ and by decreasing either the field radius $r_0$ or the frequency. However difficulties inherent with design of very stable high voltage rf generators and problems in maintaining high mechanical accuracy and reasonable cost during manufacture and assembly of rods makes a decrease in frequency the easiest method to extend the mass range for quadrupoles. The first report by Light-Wahl and coworkers\textsuperscript{25} showed extension of the $m/z$ ratio of a quadrupole mass filter up to 45,000 while decreasing the frequency from 1.2-1.5 MHz to 262 kHz. However surprisingly low resolution and a severe loss in sensitivity were reported which left significant room for further improvement. Collings and Douglas\textsuperscript{26} later reported the increase of mass range from 4000 to 8585 by lowering the radio frequency of the quadrupole power supply from 1
MHz to 683 kHz with only minor losses in sensitivity. Reduction of the frequency led to a general decrease in the transmission of low masses with little changes for intermediate and minor increases in higher mass transmission. A decrease in the transmission for all masses when the \( rf \) frequency is lowered is expected due to the reduction in the area of the acceptance ellipses. An acceptance ellipse of the quadrupole can "be thought of as a limit of values of space and velocity which a particle -focusing system will transmit so that particles with values outside the boundary will be rejected".\(^{27}\) In principle if ions are completely focused into the acceptance ellipses no decrease in sensitivity is expected at lower \( rf \) frequency.

2.4. MS/MS.

The lack of fragmentation with ESI is very useful for the unambiguous determination of molecular weights of compounds. However the resulting absence of structural information can be a drawback and serves as a major motivation for using tandem mass spectrometry (MS/MS) in bioanalytical chemistry. The term MS/MS originates from the necessity to mass analyze fragment ions in a second mass spectrometer. Tandem mass spectrometry consists of two stages of MS. The first stage occurs during the isolation step of an ion from the source, followed by the second - mass analysis stage. By coupling two mass filters together, and placing a reaction/collision cell between them for ion activation and dissociation, structural information on an ion can be obtained. Tandem mass spectrometry was originally developed for applications in ion chemistry and ion-molecule reaction dynamics. Its application to chemical analysis of complex mixtures was realized in the mid 1970s\(^{28}\). Current applications of MS/MS include quantitation of drugs and metabolites, sequencing of peptides and proteins, analysis of oligosaccharides and glycopeptides, and screening for inborn diseases. Analysis of complex biological mixtures by MS/MS
and further determination of individual components required incorporation of solution-based separation techniques like liquid chromatography (LC) and capillary electrophoresis (CE).

2.4.1. General concepts

The general idea of tandem mass spectrometry may be presented in terms of ion-molecule reactions of the type shown in eq.13 considering only singly charged positive ions. The generalization of this concept to other types of ions (negative and/or multiply charged) follows easily.

\[ \text{P}^+ + T \rightarrow \text{F}^+ + \text{N}_1 + \text{N}_2 + \ldots \]  \hspace{1cm} (13)

A mass-selected precursor ion \( \text{P}^+ \) possesses translational energy which transports the ion through the instrument including a collision cell. As a result of collisions with a target gas, \( T \), precursor ions may be converted into fragment ions (\( \text{F}^+ \)), accompanied by one or more neutral species (\( \text{N} \)) which are not normally detectable. Fragmentation of a parent ion in collision with target gases, such as neon, argon, krypton is the most commonly studied reaction of this type.

2.4.2. Ion Activation

Collisional activation transfers translational to internal energy sufficient to force fragmentation of the stable molecular ion formed in the initial ionization process. The necessary energy can be acquired by the precursor ion by multiple low energy (10-40 eV) collisions with a neutral gas causing much more extensive fragmentation than that occurring during the ESI process alone. This process will be discussed in detail in the section 2.5.
2.4.3. Scan modes

It is possible to operate any tandem mass spectrometer in one of three basic MS/MS modes. Experimental parameters can be chosen in order to fix either the mass of the precursor ion, or the mass of the product ion, or the mass of the neutral fragment. For example one can generate a product ion spectrum of the mass-selected precursor ion by setting the precursor ion analyzer to transmit parent ions of fixed m/z to the reaction region. Such a "fixed-precursor ion" experiment is the operational mode most frequently used in tandem mass spectrometry and was employed in this thesis. In the precursor ion scan the second analyzing mass filter is fixed and the first analyzer is scanned to detect all precursor ions that give a fixed product ion. In neutral loss scans both analyzers are scanned with a constant mass difference to detect all ions that lose a certain mass.

Applications of tandem mass spectrometry to mixture analysis often employ experiments which set both mass analyzers to fixed masses, resulting in the detection of precursor and product ions of interest. This is referred to as single reaction monitoring (SRM). In multiple reaction monitoring (MRM) the mass analyzers switch rapidly between two or more pairs of m/z values which can be detected in rapid succession.

2.4.4. Triple Quadrupole Mass Spectrometers

The triple quadrupole instruments transformed MS/MS from a research technique for physics and physical chemistry into a widespread, popular technique for structural analysis in biochemistry. The first tandem quadrupole instruments were designed for studies of ion-molecule reactions. These were double quadrupole instruments, with a field-free collision region situated between the two mass filters. Triple quadrupole instruments soon followed, employing an rf-only quadrupole as a photodissociation region. Yost and Enke designed and developed the first
computer controlled triple quadrupole system for analytical MS/MS; their early studies were performed on one of the early photodissociation systems, modified to perform collision induced dissociation (CID). Direct mixture analysis and structure elucidation were among the earliest applications of this instrument. The commercial introduction of computer controlled triple quadrupole instruments soon followed.

In triple quadrupole MS/MS systems precursor ions of a particular mass-to-charge ratio are mass selected by a first quadrupole (Q1). While passing through the collision cell (Q2) precursor ions collide with a neutral gas at a pressure of $10^{-4}$ to $10^{-2}$ torr, and transfer translational energy to internal. Fragment ions are formed as a result of unimolecular dissociation of excited precursor ions. The third quadrupole (Q3) is used for mass analysis of the product ions leaving the collision cell.

The second quadrupole (Q2) operates in rf only mode in order to function as a high pass filter and to efficiently contain ions of broad ranges of mass-to-charge ratios. Furthermore, enclosing the quadrupole in a cell allows the pressure to be high enough for multiple, low energy collisions. The ability to refocus ions scattered from collisions with neutral gases is the principal benefit of a quadrupole collision cell. One of the important features of rf-only quadrupole cells (q) is that the translational energy of the precursor ions can be kept low enough ($\sim 1$eV) that fragmentation of the precursor ion becomes insignificant compared to chemical reactions between the precursor ion and the chosen collision gas. The ability to study the energy dependence of collision-induced dissociation is another advantage of low-energy reaction cells.

The major advantages of the triple quadrupole approach to MS/MS include instrumental simplicity, ease of computer control and relatively low cost. Among the disadvantages are a limited mass range compared to time-of-flight (TOF) instruments, the inability to separate precursor ions with high resolution or to provide accurate mass measurements of ions formed in the ion source, and a restriction to low-energy CID.
The triple quadrupole system offers a degree of instrumental simplicity not found with sector tandem mass spectrometers. Quadrupole mass filters employ linear mass scan functions which greatly simplify the implementation of the variety of scan modes useful for analytical MS/MS. Rapid scan speeds and the ability to quickly switch between masses facilitate experiments on multiple reaction monitoring. Separation of ions in quadrupoles is a function of \( m/z \) only, greatly simplifying the control in MS/MS, in which the conversion of precursor ions to product ions involves a changes in both \( m/z \), and ion energy.

The combination of instrumental simplicity with the ease of automation led to the early development of computer control and data processing software. Modern triple quadrupole systems generally offer a mass range up to about 3000 amu, complete computer control and automation of all instrumental parameters.

2.5. Collision Induced Dissociation

2.5.1. General concept

In order to determine the structure of a molecule by mass spectrometry it is necessary to ionize it and induce structurally informative fragmentation by the ionization process itself or by some other means. The amount of energy deposited during ion formation often is not sufficient to cause significant fragmentation of the molecular ion. Collisional activation (CA) may be used to increase the internal energy of the ion in this case. From its introduction\(^6\)\(^\_,7\) in mass spectrometry in 1968, collision activated dissociation (CAD) has become a widely used technique in chemical and biochemical applications of MS/MS.

Two consecutive steps occurring on well-separated time scales can be distinguished in the overall CAD process. The first step, in which some portion of the initial translational energy of the accelerated ion is converted into internal energy of
both ion and target, is very fast ($\sim 10^{-15} - 10^{-14}$ sec). Dissociation of an activated ion is the second step in this process. The probability of unimolecular decomposition of the precursor ion after excitation is one of the major factors affecting the yield of product ions after CA. The rates of such reactions are usually explained by the quasi-equilibrium theory (QET)\textsuperscript{36,37} which postulates that unimolecular decomposition reactions depend upon the random distribution of the internal energy of the ion among all the vibrational modes of that ion. The probability of a given vibrational mode or modes accumulating enough energy to break bonds is related to the rate of decomposition.\textsuperscript{38}

Differences in the acquired internal energy, which in turn depend on the chosen method of activation, cause variations in fragment ion distributions.\textsuperscript{39} Factors such as dissimilar reaction times, the form of energy deposited, the amount of ion scattering upon excitation, and the different angles over which the ions are collected can also contribute to variations in fragment ion distributions.\textsuperscript{40,41}

It is important to control the amount of internal energy deposited in an ion during CID. Methods such as low\textsuperscript{33,42,43} and high-energy\textsuperscript{44} collision activation (eV and keV respectively), surface-induced dissociation\textsuperscript{45}, and photodissociation\textsuperscript{46,47} allow some measure of control over the degree of excitation of a selected ion. Further progress in the development of tandem mass spectrometry as a tool for the analysis of complex mixtures\textsuperscript{48} and for structural characterization of ions\textsuperscript{49,50} may come from an increase in the number of methods that can be used for ion activation. These include blackbody infrared radiative dissociation (BIRD)\textsuperscript{51}, infrared multiphoton dissociation (IRMPD)\textsuperscript{52}, and sustained off resonance irradiation (SORI-CAD)\textsuperscript{53} in ion cyclotron resonance (ICR) mass spectrometers.
2.5.2. Collision dynamics

The center of mass coordinate (CM) system which moves with the center of mass
of the collision partners is used for describing collisions between ions and a neutral
target. The maximum amount of energy (center-of-mass kinetic energy, \( E_{CM} \)) available
for internal excitation in an inelastic collision is given by\(^{54}\),

\[
E_{CM} = \frac{m_2}{m_1 + m_2} E_{lab}
\]

(14)

where \( m_1 \) is the mass of the ion, \( m_2 \) is the mass of the neutral gas and \( E_{lab} \) is the energy in
laboratory coordinate system. Heavy targets are preferable for efficient ion dissociation
due to more efficient use of the laboratory energy compared with lighter targets.\(^{54}\)

2.5.3. Collision energy: High vs. Low

Decomposition of the precursor ions to produce the secondary mass spectrum
can occur spontaneously. However the abundance of the product ion in such a
metastable spectrum will typically be 100-10000 times lower than that of the precursor
ion. To increase the number and absolute abundance of the product peaks it is
necessary to add energy to the precursor ions. Collision of the ion with the neutral gas
is the most convenient and widely used method. Collisional excitation is usually carried
out in one of two different energy regimes. CAD at high energies (\( >1 \) keV) is commonly
used in magnetic sector MS/MS instruments. For ions with such energies, excitation
results from a single grazing collision. However overall efficiency for converting
primary ions into secondary ions in magnetic sector instruments is reported to be about
1-10\%.\(^{55}\)

There is growing interest in dissociating large biomolecules such as proteins in
MS/MS. These large biomolecules possess a large number of internal degrees of
freedom. It has been shown that approximately 100 eV or more of internal energy is
required to dissociate 1eV bond of biomolecule on the millisecond time frame of ion passage through the collision cell of a triple quadrupole system. The calculated center-of-mass energy for the collision of a protein ion (MW 20,000) with neutral gas (MW 40) is equal to 1.99 eV. The internal energy required for ion dissociation ($E_{int}$) can be roughly estimated from the expression for the unimolecular rate constant for dissociation, $k$

$$k = v \left( \frac{E_{int} - E^0}{E_{int}} \right)^p$$

(15)

where $v$ is a frequency factor, $E^0$ is the dissociation energy, and $p$ is the number of degrees of freedom. Taking $v = 10^{12} \text{ s}^{-1}$, $E^0 = 1 \text{ eV}$, $p = 4000$ and $k = 10^3 \text{ s}^{-1}$ results in the value of $E_{int}$ equal to 193.5 eV. The thermal energy at 295 K accounts for 100 eV. Thus we need to acquire an additional 93.5 eV of internal energy through collisional activation which requires 50 collisions.

Triple quadrupole instruments are characterized by relatively low translational energy (5-200 eV) of the ions. Experiments showed that the low-energy quadrupole CAD process is characterized by very efficient fragmentation. Conversion of up to 65% of the ions into fragments, virtual elimination of ion losses even at collision pressures of 0.2 mtorr (Ar) and overall fragmentation efficiencies of 5-100% have been reported for the quadrupole confined low-energy collisions. The ability to independently control the ion energy in the low-energy CAD process provides an additional dimension in the determination of fragmentation pathways and the structures of ions. The versatility of low-energy collisions is another advantage which is not available with high-energy collisions. It includes the direct acquisition of energy resolved CID spectra and detection of ion-molecule reactions in triple quadrupole instruments. The change in the appearance of CID spectra with changing collision energy is generally more
pronounced at low energies. Varying the pressure of the collision gas also affects the appearance of the CID spectra, due to changes in the average number of collisions. The dramatic changes observed in the low-energy CID spectra with increasing ion kinetic energy result from the highly efficient transfer of kinetic energy to ion internal energy.

2.5.4. Instrumentation.

Low-energy collision-induced dissociation takes place in quadrupole reaction chambers of triple quadrupole, ion traps and hybrid sector-quadrupole mass spectrometers. The radio frequency field in the quadrupole collision chamber is typically used to contain and transmit ions. The strong focusing properties inherent in operating in the rf-only mode contribute to the very high transmission efficiency of the rf-only quadrupole. The trajectories of the ions after collision and fragmentation are stabilized due to a “collisional focusing” or “collisional cooling” effect. It was surprisingly found that in some cases ion transmission increases with an increase in pressure in an rf quadrupole. Following a series of low energy collisions, ions lose their radial and axial energy. Ions move to the minimum of the effective potential in the center of the quadrupole, followed by a loss in radial kinetic energy. Therefore more efficient transmission of ions through the exit aperture is achieved. Ions lose a large fraction of their kinetic energy and leave the rf quadrupole with a few eV spread of axial translational energy. Collisional cooling causes fragment ions to be effectively transmitted into Q3 with simultaneous improvements in resolution and sensitivity. High efficiency of ion fragmentation can be attributed to the many collisions which ions undergo.
2.5.5. Related applications.

2.5.5.1. Collision cross section measurements.

Collision cross sections allow estimates of the size of gas-phase protein ions. Information obtained via measurements of collision cross sections can be used in further studies of conformations of gas-phase proteins, thus providing insights into their structures. Modeling of the transport of ions through low density gases also requires evaluation of collision cross sections. Currently there are two methods being used routinely to determine collision cross sections of protein ions. The first, an energy loss method, originated with the work of Covey and Douglas in 1993\textsuperscript{60}, and was refined by Douglas\textsuperscript{61} in 1994 and Douglas and coworkers\textsuperscript{62} in 1997. In this method protein ions are injected into a radio frequency quadrupole where they collide with a neutral bath gas at a pressure ranging from 0.2 to 2 mtorr. The loss of the kinetic energy of the ions is measured and collision cross sections are determined as described below. The second, the ion-mobility method\textsuperscript{63,64}, is capable of resolving protein conformers on the basis of their different collision cross sections and provides an estimation of cross sections for the conformers that are present. Application of this method to measurements of collision cross sections for gas-phase protein ions was described by Jarrold and coworkers\textsuperscript{65} in 1995 and later.\textsuperscript{66,67} Protein ions are injected into a cell at a pressure of about 2-5 torr and drift under the influence of an electric field. Their arrival time distribution is recorded. "Reduced mobilities" are determined from the drift time. Measurements of the size of the ions are made possible through the relationship of the reduced mobility to the average collision integral. However the averaged collision integral is equal to the averaged cross-section only for hard sphere collisions. Moreover, the ion mobility method assumes a rigid geometry and elastic collisions. However the structure and conformations of the proteins and other biomolecules are more complex than those of simple molecules. Use of the hard sphere approximation can lead to a
substantial error in the cross section calculations. The averaged collision integral calculated using the hard-sphere approximation can be up to 20% larger than averaged collision cross sections calculated using the projection area approximation.\(^6\)

In this thesis, collision cross sections were measured using the energy loss method with a triple quadrupole mass spectrometer.\(^6\) A collision cross section is obtained by taking the negative slope of a plot of \(\ln(E/E_0)\) vs. target gas thickness \((S)\), where \(E\) is the translational energy of the ion after collisions with a target gas, \(E_0\) is the initial collision energy and \(S\) is the gas number density times the cell length.\(^6\) Results were interpreted using a diffuse scattering model.\(^6\) In this model there is complete memory loss of the incident momentum.\(^6\) The gas leaves the surface in a cosine distribution perpendicular to the surface of the object.\(^7\) In contrast, during specular scattering the component of the momentum parallel to the surface is unchanged and the component perpendicular is reversed. Diffuse scattering applies in two cases. In the first case, the gas is accommodated on the surface of the object before its re-emission. In the second, multiple collisions with concave regions of a rough surfaces lead to a pseudorandom distribution of scattering angles. Diffuse scattering has generally given the best fit to experimental data for small aerosol particles.\(^7\)

The ratio of lab energy of an ion before \((E_{\text{lab}})\) and after collision \((E'_{\text{lab}})\) with a neutral gas is given by\(^7\)

\[
\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2} - \frac{m_2 E_{\text{int}}}{M E_{\text{lab}}} + \frac{2 m_1 m_2}{M^2} \left(1 - \frac{E_{\text{int}} M}{E_{\text{lab}} M_2}\right)^{1/2} \cos \theta_{\text{cm}} \tag{16}
\]

where \(m_1\) is the ion mass, \(m_2\) is the mass of the neutral collision gas, \(E_{\text{int}}\) is the energy transferred to internal energy of the collision partners, \(\theta_{\text{cm}}\) is the scattering angle in center-of-mass coordinates, and \(M=m_1+m_2\). The simple model which was used by Covey and Douglas in the original work\(^6\), made the following approximations.
1) Collisions are elastic, so that $E_{\text{int}} = 0$

2) The average scattering angle is $90^\circ$

3) The collisions are between hard spheres

4) The thermal motion of the collision gas is neglected

Thus equation (16) transforms to

$$\frac{E'_{\text{lab}}}{E_{\text{lab}}} = \frac{m_1^2 + m_2^2}{M^2}$$  \hspace{1cm} (17)

Calculations of the energy loss of protein ions passing through a collision cell give the average ratio ($\alpha$) before and after a collision as:

$$\alpha = E_1/E_0 = E_2/E_1 = \ldots E_N/E_{N-1}$$  \hspace{1cm} (18)

so that

$$E_N = \alpha^N E_0$$  \hspace{1cm} (19)

where $E_N$ is the laboratory energy after $N$ collisions and $E_0$ is the energy with which an ion enters the collision cell.

The number of collisions $N$ according to gas kinetic theory is given by the ratio of the cell length $l$ to the mean free path $\lambda$,

$$N = \frac{l}{\lambda}$$  \hspace{1cm} (20)

where the mean free path $\lambda$ is defined as

$$\lambda = \frac{n \sigma}{1}$$  \hspace{1cm} (21)

where $n$ is the collision gas number density and $\sigma$ is the collision cross section ($\text{Å}^2$).

The energy of the ion leaving the collision cell is

$$\frac{E}{E_0} = \alpha^{\sigma nl}$$  \hspace{1cm} (22)

An exponential loss of energy with increasing target thickness is predicted by this model. To determine collision cross sections, measured energy ratios $E/E_0$ are fit to eq.(22).
The simple collision model was refined later by introduction of an aerodynamic drag model concept.\textsuperscript{61} In this concept the particle cross sectional or projection area, $A$, of an object moving through a gas with speed $v$ relates to the force through the drag coefficient ($C_D$). This led to the refinement of the calculation of the particle projection area ($A$),

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

In general the drag coefficient is dependent on such parameters as the Reynold's number (gas density, viscosity, ion speed), the Knudsen number (the ratio of the mean free path of the collision gas to the object size) and the speed ratio (ratio of the object speed to the thermal speed of the gas).

This concept was investigated and evaluated in work by Douglas and co-workers\textsuperscript{62} in 1997 by use of different collision gases and different expressions for drag coefficients from the literature. It was found that for a given charge state, collision cross sections determined with the diffuse model for the drag coefficients show a slight increase from Ne to Ar to Kr. This increase can be equally attributed either to the difference in polarizabilities for the collision gases or to a small contribution to the collision cross section from the size of the collision gas. The experimental values for protein ions measured using the ion mobility method consistently exceeded by ~25% those measured by the energy loss method interpreted with drag coefficients based on specular scattering.\textsuperscript{62} The best agreement (~5%) between cross sections measured by both methods was achieved by analysis of experimental results of both with the diffuse scattering model.

For diffuse scattering, the expression for the drag coefficient is,

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

(24)
where the speed ratio $s$ is given by the ratio of the object speed ($v$) to the thermal speed of the gas,

$$s = \frac{v}{\sqrt{2k_B T / m_2}}$$

(25)

where $k_B$ is Boltzmann's constant, and $T$ is the gas temperature.

Due to the improved agreement between cross sections obtained by the energy loss method and by the mobility methods interpreted with the "diffuse" scattering model it was suggested that this model is the most suitable to describe collisions between protein ions and a neutral collision gas.

**2.5.5.2 Simple collision model for ion activation**

Douglas and co-workers\textsuperscript{73} presented a new collision model to calculate the relative internal energies transferred to large molecules in tandem mass spectrometry. This model allows comparison of the change in the internal energy required to dissociate different charge states of a parent ion for a number of different pressures or different collision gases. The following considerations were taken into account:

1) due to a greater number of collisions at higher pressures, lower initial ion energies are needed to induce dissociation

2) collision cross sections determine the number of collisions of a precursor ion

3) For a given accelerating potential, higher absolute energies are attained by higher charge states

4) Loss of kinetic energy by the ions passing through a collision cell reduces the total energy transferred to an ion. Collision cross sections determine the extent of energy loss.

In MS/MS experiments, ions of protein complexes are injected into Q2 with increasing energies to dissociate them to individual proteins. This process is shown
schematically in Fig.6. The voltage differences Q0 - Q2 giving a 50% loss of precursor complex ions (the "dissociation voltages") are measured.

\[ E_{CM}(Z) \]

\[ E_0 \]

\[ z \]

\[ m_1 \]

\[ m_2 \]

\[ n \]

\[ l \]

**Fig. 6. Collision model for an ion activation.** \( E_0 \) is the initial kinetic energy of an ion at the cell entrance, \( m_1 \) is the ion mass, \( m_2 \) is the collision gas mass, \( Z \) is the distance travelled by the ion from the cell entrance, \( n \) is the gas number density, and \( l \) the collision cell length.

Relative internal energy, i.e. the energy transferred to an ion to cause dissociation, \( E_{int} \), is proportional to the sum of the center-of-mass collision energies for each of a series of low energy collisions and is given by:

\[
E_{int} = \frac{m_2}{M} E_0 \frac{m_1}{m_2} \frac{\phi}{C_D} \left[ 1 - \exp \left( - \frac{C_D n m_2 \sigma l}{m_1} \right) \right] \tag{26}
\]

where \( m_1 \) is the ion mass, \( m_2 \) the collision gas mass, \( M = m_1 + m_2 \), \( n \) the gas number density, \( l \) the cell length, \( C_D \) a drag coefficient, \( \sigma \) the collision cross section, \( \phi \) the average fraction of center-of-mass energy transferred to internal energy of the ion in a collision and \( E_0 \) is the energy of ion injection into Q2 (number of charges times dissociation voltage). This equation corrects for energy loss as the ions pass through the cell and also corrects for the greater number of collisions for ions with greater cross sections.
3. Materials and Methods

This chapter of the thesis describes materials, biochemical, mass spectrometric and analytical procedures which were used in the further chapters of this thesis.

3.1. Materials.

Trypsin, trypsinogen and chymotrypsin were from Worthington Biochemical Corporation (Lakewood, NJ), trypsin inhibitor from bovine pancreas (cat. # 236 624) from Boehringer- Mannheim (Indianapolis, IN, USA), α-amylase from porcine pancreas (cat. # A-3176 ), glycogen (cat # G 8751 ) and reserpine (cat. # R-0875) were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Recombinant wild type BPTI and recombinant mutant [5-55]Ala BPTI were a kind gift of M. Milhollen of the P. Kim lab. Tendamistat was produced in laboratory of Steven Withers. Methanol, acetic and hydrochloric acid, urea (cat # U15-500), TRIS (cat # BP152-1), sodium phosphate (cat # S374-500), NaCl ( cat # S271-50), CaCl₂ (cat # C79-500), ethyl ether (cat # E138-1) and acetone (cat # A949-1 HPLC grade) were from Fisher Scientific (Nepean, Ontario). Poly(propylene glycol) and DTT were from Aldrich-Chemie (Steinheim, Germany). Ethanol was from Commercial Alcohols Inc (Richmond, B.C.). All the above mentioned materials except α-amylase were used without further purification. Gases were Praxair (Mississauga, Ontario,) Ne UHP grade (99.996% manufacturer' stated purity), Ar Linde grade (99.9995% manufacturer' stated purity ), Kr research grade ( 99.995% manufacturer' stated purity ), and N₂ UHP grade ( 99.999% manufacturer' stated purity ). The pressure in the collision cell was measured with a precision capacitance manometer (MKS model 120, manufacturer's stated precision 0.12% of reading).

3.2. Reduction of disulphide bonds
BPTI was reduced by incubating a mixture of 0.4 milliMolar (mM) protein, 5 mM Tris-HCl (pH 8.7), 40 mM urea and 40 mM DTT at 45°C for 15 minutes. Because salt concentrations greater than ~ 0.1 mM can interfere with the ionization process, the solution was diluted for ESI. A 100 ml sample from the reaction mixture was resuspended in 900 ml of a water/methanol (9/1) buffer (pH 3, glacial acetic acid buffer). ESI mass spectra were obtained immediately after the reduced protein was added to the water/methanol solution.

3.3. Purification of α-amylase

Highly purified amylase, in the form of the enzyme-glycogen complex, can be isolated on a microscale, based on the highly specific binding of the enzyme to its substrate and isolation of the resulting complex\textsuperscript{74,75}. Enzyme essentially free of other proteins is thus obtained in a single purification step. Glycogen and its hydrolysis products which are still bound to the enzyme can be subsequently removed by enzyme precipitation and washing. The overall procedure is as follows. Most of the operations are carried out at 0-4 °C.

\textit{Step 1.} One gram of crude sample of α-amylase was dissolved in 16 ml of water, stirred and centrifuged for 10 minutes at 10,000 g at 3 °C. The clear supernatant containing the enzyme was decanted and kept on ice. A 40\% ethanol/water mixture was added to reduce the amylase concentration to 2000 units/ml.

\textit{Step 2.} Phosphate buffer 0.2 M (pH 8.0) was added to a final concentration of 0.01 M. Glycogen reagent (110 mg of glycogen in 987 μl of water) was then introduced in the ratio 1 mg glycogen/500 enzyme units, followed immediately by 96\% ethanol to a final concentration of 40\%. Thorough stirring maintained throughout the various additions was continued for 5 min after addition of the ethanol. The mixture was centrifuged for 5 minutes at 2600g. The precipitate containing enzyme-glycogen complex was washed
twice with 40% ethanol containing 0.01M phosphate buffer (pH 8.0). About 1 ml of buffered ethanol was added per mg glycogen for the first wash and 0.5 ml/mg glycogen for the second wash.

**Step 3.** The washed precipitate of the enzyme-glycogen complex was suspended in 0.02 M phosphate buffer (pH 6.9) containing 0.007 M NaCl and 0.003 M CaCl₂ to a final volume of 5 ml. The mixture was kept at 25 °C for 1 hour to digest glycogen. The mixture was centrifuged at 20 °C for 5 minutes at 6000 g. A small amount of precipitate of unknown nature was removed. The mixture was cooled and kept at 0 °C for 30 minutes. The enzyme precipitate obtained after centrifugation at 9000 g for 20 minutes at 2 °C was washed with distilled water and resuspended at room temperature.

**Step 4.** For each 10 ml of resuspended enzyme solution, 9 ml of acetone was added followed by 21 ml of an ethanol-ether (1:1) mixture. Solvent addition was performed with mixing over a period of 20 minutes, the mixture being kept at -10 °C. Stirring was subsequently continued for another 15 minutes. The resulting suspension was centrifuged at -5 °C for 20 minutes at 2600 g. Ten milliliters of distilled water was added to resuspend the pellet containing the enzyme. The suspension was stirred for about 15 minutes at room temperature. The mixture was centrifuged at 20 °C for 5 minutes at 6000 g. The precipitate, which was apparently of lipid nature, was removed.

**Step 5.** The enzyme solution was precipitated at 0 °C, extracted in water and reprecipitated as described in the fourth step. The enzyme precipitate in the centrifuge tubes was thoroughly drained. Distilled water was gradually added and suction was applied for further removal of solvent. Distilled, deionized water was added to resuspend the protein to a final concentration of 100 µM. The solution was stored at 4°C.

### 3.4. Formation of Noncovalent Complexes
Trypsin, chymotrypsin, trypsinogen and α-amylase (100 μM) were incubated overnight in the refrigerator at 4 °C in aqueous solution at pH 7 with a 5-fold excess of BPTI and tendamistat respectively. ESI mass spectra were obtained immediately after the solutions were diluted by a factor of 10 with water.

3.5. Electrospray Ionization-Mass Spectrometry

![Diagram of a triple quadrupole mass spectrometer](image)

Fig. 7. Schematic of the triple quadrupole mass spectrometer. Q0, rf only quadrupole; PF, prefilter; Q1, mass analyzing quadrupole; Q2, rf only quadrupole; Q3, mass analyzing quadrupole.

ESI-MS was performed with the triple quadrupole mass spectrometer shown in Fig. 7. Protonated ions formed by pneumatically assisted ESI\textsuperscript{9,15} pass through a flow of dry nitrogen gas and a sampling orifice into a region with a background pressure of 2 torr. Ions are collisionally cooled to an energy spread of 1 eV or less in a radiofrequency only quadrupole Q0, maintained at a pressure of 7 mtorr. Quadrupole Q1 was used for mass analysis of the ions. The pressure in Q1 was about 8 μtorr. Mass calibration was performed using poly(propylene glycol) ions and confirmed by the mass measurement of reserpine (MH+, 609.7). For MS experiments samples of BPTI were infused using a syringe pump (Harvard 22, South Natic, USA) at 3 μl/min. The mass spectrometer was
scanned from $m/z = 600$ to 1800 in 1 $m/z$ unit steps and at 10 ms per step. To detect the 6 Dalton (Da) increase in mass of reduced BPTI, the step size was reduced to 0.1 $m/z$. The same solution conditions were used for each protein: water/methanol (9/1) adjusted to pH 3, 4 mM urea, 0.5 mM Tris-HCl, 0.04 mM BPTI. In addition, the solution of reduced BPTI contained 4 mM DTT. Samples were run as native BPTI, recombinant wild type, recombinant [5-55]Ala BPTI, and reduced BPTI. Solutions of the protein-protein complexes were infused at 1 µl/min. The mass spectrometer was scanned in 2 $m/z$ unit steps and at 5 ms per step from $m/z = 900$ to 4000 for BPTI-tryptic protein complexes and from $m/z$ 800 to 6000 for the α-amylase-tendamistat complex. MacSpec and Bio-MultiView software packages (Perkin-Elmer SCIEX, Concord, Ontario) were used in the data analysis and in reconstruction of the molecular weight from the spectra.

3.6. *Tandem Mass Spectrometry*

Tandem mass spectrometry experiments were performed on the triple quadrupole mass spectrometer described above. The potentials of the ion path during MS/MS experiments are shown in the Table 1.

Ions of a particular mass-to-charge ratio were mass selected in quadrupole Q1 and injected into the collision cell (Q2) and a collision gas at a pressure of 0.1-1 mtorr, where they undergo collisional activation followed by dissociation. Fragment ions are mass resolved in quadrupole Q3 before reaching the detector. The collision gases used were neon, argon and krypton and the collision energies (determined by the difference between the Q0 and Q2 potentials) for precursor and fragment ion scans were adjusted for each experiment.
Table 1. Voltages used in MS/MS experiments

<table>
<thead>
<tr>
<th>Element</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprayer needle</td>
<td>4500</td>
</tr>
<tr>
<td>Curtain plate</td>
<td>1000</td>
</tr>
<tr>
<td>Orifice</td>
<td>120</td>
</tr>
<tr>
<td>Skimmer</td>
<td>110</td>
</tr>
<tr>
<td>Q0 rod offset</td>
<td>103</td>
</tr>
<tr>
<td>Q0/Q1 lens</td>
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</tr>
</tbody>
</table>

Samples were infused using a syringe pump at 1 μl/min and analyzed in the positive ion mode. The mass spectrometer was scanned from m/z = 900 to 4000 in 2 m/z unit steps and at 5 ms per step for all MS/MS experiments. MS/MS signals were optimized for parent ions.

3.7. Nano-ESI-MS

The pneumatically assisted electrospray source was replaced by the nanoES source from Protana (Odense, Denmark). No nebulizer gas or sheath flow was employed. A low solution flow rate was achieved with gold-coated capillaries drawn with a short taper to a fine tip of ~1 μm inner diameter. Clean (manufacturer's stated purity 99.999%) nitrogen was used as the curtain gas at a flow rate of 0.2-0.6 L/min. The
sprayer voltage was 1600-1700 V, the skimmer voltage was typically 110 V, and the orifice potential was 120 V.

The sample (7-10 μL) was injected directly into the open end of the glass capillaries using long, thin, gel-loader tips (Eppendorf, Hamburg). The capillary was mounted in an air-tight stainless steel capillary holder with O-ring fittings to hold the capillary without damaging it. The holder was mounted on a small bracket which in turn attached to the arm of the Sciex "articulated ion spray" source and pushed forward to position the spraying needle 1-2 mm from the front of the curtain plate.

To prepare for spraying, the needle was briefly touched against the curtain plate with all potentials at ground and then centered in front of the mass spectrometer's orifice. A static air pressure of ~0.8 bar was applied at the capillary holder via an air-filled syringe which caused a small droplet to appear at the tip of the needle. Experiments are started with the instrument scanning and the voltage at about 1 kV. The voltage was gradually increased to 1600-1700 volts until a stable signal was observed and then it was adjusted for maximum signal-to-noise ratio. The air pressure was then reduced to avoid forcing the flow for the Nanospray. The necessary pressure was adjusted according to the mass spectrometric signal so that a constant stable flow was achieved. A new capillary was used for each experiment.

3.8. Cross section measurements

Collision cross sections were measured with a triple quadrupole mass spectrometer as described previously. Ions formed by electrospray are collisionally cooled in the radio frequency quadrupole (Q0) to an energy spread of 1 eV or less. Exiting the quadrupole Q1 (set to transmit all ions) the ions enter a collision gas cell (Q2) filled with Ne. The Q2 rods are set at an offset potential 10 volts less than the potential of Q0 to give an initial collision energy $E_0$ of 10 eV/charge. Quadrupole Q3 is
used to select the ion mass-to-charge ratio and the rod offset is used to determine the translational energy lost due to collisions. The signal from the ions leaving Q2 was reduced by approximately two orders of magnitude by increasing the Q3 rod offset voltage in steps of 1V. Energy distributions were determined from stopping curves obtained for several values of target gas thickness. The rod offset on Q3 required to stop the protein ions is measured at several different pressures of neon. The potentials of the ion path during energy loss experiments are shown in the Table 2.

Table 2. Voltages used in energy loss experiments

<table>
<thead>
<tr>
<th>Element</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprayer needle</td>
<td>4500 - 5500 (ESI)</td>
</tr>
<tr>
<td></td>
<td>1600-1700 (NanoESI)</td>
</tr>
<tr>
<td>Curtain plate</td>
<td>1000</td>
</tr>
<tr>
<td>Orifice</td>
<td>120</td>
</tr>
<tr>
<td>Skimmer</td>
<td>110</td>
</tr>
<tr>
<td>Q0 rod offset</td>
<td>103</td>
</tr>
<tr>
<td>Q0/Q1 lens</td>
<td>102</td>
</tr>
<tr>
<td>Prefilter 1</td>
<td>0</td>
</tr>
<tr>
<td>Q1 rod offset</td>
<td>101</td>
</tr>
<tr>
<td>Q1/Q2 lens</td>
<td>100</td>
</tr>
<tr>
<td>Q2 rod offset</td>
<td>93</td>
</tr>
<tr>
<td>Q2/Q3 lens</td>
<td>83</td>
</tr>
<tr>
<td>Prefilter 2</td>
<td>83</td>
</tr>
<tr>
<td>Q3 rod offset</td>
<td>Varied</td>
</tr>
<tr>
<td>Exit</td>
<td>0</td>
</tr>
<tr>
<td>Deflector</td>
<td>-270</td>
</tr>
<tr>
<td>Detector</td>
<td>-5000</td>
</tr>
</tbody>
</table>

42
Energy losses of the ions were related to their cross section, $\sigma$, by the expression\(^{60}\)

$$\frac{E}{E_0} = \exp (-\sigma S \ln \alpha) \quad (27)$$

where $\alpha$ is the average ratio of energy loss before and after collision, $\sigma$ is the collision cross section, $E_0$ is the kinetic energy of the ion entering the collision cell, $E$ is the kinetic energy of the ion leaving the collision cell, $S = n l$ is the target thickness, $n$ is the gas number density and $l$ is the cell length.

Determination of the collision cross section was performed by fitting the experimental energy loss to this equation at each target thickness. The energy at which the ion intensity was decreased to one/tenth of its original value, i.e. with no stopping potential, was taken as a measure of the average ion energy. In interpreting the experimental data, this value was interpolated from the stopping curves and is referred to as $E_{1/10}$. The values of $E_{1/10}$ taken from stopping curves were fit to equation (27) and a cross section was obtained. The particle projected area ($A$) was calculated by multiplying the collision cross section value by $2/C_{Dd}$ (eq.23). Drag coefficients ($C_{Dd}$) were calculated from the equations of Stalder and Zurick.\(^62\)

The collision gas was neon. The time from ion formation to ion detection was a few milliseconds. Conformation changes of the gas phase protein ions taking place on much longer time scales would not be detected with this apparatus. All experiments were done with room temperature samples, 21± 2 deg. C.

**3.9. Analysis of MS/MS experiments and Calculations**

Analysis of the tandem MS results was done using the Kaleidagraph\(^\circledR\) program (Synergy Software, PA). Spectra were exported into it and areas under the peaks were
integrated. Due to a significant level of background in most MS/MS spectra the following procedure was employed for analysis of experimental data. Areas of peaks of product ions corresponding to individual proteins were summed and normalized to 1 at a value of the difference between R0 and R2 corresponding to the full decomposition of the precursor ion. Subtraction of the normalized areas of the product ions from 1 gives an estimate of the area corresponding only to precursor ions at different experimental values of the accelerating potential (difference between R0 and R2). Non-linear regression analysis was used to draw the best fit curves for data presented in the figures. Calculations of the changes in internal energy were performed using a program written in Matlab® software (The MATH WORKS Inc., MA).
4. PROBING PROTEIN CONFORMATIONS BY ESI-MS.

One of the challenges facing application of ESI-MS to the study of biomolecules is the question of the correlation of the solution and gas phase structures. The first approach to this problem was to study individual biomolecules, such as proteins, by ESI-MS and see whether it is possible to detect changes occurring in their structure in the gas phase using folding and unfolding conditions in solution. This chapter of the thesis describes the study of individual proteins by ESI-MS. Following the introduction and description of the proteins used in this study (Sections 4.1 and 4.2 respectively), the effect of the changes in the pH of the solution and addition of denaturing agents (methanol, urea) on the charge state distributions of the individual proteins (BPTI, ubiquitin) are described in Section 4.3 and 4.4. Studies of the changes in charge state distributions and the collision cross sections of BPTI upon disulphide bond reduction follow in the Section 4.5. Finally, the collision cross sections of gas phase ions of native, reduced and mutant BPTI were measured and the degree of BPTI unfolding was estimated and compared with data from the literature for BPTI in solution (Section 4.6).
4.1. ESI-MS as a tool to study protein conformation

In order to understand and correctly predict the role of proteins in biological systems it is important to know their three-dimensional structures which are dictated by the amino acid sequence. A number of denaturation techniques have been employed to affect native protein conformation in ESI studies. These include reduction of disulphide bonds\cite{76,77}, change of the solution pH\cite{78,79}, increases in temperature\cite{80,81,82} and addition of organic solvents\cite{83,84}. Changes in higher-order protein structure in solution affect the charge state distribution of gas phase protein ions formed by ESI\cite{77,85}. Denatured proteins in solution usually produce higher charge states in the gas phase than proteins in the native state\cite{86,87,88}. However the physical basis for this relationship is not clear. Attempts have been made to rationalize, that for an unfolded protein in solution, an increase in the surface area\cite{89} leads to a greater number of accessible protonation sites. Changes in their steric accessibility, along with changes in $pK_a$ values\cite{77}, and reduced Coulombic repulsion of the protons on the proteins\cite{83,88} can also play a significant role in this event.

The net charge of proteins in solution depends on the combination of several factors. The number of basic and acidic residues in the particular protein, the extent to which the protein conformation affects the ionization of these residues, and the pH of the solution are among them. Although the basic residues are the most probable sites for protonation in solution, the situation in the gas-phase may be different. Evidence has been presented that sites with lower apparent proton affinity (glutamine residues) may be protonated depending upon protein conformation\cite{77}. It has been shown that proton transfer reactions taking place during the ESI process\cite{90,91} can also influence the appearance of charge state distributions. Reactions between a gas phase base (dimethylamine) and multiply charged protein ions demonstrated that deprotonation
rate constants increased with increasing charge state of the reactive ion. An experiment using 1,6-diaminohexane as a reactive base showed that ions bearing the highest number of charges were the most reactive. It was noted that following the transfer of a certain numbers of protons from the analyte to the base, the remaining analyte bound protons became unreactive. This was attributed to reduced coulombic repulsion between neighboring charges, and an increase in basicity of the remaining proton-bearing sites on the analyte molecule.

The number of possible protonation sites is the principal factor affecting the multiple charging observed in ESI mass spectra. Increases in relative molecular mass ($M_r$) of the analyte protein typically lead to an increase in the number of recorded charges within the same mass/charge ratio range, thus extending the mass range of the mass spectrometer. Most peptides and proteins showed relatively good correlation between MW and the maximum positive charge state observed in the ESI mass spectrum. The larger the MW of the protein the higher the charge state that is observed. An approximate linear correlation was observed initially between the number of charges and the number of basic amino acid residues (e.g. arginine, lysine, and histidine). Gas-phase proton affinity data also supported this observation as such basic amino acids as arginine, histidine, and lysine have the highest proton affinities. However poor correlation was lately shown for disulphide-rich proteins due to the presence of cysteine-cysteine linkages. For example mass spectra of hen egg lysozyme and bovine α-lactalbumin each having four disulphide bridges, and bovine albumin possessing 17 disulphide bridges showed a lower number of charges than was expected based on the number of basic residues available for protonation. This was attributed to the inaccessibility of some basic residues which were “buried” inside a tighter conformation imposed by disulphide bonds. This argument was supported by a drastic shift in the charge state distribution of the same proteins towards higher
numbers of charges and lower mass/charge ratio upon reduction of the disulphide bonds.\textsuperscript{77} The maximum protein charge state may be constrained by limitations imposed by the secondary or tertiary structure. Accordingly, reduction of disulphide bonds allows the protein to extend its conformation and acquire more positive charges.\textsuperscript{77}

Negative ion formation by ESI has been demonstrated for a variety of molecules with carboxylic, phosphoric, and sulfonic acid groups.\textsuperscript{97,98} Proton abstraction produces negative, multiply charged protein ions. ESI mass spectra of mono- and oligonucleotides\textsuperscript{98} in the negative mode have been reported in the literature and a distribution of multiply charged molecular anions for proteins with $M_r$ up to 66,000 have also been reported.\textsuperscript{96} Initially it was thought that ESI of most polypeptides in neutral and acidic aqueous solutions would not afford multiply charged anions in the negative mode because the $pK_a$ for such acidic residues as glutamic and aspartic acid is 5 or less.\textsuperscript{96} However recent studies show that one can choose the operation mode (negative or positive) irrespective of the charge state of the analyte in solution.\textsuperscript{99,100} Positive ions have been generated from negatively charged proteins in solution and vice versa.\textsuperscript{101} The detailed mechanism of ion formation under such conditions is still unclear.

Ultimately the number of charges on the positive or negative protein ions produced in ESI-MS is related to the number of basic or acidic amino acid side chains, respectively, on the surface of the protein. The charge states of these amino acid residues in solution are related to their $pK_a$ values and solvent accessibility which both depend to a large extent on the conformational state of the protein. It is now generally believed that instrumental conditions, solvent effects, types of counterions present, analyte concentration and conformation along with gas-phase modifications and solution-phase equilibrium considerations play an important role and can significantly influence the maximum charge state in the spectra.\textsuperscript{102}
The molecular structure and the three-dimensional conformation of an ion can also significantly influence the ability to retain charge. Hydrogen/deuterium (H/D) exchange along with such methods as circular dichroism\textsuperscript{103,104}, fluorescence\textsuperscript{105,106}, and NMR\textsuperscript{107,108,109} have been used to probe protein structure and conformation in solution. Monitoring H/D exchange in solution in real time by mass spectrometry can provide important information\textsuperscript{110,111} about solution protein structure due to the fact that external hydrogens are more likely to be exposed to the deuterated environment. Katta and Chait demonstrated the difference in a number of exchangeable hydrogens between native and reduced lysozyme in the gas phase\textsuperscript{76,83}. Wagner and Anderegg demonstrated the complementarity of hydrogen exchange and ESI-MS charge state distributions for detecting protein conformations of cytochrome c\textsuperscript{112}. The mass spectra of both thermally denatured in water solution and native cytochrome c at pH 2.7 produced bimodal charge state distribution suggesting the co-existence of folded and unfolded forms. These studies demonstrated that different solution conformations of proteins resulted in different charge state distributions.

However it can be wrong to draw conclusions about conformational changes based solely on the shift in charge state distribution in ESI mass spectra. It has been reported that a substantial change in protein conformation resulted in only a slight change in charge state distribution.\textsuperscript{113} Studies on cytochrome c showed that "despite the different conformations of the protein in solution" confirmed by circular dichroism, fluorescence and absorption spectroscopy "the acid denatured states for the two" different "methanol concentrations show very similar mass spectra. This indicates that the charge state distribution generated during ESI is not sensitive to the differences in the secondary structure of the denatured protein. The observed transition from low to high charge states is due to the breakdown of the tertiary structure in both cases."\textsuperscript{113}
The study of conformational changes of the protein in the presence of organic solvent and solvents of different pH can also be complicated by so-called "secondary solvent effects". Changes in the solvent properties during ESI can significantly influence the spraying process and impose changes in charge state distribution which may not be related to conformational changes in the protein.

From the discussion of results presented above, it is evident that the charge state distributions found in ESI mass spectra of proteins may be directly related to the net charge they carry in solution. Denaturation studies of proteins as a function of pH, temperature, or solvent conditions demonstrated the ability to differentiate between the native and denatured states of the proteins. H/D exchange and cross section measurements suggested that certain elements of native protein conformation are preserved in the gas-phase. Thus ESI charge distributions may be used in some cases to detect conformational changes in proteins.

The production of intact gas-phase protein ions during the ESI process initiated discussion of the structures of these ions, such as the extent to which the conformations of gas-phase protein ions resemble their solution conformations. Recent studies, probing the gas-phase conformations of ions by H/D exchange, proton transfer reactivity and high-energy surface-impact collisions present evidence for tight, highly compact conformation of the studied gas phase proteins, although the exact nature of these conformations is still a matter of debate. Collision cross section measurements can provide valuable information about gas-phase conformation of protein ions. Covey and Douglas measured collision cross sections for a number of proteins ranging from motilin (2.7 kDa) to bovine serum albumin (66 kDa). Jarrold and co-workers measured collision cross sections for conformers of cytochrome c. Both ion mobility and energy loss method show that cross sections of protein ions in gas-phase increase with an increase in charge. ESI mass spectra of the proteins under denaturing
conditions produced ions with larger cross sections for the same charge state which were consistent with a more denatured conformation.

One of the major conclusions from the above review is that protein conformation is of the greatest importance for determining the charge state distribution. In order to test this hypothesis simple denaturing techniques such as addition of organic reagents and reduction of disulphide bonds were used to change solution conformations of model, very well studied, proteins. Changes in the charge state distributions of the gas phase ions were correlated with protein folding state in solution. Changes in the protein conformation in the gas phase ions were evaluated using collision cross section measurements.

4.2. Selected proteins used in this study.

4.2.1. BPTI

Bovine pancreatic trypsin inhibitor (BPTI) is perhaps one of the best-characterized proteins. Its stability against denaturation (thermal, acid, or base) combined with its small size make it a particularly attractive model system for experimental and theoretical studies of globular proteins. Such studies included molecular dynamics simulations, normal mode calculations, extensive nuclear magnetic resonance analysis, amide hydrogen exchange kinetics and protein folding studies. Recent successful production of BPTI variants by site-directed mutagenesis promises the further development of BPTI as the model for small globular proteins.

Wild type bovine pancreatic trypsin inhibitor (BPTI) depicted in Fig.8 is a relatively small (58 residues, M.W. ~ 6511) very stable protein with a well characterized native structure.
The main structural elements of native BPTI are a two stranded $\beta$-sheet and an $\alpha$-helix, which are stabilized by three disulphide bonds between residues 30-51, 5-55, and 14-38.\textsuperscript{88,131} The folding of BPTI and the contributions of the disulphide bonds to its stability have been the subject of much experimental work.\textsuperscript{125,127} The crystal structure shows that BPTI binds 4 internal water molecules. The disulphide bonds in BPTI can be removed by reduction with reagents such as dithiothreitol (DTT). In addition, mutants with one or two native or non-native disulphide bonds can be prepared by site-directed mutagenesis\textsuperscript{128,129} or other techniques.\textsuperscript{126,132}

Samples of native and reduced BPTI along with recombinant wild type and recombinant mutant "$[5-55]_{\text{Ala}}$BPTI" were used in this work. Mutant $[5-55]_{\text{Ala}}$ BPTI was engineered by removing the 14-38 and 30-51 disulphide bonds. This was done by replacing the four cysteine residues with alanine.\textsuperscript{133}

4.2.2. Bovine ubiquitin

Bovine ubiquitin (Fig.9) is another tightly folded protein however without disulphide bonds. It is very resistant to denaturation and consists of 76 amino acid residues (M.W. 8565). The extreme stability of the protein has been attributed to the pronounced hydrophobic core and the fact that some 90% of the residues in the
polypeptide chain appeared to be involved in intramolecular hydrogen bonding.\textsuperscript{134} Extensive mass spectrometric investigations of conformational changes in ubiquitin induced by changes in pH, organic solvent\textsuperscript{83} and temperature\textsuperscript{86} have been carried out.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Fig_9.png}
\caption{Bovine ubiquitin.}
\end{figure}

Heat-induced conformational changes in ubiquitin have been previously investigated by nuclear magnetic resonance measurements.\textsuperscript{135,136} The absence of disulphide bonds in its structure, tight packing, relatively low MW and resistance to denaturation makes it an attractive partner for comparison to BPTI and studying changes of the protein conformations.
4.3. Effect of the pH on charge state distributions

Samples of BPTI were dissolved in aqueous solutions with pH values adjusted with hydrochloric acid between 7 and 2 in steps of 1 pH unit and ESI mass spectra were recorded. Some of these spectra are shown in Fig. 10.

![Fig. 10. ESI mass spectra of 10 μM BPTI recorded at different pH. The pH was adjusted by addition of hydrochloric acid. Numbers denote the charge states of protein ions.](image)

At pH 7 BPTI produces charge states from +4 up to +7 with a maximum at the +6 charge state. No pronounced changes in the charge state distribution were observed for pH 5 and 3. However at pH 2 only three charge states are observed instead of four and the position of the maximum shifts somewhat surprisingly towards the lower charge state, +5. Mass spectra showed several other peaks in addition to the peaks from BPTI. Their presence, especially at masses higher then protein, can be partially attributed to the relatively high level of concomitant salts. Partial decomposition and impurity of the
commercial sample may account for the other peaks, especially those with masses less than the protein.

Samples of bovine ubiquitin were dissolved in aqueous solutions of different pH between pH 7 and 2 with steps of 1 pH unit adjusted with hydrochloric acid and ESI mass spectra were recorded. Some of these spectra are shown in Fig. 11.

Fig. 11. ESI mass spectra of bovine ubiquitin recorded at different pH. Samples of ubiquitin were dissolved to 10 μM in the aqueous solutions adjusted to different pH values with hydrochloric acid. Numbers denote the charge states of protein ions.

At pH 7, ubiquitin produces charges states ranging from +5 up to +9 with a maximum intensity at +7. Slight changes in the charge state distribution were observed for pH 5 and 3, mainly the appearance of charge state +10. However the overall appearance of the charge state distribution and the position of the maximum at +7 remained unchanged. At pH 2 once more, as with BPTI, there is narrowing of the
charge state distribution. A shift to the lower charge states is observed and the maximum shifts to the +6 charge state.

4.4. Effect of organic solvents on charge state distributions

ESI mass spectra of BPTI samples dissolved in water (pH 7) and 100% methanol (pH 6.5) show almost identical charge state distributions (Fig.12). Charge states ranging from +4 up to +7 with a dominance of +6 were observed in both cases.

![Fig. 12. ESI mass spectra of 10 μM BPTI recorded in water and methanol. Numbers denote the charge states of protein ions.](image)

A narrowing of the charge state distribution along with a slight shift in the position of the maximum were observed for BPTI in the presence of urea (Fig.13).

![Fig. 13. ESI mass spectra of BPTI in different concentrations of urea. Numbers denote the charge states of protein ions.](image)
Charge states ranging from +4 up to +6 with a maximum at the +5 charge state were produced in the presence of urea in comparison with maximum at +6 for a water solution. There is an approximate ten-fold decrease in signal intensity with the increase in urea concentrations from 0 through 8 mM to 80 mM.

ESI mass spectra of bovine ubiquitin in solutions with different ratios of methanol and water showed significant changes in the charge state distributions (Fig. 14). Charge states ranging from +5 up to +9 with a maximum intensity at +7 were recorded for a water/methanol (9 vol/1 vol) solution. Drastic shifts in the charge state distribution and the position of the maximum were observed for a methanol/water (9 vol/1 vol) solution. Charge states ranging from +6 up to +13 were observed with a maximum intensity at +10.

Fig. 14. ESI mass spectra of bovine ubiquitin recorded in different methanol/water solutions.
Numbers denote charge states of protein ions. Samples of ubiquitin were dissolved to 10 μM.

4.5. Reduction of the disulphide bonds

Figures 15 (a-d) show mass spectra of native BPTI, recombinant wild type, recombinant mutant [5-55] Ala BPTI and reduced BPTI respectively.
Molecular weights determined from these spectra were 6511 ± 2 for native and recombinant wild type, 6520 ± 2 for [5-55] Ala BPTI and 6517 ± 2 for reduced BPTI (M.W. calculated from the sequence 6512, 6521 and 6518 respectively). An increase in mass of 6 ± 1 Da. for the reduced BPTI was observed, confirming that all three disulphide residues were reduced. Native BPTI produced charge states +4 to +7, recombinant wild type BPTI charge states +3 to +7, [5-55] Ala BPTI produced these charge states and in addition a minor peak at +8, and reduced BPTI produced charge states up to +9. In addition to the peaks from the protein ions, the solutions of native BPTI, recombinant wild type and reduced BPTI showed several other peaks in the mass spectrum. These probably derive from relatively high levels of concomitant salts which can give additional peaks. For example some of the additional peaks in Fig. 15 correspond to an increase in protein mass of 97 Da and are likely due to adduction of
sulfate or phosphate groups. Other peaks corresponding to masses less than that of the protein may be attributed to the partial decomposition and impurity of the commercial sample. These additional peaks did not interfere with measurements of the cross sections because they differ significantly in m/z from the BPTI ions. Native BPTI and reduced BPTI did not produce sufficiently reproducible signals for the +3 ion.

4.6. Cross section measurements

4.6.1. Typical example of collision cross section calculations

Stopping curves were obtained for ions of BPTI with charges +4 - +9 (m/z 1627, 1302, 1086, 931, 814, 724 respectively). Representative stopping curves for the +4 charge state are shown in Fig. 16.

![Stopping curves for native BPTI + 4 ions (m/z 1627). Pressures were 0, 0.25, 0.5, 0.75, 1 mtorr of Neon.](image)

Fig. 16. Stopping curves for native BPTI + 4 ions (m/z 1627). Pressures were 0, 0.25, 0.5, 0.75, 1 mtorr of Neon.

All stopping curves show a sharp decrease of intensity, with a potential change of 1V on Q3. The E_{1/10} energies for BPTI +4 to +7 charge states at each target thickness and a linear fit to the data are shown in Figure 17.
It is obvious from this figure that ions of different charge state show different energy losses which in turn derive from different collision cross sections. Cross sections for all four charge states of BPTI ions are shown in Table 3. Particle projected area ($A$) was calculated from the collision cross section obtained from the simple hard sphere model ($\sigma$) by multiplying by $2/C_{Dd}$. Drag coefficients ($C_{Dd}$) were calculated from equation 24.

**Table 3. Values of speed ratios, drag coefficients, and collision cross sections for 4 charge states of BPTI gas phase ions**

<table>
<thead>
<tr>
<th>Charge state</th>
<th>Speed ratio</th>
<th>$C_{Dd}$</th>
<th>$2/C_{Dd}$</th>
<th>$\sigma$, Å²</th>
<th>$A$, Å²</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td>2.212</td>
<td>2.9221</td>
<td>0.684</td>
<td>700</td>
<td>479</td>
</tr>
<tr>
<td>+5</td>
<td>2.473</td>
<td>2.7915</td>
<td>0.716</td>
<td>802</td>
<td>574</td>
</tr>
<tr>
<td>+6</td>
<td>2.709</td>
<td>2.6994</td>
<td>0.741</td>
<td>817</td>
<td>605</td>
</tr>
<tr>
<td>+7</td>
<td>2.926</td>
<td>2.6306</td>
<td>0.760</td>
<td>849</td>
<td>645</td>
</tr>
</tbody>
</table>
4.6.2. Collision cross sections for BPTI samples

Collision cross sections for each of the protein ions of Figure 15 are shown in Figure 18 and listed in Table 4. The averages of five separate experiments done on different days are shown. Results were reproducible to 5% (one standard deviation) for measurements over a period of three months. Typical uncertainties are shown by the error bars on the figure.

![Graph showing collision cross sections vs. charge state for BPTI samples]

**Fig. 18.** Collision cross sections vs. charge state for BPTI samples. Native BPTI (■), mutant [5-55]Ala (○), reduced BPTI (●).

For a given charge state, reduced BPTI gives greater collision cross sections than native BPTI, while [5-55]Ala BPTI gives cross sections intermediate between them.

**Table 4. Cross sections for BPTI samples**

<table>
<thead>
<tr>
<th>Charge State</th>
<th>Cross section, Å²</th>
<th>Native</th>
<th>Mutant [5-55]Ala</th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td>531</td>
<td>572</td>
<td>565</td>
<td></td>
</tr>
<tr>
<td>+5</td>
<td>535</td>
<td>590</td>
<td>655</td>
<td></td>
</tr>
<tr>
<td>+6</td>
<td>548</td>
<td>593</td>
<td>678</td>
<td></td>
</tr>
<tr>
<td>+7</td>
<td>619</td>
<td>681</td>
<td>718</td>
<td></td>
</tr>
<tr>
<td>+8</td>
<td></td>
<td>696</td>
<td>793</td>
<td></td>
</tr>
<tr>
<td>+9</td>
<td></td>
<td></td>
<td>810</td>
<td></td>
</tr>
</tbody>
</table>
Cross sections for recombinant wild type and native BPTI agreed within 3% on average. For Table 4 the cross sections for these have been averaged and are listed as "native". In all cases the cross sections increase with charge state. This increase is seen generally for gas phase protein ions and is likely due to increased Coulomb repulsion in the more highly charged ions. For reduced BPTI, the experimental cross sections are 17% greater than those of native BPTI, averaged over charge states +4 to +7 (i.e. for those charge states where comparisons are possible). Mutant [5-55]Ala BPTI gives cross sections 8% greater than native BPTI for the +6 ion or 9% averaged over charge states +4 to +7, intermediate between native and fully reduced BPTI. These observations show that removing disulphide bonds reduces the stability of BPTI ions in vacuo, but that the ions still retain compact structures.
4.7. Discussion

4.7.1 Charge states

It is now well established that proteins unfolded in solution produce higher charge states in ESI mass spectra than folded proteins.\textsuperscript{88,92,93} However this correlation is qualitative; the relations between protein charge state and solution conformation is unknown.

Effect of pH on charge state distribution

The charge state distributions for both BPTI and ubiquitin showed only minor changes between pH 7 and 3. Moreover at pH 2 the charge state distributions for both proteins surprisingly shifted towards lower charge states. The presence of the relatively high concentrations of urea (8 and 80 mM) caused a significant drop in the intensity of the BPTI signal with no effect on the charge state distribution. The stability of BPTI and ubiquitin towards changes in pH is not surprising as it is well known that both proteins are extremely resistant to denaturation. In the case of BPTI, this is largely attributed to the presence of three disulphide bonds.\textsuperscript{137,138} Dense packing of the ubiquitin’s hydrophobic core along with a tight network of hydrogen bonds is believed to be responsible for the stability of its native conformation.\textsuperscript{134,136}

A shift in charge state distribution in acidic conditions can be attributed to the combined effects of a higher degree of solution protonation of proteins in a given conformation along with the unfolding of tertiary structure of proteins at low pH. A surprising shift of the charge state distribution towards higher \textit{m/z} values at pH 2 or less has been reported previously.\textsuperscript{139} Ionization changes in this range are possible only for the protonation of a terminal carboxylic group. This cannot account for a net decrease in a positive charge state. Mirza and Chait explained this by a two step process. The first step is association of anions with protonated groups of the protein in
solution and the second collision-induced dissociation of the acid from the protein during the ion sampling process. However such phenomenon as acid-induced refolding for some proteins observed below the pH 2 can play some role as well. A decrease of the charge repulsion of the positively charged protein in solution due to binding of anions is believed to be responsible for this phenomenon.

Effect of denaturing agents on charge state distribution

Comparison of the effect of organic solvent on ESI mass spectra of both proteins showed significant differences. The ESI mass spectra of BPTI in water and in methanol were identical. In contrast mass spectra of ubiquitin recorded in a 90/10 mixture of methanol and water were different from the mass spectra in the presence of a 10/90 methanol/water mixture. A shift in the charge states distribution and in the position of the maximum towards lower m/z values was observed. Heat, extremes of pH, and the presence of guanidinium chloride have previously been shown to cause significant shifts in ubiquitin's charge state distribution towards higher charge states, indicating possible loss of ubiquitin native structure. The destabilizing effects on the structure of ubiquitin of such organic solvents as methanol in combination with pH have also been reported previously. Unfolding of ubiquitin, unlike BPTI, was recorded in 60 % water/40 % methanol mixtures at pH 4.0.

Effect of protein conformation on charge state distribution

It is known that both BPTI and ubiquitin undergo substantial changes in charge state in solution within the pH range studied here. Protonation of amino-acid side-chains is attributed to the acid-induced unfolding of proteins in solution. It is also known that in many cases the protonation pattern in solution does not correlate with charge states observed in ESI MS. Generation of positively charged gas phase ions from negatively charged analyte solution is the most dramatic example of this. The disparity between ESI and solution charge states is clearly illustrated in the following
example. ESI mass spectra of ubiquitin at neutral pH produces charge states ranging from +5 up to +9 with the maximum at +7. Because the isoelectric point of ubiquitin is 6.7 the overall charge in the solution protein is close to zero at this pH. Thus it has been suggested that unless the protein conformation remains unchanged, one can't expect changes in the ESI charge state distribution due to the changes in solution charge states. In other words, the positive ion ESI charge state distribution of a protein is not determined by the charges the protein carries in solution, but rather by the conformation of the protein in solution. Consequently the ESI charge state distribution in the positive mode reflects the changes of the protein conformations in solution and can be used to detect and study those changes.

Recent work with cytochrome c suggests that the charge state distribution in ESI is most sensitive to the tertiary structure of the protein. Native and recombinant wild type BPTI produced charge states +4 to +7 in the spectrum. Mutant [5-55] Ala BPTI produced spectra very similar to wild type BPTI suggesting it has a similar, presumably near-native, conformation in solution. This is in accord with nuclear magnetic resonance (NMR) measurements that show that this single disulphide bond is sufficient to cause BPTI to fold into its native conformation. However [5-55] Ala BPTI did show an additional small peak for the +8 ion, suggesting it may be slightly unfolded in solution. Reduced BPTI shows the additional charge states +8 and +9 indicating that it has partially unfolded under the solution conditions used here.

4.7.2. Comparison of cross sections to mobility data

Cross sections, or more correctly "collision integrals", "Ω", for native BPTI have been determined by ion mobility. The best agreement between cross sections derived from collision integrals determined by mobility and cross sections derived from energy loss experiments is obtained if a "diffuse scattering" model is used to interpret the data.
in each case. For the mobility experiments this requires reducing the collision integral \( \Omega \) by a factor of 0.74 to obtain a geometric cross section or projection area. For the energy loss experiments it requires using a drag coefficient for diffuse scattering, suitable for a "rough" surface. For both mobility and energy loss experiments this procedure gives values for cross sections that can be compared directly to projection areas of proposed structures. Using this procedure, the cross sections measured here agree reasonably well with the mobility measurements, the values here being about 12% smaller (Table 5).

**Table 5. Comparison of cross sections for native BPTI measured by Energy loss and Ion Mobility methods**

<table>
<thead>
<tr>
<th>Charge State</th>
<th>Cross section, ( \text{Å}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>+4</td>
<td>531</td>
</tr>
<tr>
<td>+5</td>
<td>535</td>
</tr>
<tr>
<td>+6</td>
<td>548</td>
</tr>
<tr>
<td>+7</td>
<td>619</td>
</tr>
</tbody>
</table>

Differences may arise from the combined uncertainties of the experiments, different approximations to the collision dynamics in the different energy regimes of the two experiments, or differences in the degree of collisional activation of the ions in the ion sampling region of the different instruments.

**4.7.3. Comparison to cross sections from the crystal structure and from radii of gyration**

For native BPTI, the measured collision cross sections can be compared to those expected from the crystal structure and to those calculated from the radius of gyration, \( r_g \), determined by either x-ray scattering from solutions or from molecular dynamics (MD) simulations. These estimates are summarized in Table 6.


Table 6. Comparison of $r_g$ and cross sections

<table>
<thead>
<tr>
<th>Method</th>
<th>$r_g$, Å</th>
<th>Cross section, Å$^2$</th>
<th>Native</th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal projection</td>
<td></td>
<td>767$^{67}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>x-ray (solution)</td>
<td>10.4$^{149}$</td>
<td>566</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.D. (solution)</td>
<td>10.65$^{120}$</td>
<td>11.9$^{151}$</td>
<td>594</td>
<td>741</td>
</tr>
<tr>
<td></td>
<td>11.5$^{121,123}$</td>
<td>12.3-12.6$^{122,150}$</td>
<td>692</td>
<td>792-831</td>
</tr>
<tr>
<td>random coil</td>
<td></td>
<td>3142-</td>
<td>5031</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.5-31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.D. (in vacuo)</td>
<td>10.12$^{120}$</td>
<td>536</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.93$^{121,123}$</td>
<td>625</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Shelimov et al.$^{67}$ have calculated the projection area from the crystal structure II (SPTI) as 767 Å$^2$, significantly greater than the cross sections observed here. The mobility experiments also give cross sections less than this value. The radius of gyration $r_g$ calculated from solution x-ray scattering is 10.4 Å which gives a calculated cross section $(5/3\pi r_g^2)$ of 566 Å$^2$. The experimental cross sections here are in reasonable agreement with this value and show that gas phase native BPTI retains a compact structure similar in size to that of BPTI in solution. Recent measurements show that addition of a first water of hydration to gas phase BPTI gives a significant reduction in the entropy of the protein. It would be of interest in future work to see if this is accompanied by a change in collision cross section.$^{153}$

Molecular dynamics simulations$^{120}$ give values of $r_g$ for native BPTI in solution of 10.65 Å or about 11.5 Å which correspond to cross sections of 594 Å$^2$ and 692 Å$^2$. 

67
respectively. These molecular dynamic simulations are for an ion with +6 charges and can be compared directly to the experimental data for the +6 ion, although the charge sites may differ. Simulation of BPTI \textit{in vacuo}\textsuperscript{120} with 6 charges shows that the radius of gyration decreases from 10.65 to 10.12 Å or from 11.5 to 10.93 Å. These values of \( r_g \) give cross sections for BPTI +6 \textit{in vacuo} of 536 Å\textsuperscript{2} and 625 Å\textsuperscript{2} respectively.\textsuperscript{121,123} The experimental cross section for native BPTI +6 is 548 \( \pm \) 30 Å\textsuperscript{2}, at the low end of these estimates from theoretical models. The simulations include four bound waters within the protein whereas the experimental value here applies to the protein without water. It is possible that without bound water the protein can adopt a somewhat more compact configuration \textit{in vacuo}.

For reduced BPTI in solution, radii of gyration from molecular dynamics calculations\textsuperscript{149} are 11.9 Å and 12.3-12.6 Å giving cross sections 741 Å\textsuperscript{2} and 792-831 Å\textsuperscript{2} respectively.\textsuperscript{122,149} These are 5% to 9% greater than those of native BPTI calculated with the same procedures and correspond to an increase in area of 10% to 19% over native BPTI. The experimental cross sections show an increase of 24% for the +6 ion, and 17% averaged over charge states +4 to +7. Thus the gas phase ion shows an increase in cross section similar to that calculated in molecular dynamics simulations.

There are several estimates for the radius of gyration of BPTI in a random coil. The results of Miller and Goebel\textsuperscript{154} give \( r_g = 24.5 \) Å. Using the relation \( 6 \, r_g = 130 \times \text{number of residues} \), of Tanford\textsuperscript{155} and correcting for the finite length of the chain\textsuperscript{155}, Pan \textit{et al.}\textsuperscript{156} calculated \( r_g = 31 \) Å. BPTI contains 6 glycine residues. For a coil with 10% glycine residues Tanford\textsuperscript{155} gives \( 6 \, r_g = 90 \times \text{number of residues} \). Correcting for finite length then gives \( r_g = 25.8 \) Å (similar to the result of Miller and Goebel). Estimates of the cross section for a random coil then range from 3143 Å\textsuperscript{2} to 5032 Å\textsuperscript{2}. These are much greater
than the observed cross sections and further demonstrate the compact nature of the gas phase ions.

4.7.4. Comparison to Areas Determined from Hydrodynamic Radii

Hydrodynamic radii, $r_h$, for BPTI, have recently been measured by NMR.\textsuperscript{156} This has been done for native BPTI, for [14-38]Abu BPTI which retains the 14-38 disulphide bond but has the other cysteine residues replaced by $\alpha$-amino-$n$-butyric acid (Abu), and [R]Abu BPTI, where all the disulphide bond have been removed by replacing cysteine residues with Abu. Hydrodynamic radii were found to increase at pH 2.5 relative to pH 4.5. The radii increase in the order native BPTI<[14-38]Abu<[R]Abu and demonstrate that the single 14-38 bond can contribute to a more collapsed structure in solution.

Table 7. Relative areas calculated from gas phase cross sections and hydrodynamic radii in solution

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Cross Sections</th>
<th>Hydrodynamic Radii</th>
</tr>
</thead>
<tbody>
<tr>
<td>native BPTI</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>[5-55]$_{\text{Ala}}$ BPTI</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>reduced BPTI</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>[14-38]$_{\text{Abu}}$ pH 4.5</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>[14-38]$_{\text{Abu}}$ pH 2.5</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>[R]$_{\text{Abu}}$ pH 4.5</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>[R]$_{\text{Abu}}$ pH 2.5</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>random coil</td>
<td>5.6-9.0</td>
<td>1.67-2.68</td>
</tr>
</tbody>
</table>

Direct comparison of $r_h$ to $r_g$ or cross sections is difficult because the relationship depends on protein structure. For spheres $r_h > r_g$ but for a random coil $r_h < r_g$.\textsuperscript{156} Despite this limitation, relative areas calculated from $r_h$ are compared to relative gas phase cross sections in Table 7. Protein areas were calculated from\textsuperscript{157} $A = \pi(r_h - 2.8)^2$ to allow for the
contribution of a monolayer of solvent to the hydrodynamic radius.\textsuperscript{156} If the hydrodynamic radii can be used to calculate areas in this way, the results suggest BPTI unfolds less in the gas phase than in solution when disulphide bonds are removed.

4.7.5. Comparison to lysozyme

These results for BPTI can be contrasted with the behavior of lysozyme ions \textit{in vacuo}. Lysozyme (M.W. 14,306) contains four disulphide bonds. Reduced lysozyme produces much higher charge states in ESI (+10 to +17) than native lysozyme (+8 to +10) showing that in solution the reduced form has unfolded substantially.\textsuperscript{157,158} There is little overlap between the charge state distributions of the reduced and native proteins. For the +10 charge state, where a direct comparison is possible, the reduced protein gives a cross section about 70\% greater than the native protein.\textsuperscript{158} In addition, gas phase basicities of reduced lysozyme correspond to those expected for a fully denatured protein.\textsuperscript{157} Both these studies indicate a greater degree of unfolding in the gas phase for lysozyme than BPTI upon reduction of the disulphide bonds. However reduced lysozyme could be refolded to more compact structures by lowering its charge state in the gas phase.\textsuperscript{158}

4.8. Summary

- The data presented here once more demonstrate the stability of BPTI and ubiquitin towards changes in pH. This stability is evident from the lack of the changes in charge state distributions. However comparison of the effect of methanol on ESI mass spectra of ubiquitin and BPTI showed significant differences. Mass spectra of BPTI were identical both in water and in methanol, while mass spectra of ubiquitin show essential shifts in the charge state distribution.
- The results of collision cross section measurements show that the disulphide bonds of BPTI contribute to the folding of the gas phase ions, but that even in the absence
of disulphide bonds, the protein ions maintain compact structures. In solution the disulphide bonds contribute to the stability of the native conformation of BPTI. Removal of the disulphide bonds progressively lowers the midpoint temperature of the unfolding transition.\textsuperscript{159,160} However the remaining van der Waals forces, hydrogen bonds, electrostatic and hydrophobic interactions also contribute substantially to the stability of this protein. Fluorescence measurements show reduced BPTI retains a compact structure even in strongly denaturing solutions such as 6 M guanidine hydrochloride.\textsuperscript{161,162} NMR studies demonstrate that this form of BPTI contains non-random structure.\textsuperscript{155,162}

- The ESI spectra show an increase in the charge on the protein suggesting reduced BPTI has partially unfolded under the solution conditions here. Only a modest increase in the size of the gas phase ions is seen upon removal of the disulphide bonds. Molecular dynamics simulations however show that this compact structure in solution can undergo comparatively large fluctuations\textsuperscript{149} and that the structure is stabilized by non-native salt bridges.

- Comparison to areas calculated from hydrodynamic radii suggest that in solution BPTI unfolds more than the gas phase ion when disulphide bonds are removed. Future molecular dynamics simulations comparing the protein behavior \textit{in vacuo} and in solution may contribute to understanding this difference between solution and vacuum behavior and provide additional insights to the folding of BPTI.

- The results presented in this chapter demonstrated the ability of ESI-MS to detect and evaluate changes occurring in protein conformations in solution. It was demonstrated that the relative changes in charge state distributions of gas phase ions are related to changes in their tertiary structure in solution. Consequently the charge state distribution of the gas phase protein ions formed by ESI reflects the changes of the protein conformations in solution and can be used to detect and
study those changes with advantages in speed, sensitivity and selectivity comparing to other techniques.

5. Noncovalent Interactions studied by Nano- & ESI-MS.

The results presented in Chapter 4 demonstrate that ESI - MS can be used to study protein conformation in solution. The next step in the application of ESI - MS to the study of biomolecules is to look into the interaction between them. In order to do this it is necessary to detect complexes between biomolecules in the gas - phase. This chapter shows the potential of Nano- and ESI-MS to detect a number of noncovalent protein-protein complexes. A brief introduction to the subject of noncovalent interactions (Section 5.1 ) is followed by the specifics of the detection of noncovalent complexes in the gas phase and the particular examples of noncovalent complexes detected. (Section 5.2. and 5.3 respectively). Noncovalent complexes used in this study are introduced in the Section 5.4. of this chapter followed by their detection by Nano- and ESI-MS described in Section 5.5 of this chapter.
5.1. Protein - ligand noncovalent interactions

Ionic interactions, hydrogen bonds, hydrophobic effects, and Van der Waals interactions are four types of noncovalent forces which are generally involved in noncovalent interactions. Proteins, peptides, small molecules, metal ions, lipids, polysaccharides, nucleic acids and oligonucleotides interact with each other thus governing the activity and function of a living system. Specific, non-covalent interactions between these biomolecules are crucial to maintain proper functioning in a number of processes inside a cell. These include gene transcription and translation, cell division, cell signaling, ion transport, and homeostasis.

The interaction of proteins and ligands is characterized by their affinity towards each other, which can be used as a measure of the overall free energy change of the interaction. The affinity between a protein and a ligand can be estimated using the dissociation equilibrium constant \( K_D \) for the binding reaction.

\[
P + L \rightleftharpoons P\cdot L \\
K_D = \frac{[P\cdot L]}{[P][L]} \tag{28}
\]

The lower the value of the dissociation constant the higher the affinity. The observed affinities of proteins for ligands vary drastically from very high \(10^{14} \text{ M}\) to very low values (less than \(10^6 \text{ M}\)).

The energetics of binding is expressed by the Gibbs free energy of binding.

\[
\Delta G^0 = -RT\ln K_D \tag{29}
\]

The values of the dissociation constant \( K_D \) or the standard free energy of dissociation \( \Delta G^0 \) are measures of the thermodynamic stability of protein-ligand complexes. The balance of several large terms favoring or opposing complex formation is reflected by the free energy of dissociation. Loss of translational, rotational and internal degrees of freedom resulting in entropy losses combined with the cost of the free energy from any
conformational rearrangements that might occur are among the major factors resisting protein association. Hydrophobic energy, gained from the surfaces buried in the recognition sites and electrostatic energy from the hydrogen bonds contribute to the formation of the complex.\textsuperscript{164} Considerable discussion and some dispute are devoted to the magnitude and relative importance of the different interactions.\textsuperscript{165,166}

One of the very interesting types of noncovalent interactions is the specific and tight binding of proteins with each other generating large complexes. Some of the binding proteins may act as inhibitors, regulating the activity of their target proteins. Each inhibitor is characterized by its inhibition constant $K_i$ which is usually the same as the dissociation constant of the inhibitor from the enzyme. The stability of a noncovalent complex formed between two proteins derives from the burying of hydrophobic surface residues and the formation of favorable polar interactions between the proteins. The interface region between two proteins is packed as tightly as the interior of the interacting proteins.\textsuperscript{164}

Several kinetic studies have been performed on a number of different protease-inhibitor complexes.\textsuperscript{167,168} Association rates in these studies varied little, and affinity changes appear to depend mostly on changes in dissociation rate constants.\textsuperscript{169,170} Association rate constants can be compared with the collision rate between protein and inhibitor molecules. One in 100 of the collisions leads to association in the complex with the highest affinity and one in 10,000 in the complex with lowest affinity. It was implied that most collisions at the target surface for such proteins as trypsin, BPTI and lysozyme lead to stable association due to the fact that the interface covers only 5-20\% of the protein surface. The following mechanism for formation of a complex was proposed as a result of the kinetic studies.\textsuperscript{171} Initially, a loose complex is formed by a fraction of the possible interactions followed by isomerization of the complex to the stable structure with the correct interface packing.
In this thesis, protease-inhibitor complexes are studied. In all the protease-inhibitor complexes studied so far, generally 10-15 residues in the inhibitors form contacts with 17-29 residues in the proteases.\textsuperscript{164} This asymmetry arises from the shapes of the surfaces involved. The active sites and specificity pocket in the protease surface form a long groove which fits to an extended loop of the inhibitor.\textsuperscript{172} The buried surfaces of the individual proteins in the recognition sites form about 20\% of the total accessible area of the smaller proteins (BPTI)\textsuperscript{165} and some 5\% of larger proteins, such as carboxypeptidase.\textsuperscript{173} The total buried areas in all the studied protease-inhibitor complexes is about 1600 ± 350 Å\textsuperscript{2}. Generally the areas involved in subunit interfaces in oligomeric proteins are much larger than those for protease-inhibitor complexes.\textsuperscript{174} The largest reaches 5000 Å\textsuperscript{2} per monomer,\textsuperscript{175} while the smallest is comparable in size to protease-inhibitor complexes.\textsuperscript{174} Both subunit association and the tertiary structure of the individual subunits are stabilized as a result of such large interface areas.\textsuperscript{175} More hydrophobic residues and fewer hydrogen bonds are involved in the interfaces in oligomeric proteins compared with those proteins involved in functional recognition.\textsuperscript{174}

One of the important characteristics of a protein-protein complex is its flexibility, or the lack of it. The presence of rigid preformed binding loops on inhibitors promote their efficient inhibition of proteases.\textsuperscript{165,172} The binding loops become partly immobilized in the complexes while their flexibility is comparable to the rest of the polypeptide chain for a free inhibitor.\textsuperscript{176} In contrast mobile regions on the antigens' protein surface are easily adaptable to the antibodies' binding sites. Several structures of the individual proteins forming protease-inhibitor complexes have been determined. Comparison of these structures with the structures of the same components in the complex shows that protein-protein association involves small conformational changes although recognition sites lack high mobility.\textsuperscript{173,177} Close packing and hydrogen bond formation is facilitated by these changes. Significant conformational changes are seen in the formation of the
trypsinogen-inhibitor complexes. Trypsinogen is characterized by a partially disordered region that forms the specificity pocket. This region becomes fully ordered following either the proteolytic activation of trypsinogen or upon inhibitor binding.\textsuperscript{178}

5.2. Detection of noncovalent complexes by ESI-MS

ESI-MS\textsuperscript{179,180} is complementing such widely established methods as NMR and X-ray crystallography\textsuperscript{181}, spectroscopy\textsuperscript{182}, analytical ultracentrifugation\textsuperscript{183,184} and differential scanning calorimetry\textsuperscript{183} for studying non covalent interactions with advantages in speed, sensitivity and mass accuracy using significantly less quantities of the material.

Figure 19 depicts the process of solvent evaporation during transfer of a noncovalent complex from solution to the gas-phase.

![Fig. 19. ESI-MS of noncovalent protein complexes. The process of solvent evaporation during transfer of a noncovalent complex to the gas-phase.](image)

Spectroscopic and fluorescence data can indicate changes in three-dimensional structure. Unparalleled high resolution structures of biomolecules and noncovalent complexes from the solution and solid phase can be produced by NMR and X-ray crystallography respectively. However these techniques require relatively large quantities of precious materials and in addition are slow. Furthermore NMR is limited to analysis of the proteins with molecular weights less than 50 kDa and not all proteins
are readily crystallized which imposes some limitations on the application of X-ray crystallography. Analytical ultracentrifugation is also capable of measuring the MW of the analyte material. However its mass accuracy does not compare well with MS. At the same time MS is in principle capable of providing important stoichiometric information about non covalent interactions with great accuracy using much less material and in a much shorter time.

ESI interface conditions are important for the observation of gas phase ions of noncovalent complexes as most complexes are relatively fragile. Typically, the gentlest possible conditions are required to observe non-covalent complexes. Orifice-skimmer voltages and, in some instruments the temperature of the ion inlet capillary can be adjusted to preserve noncovalent complexes in the interface region. An increase in the orifice potential results in an increase in the internal energy of the complex during the desolvation process. This leads to desolvation of the molecular ions along with dissociation of noncovalent complexes. Generally, detection of the noncovalent complexes involves a compromise between providing adequate ion desolvation while trying to minimize the effects of complex dissociation i.e. there is a fine line between sufficient desolvation and dissociation of the gas-phase complex. The nature of the interactions of noncovalent complex, the rate of heating, the time spent by the ion in the interface region and the relative stabilities of the nonspecific complexes compared to specific associations are among factors affecting the extent to which a complex may be activated and preserved at the same time.

Any higher-order structure must be examined close to physiological conditions, near neutral pH, as the presence of organic solvents and acidic conditions generally used in conventional ESI-MS experiments may lead to denaturation of biopolymers. One of the primary questions concerning noncovalent associations is the determination of the specificity of observed complexes. It is of great importance to distinguish
structurally specific noncovalent interactions present in solution from nonspecific interactions which may occur during the electrospray ionization process itself. A number of different approaches can be used to determine the specificity of noncovalent interactions and distinguish possible artifacts. Among them are changes in solution conditions, competition experiments, and analysis of specificity based on changes in pH and concentrations. Any disruption of the complexes occurring in the solution due to the change in the solution conditions should be reflected in the mass spectra. A particular stoichiometry of the gas-phase complex is also a major condition for recognizing the specificity of a noncovalent complex. Modifications of the interface conditions can also be effectively used to positively identify specific non-covalent interactions by observing ions of specific complexes of a particular stoichiometry over ions formed due to random aggregation. Chemical modification of one of the complex components should provide further convincing evidence of the specificity of observed noncovalent interactions. Chemical modification affects the formation of the complex in solution and results in changes to the mass spectra. MS/MS experiments can provide valuable information based on the ease of dissociation of observed complexes and additional information on composition can be gained based on the dissociation of a complex to distinct subunits.

5.3. Brief survey of the literature

A number of excellent reviews has been written on the detection of noncovalent complexes by ESI-MS. Protein-ligand, protein-protein, protein-nucleic-acid, protein multimers, and antibody-antigen complexes are examples of intermolecular noncovalent complexes which have been studied by ESI-MS. Detection of conformational changes, measurement of relative dissociation constants, and discrimination between cooperativity and sequential metal binding to
metalloproteins\textsuperscript{207} along with accurate mass measurement and stoichiometry of noncovalent complexes open new horizons for application of ESI-MS. A few selected examples that illustrate the types of interactions that can be observed will be briefly discussed here.

Observation of intact noncovalent complexes in the gas phase raised the question of whether the structures of the gas phase and solution complexes resemble each other and in particular how well gas phase binding energies correlate with solution binding constants. A number of different approaches using MS have been used to provide an answer on this question. Titration\textsuperscript{208,209,210} and competitive binding experiments\textsuperscript{208,211,212} on a number of systems have shown that the relative abundances of the gas-phase ions reflect solution binding constants.

Work of Collings and Douglas on conformations of gas-phase holomyoglobin (Mb) ions showed a relatively compact structure which unfolds upon loss of a heme group.\textsuperscript{152} Unfolding of the Mb was achieved by the increase in orifice-skimmer potential (section 2.1.1) from 30V through 110V and up to 180V. However the extent of unfolding is rather moderate (ca.1.2 times in area) compared to the increase (ca 2x) seen for the higher charge states formed from denatured apomyoglobin (apoMb) in solution. For a given charge state holomyoglobin ions have smaller cross sections than those measured for apoMb ions. Thus cross sections provided direct evidence of unfolding of the gas-phase Mb ion when heme is lost. "The moderate increase in cross section on loss of heme from gas-phase Mb ions is qualitatively similar to the moderate increase in cross section observed on loss of heme from Mb in solution determined by small-angle X-ray scattering and size exclusion chromatography."\textsuperscript{152} However the authors pointed out that "detailed comparison between solution and gas-phase cross sections is difficult, particularly for apoMb, because the mass spectral data describe individual charge states and it is not clear how to compare these directly with solution measurements."\textsuperscript{152}
The stability of the binding of the heme group in wild type and variant forms of Mb in the gas phase was studied and compared to the solution behaviour. A series of protein - heme complexes was constructed in which the hydrogen-bonding interactions between the heme and apoprotein were removed systematically. A positive linear correlation of the orifice-skimmer potential required to dissociate the heme-protein complexes with the activation energy for dissociation in solution was observed for wild type holoMb and series of proteins with a reduced number of hydrogen bonds between the heme propionate groups and protein residues. This correlation suggested that heme-protein interactions known to exist in solution are retained in the gas-phase ions in these experiments. However the opposite behaviour i.e. destabilization in solution and stabilization in the gas phase was observed for the protein-heme complex with hydrogen bonds to the proximal histidine removed. It was suggested that "this difference could conceivably reflect a different dissociation mechanism in the gas phase".

Studies of noncovalent interactions using competitive binding experiments have been reported previously. Investigation has been carried out on a number of different systems. Among them are protein-peptide, protein-drug, oligonucleotide-drug. In all three cases peaks representing gas phase ions of the host-ligand complex with the higher affinity were noticeably more abundant. This method provided an estimate for the relative binding affinity. In addition absolute binding affinity can be determined by titration experiments. In these experiments the relative abundance of the noncovalent complexes is monitored with increasing ligand concentrations. Protein-peptide, protein-oligonucleotide and antibiotic-peptide systems were used in these studies. Data from ESI-MS experiments were used to construct Scatchard plots to determine the binding constants. In all three cases a reasonable agreement with previously reported solution based values was reported for gas phase measurements. Reported agreement
validated the application of ESI-MS to study noncovalent interactions and provided further evidence and support to the structural correlation between solution and the gas-phase complexes.

Surprisingly the study of protein-protein interactions is a relatively new application for ESI-MS despite the increasing number of publications describing noncovalently bound enzyme complexes. These studies have principally been concerned with the binding of relatively small molecules to enzymes, or with protein-protein interactions in natural oligomers. The binding of peptides to calmodulin is among the rare examples of protein-peptide interactions studied by ESI-MS. Calmodulin is a well characterized Ca\(^{2+}\)-signaling protein which binds some peptides in a Ca\(^{2+}\)-dependent manner. ESI-MS was used to examine the Ca\(^{2+}\)-dependent noncovalent complex formed between calmodulin and mellitin and calmodulin binding peptide from calmodulin-dependent protein kinase II. In both cases it was observed that binding of Ca\(^{2+}\) by calmodulin was necessary for the formation of the protein/peptide complex.

The determination of the number of subunits in the quaternary structure of proteins can be achieved easily by ESI-MS. For example intact specific complexes for multimeric complexes for concavalin and streptavidin were detected using solution conditions close to physiological (pH 5.7-6.8) and mild ESI interface conditions. Observations of tetrameric complexes were consistent with predicted solution behavior. Surprisingly these proteins exhibit relatively low charge states, considering their large MW. This can be attributed to the compact higher order structure of the multimer protein complex. Nonspecific forms of aggregation such as trimers and pentamers which might be formed under some interface conditions during the ESI process were not observed. It was found that thermal or collisional activation in the interface region readily dissociates these tetrameric species, confirming their noncovalent nature. The
detection of the inactive hexameric structure of insulin stored in the pancreatic β-cells is among more recent examples of the application of ESI-MS to the study of multimeric proteins. The charge acquired by a complex may be much lower than the charge observed under denaturing conditions, resulting in m/z values beyond the range of most quadrupole or ion traps instruments. Use of unlimited m/z range of TOF spectrometers was particularly helpful in this case. Standing and coworkers used a TOF instrument coupled with ESI to study the oligomeric structure of the enzyme 4-oxalocrotonate tautomerase (4OT), monomer with MW 6810 Da. Preliminary gel-permeation and ultracentrifugation experiments indicated the presence of the pentamer in solution. In contrast ESI-TOF studies demonstrated the existence of the hexamer in solution at a neutral pH consistent with X-ray crystallography data. However modification in solution pH and declustering voltage resulted in a loss of the hexamer and appearance of a relatively stable pentamer in addition to dimers, trimers, and tetramers. Quadrupole mass analyzers which have been modified for high m/z transmission, FTMS and magnetic sector instruments have been used to detect multimeric protein complexes as well.

The binding of BPTI and soybean trypsin inhibitor (SBTI) with trypsin has been investigated by ESI-MS in a study of protein-protein interactions between two different proteins. Partial dissociation of the noncovalent complexes was observed at high interface "cone voltages". However lowering of this voltage produced good signals from the noncovalent complexes. The charge state distribution on the trypsin and trypsin inhibitors (BPTI, SBTI) produced by gas-phase dissociation of the complexes were "markedly different" from those of the components alone. It was suggested that ESI-MS can provide a novel probe for exploring the ionic interactions at the contact surface of the proteins. At the same time, the question of the gas-phase structure of these complexes was not addressed.
Although detection of noncovalent protein complexes formed between multimeric complexes was routine, detection of noncovalent complexes formed between two different proteins has been reported only once. Thus it was of real interest to evaluate detection of noncovalent protein-protein complexes formed between two different proteins by Nano- and ESI sources. This allows evaluation of the properties of these ions in the gas phase using tandem mass spectrometry methods.

5.4. Selected noncovalent complexes used in this study

5.4.1. Noncovalent interactions between BPTI and its target enzymes.

The interaction of BPTI and its target enzymes (trypsin, chymotrypsin, trypsinogen) has been studied extensively. The target specificity of BPTI, belonging to the serine protease family of inhibitors, is a function of the chemical nature of the amino acids present in the regions of the active-site loops that form the enzyme interface. Trypsin, chymotrypsin and trypsinogen have similar molecular weights but different affinity for BPTI in solution thus forming a system of natural mutants which become an attractive model for studying protein-protein interactions. Figure 20 depicts crystal structures of noncovalent complexes formed between BPTI and trypsin, chymotrypsin, and trypsinogen.

Dissociation constants show that the trypsin-BPTI complex is the most stable in solution, trypsinogen-BPTI complex has the weakest binding, and the complex between chymotrypsin and BPTI is of intermediate stability. The main chain of the principal binding loop of BPTI (residues 11-19) forms a section of intermolecular antiparallel β-sheet structure with the enzyme binding pocket. Five main chain H-bonds stabilize this sheet structure. Productive contact of the inhibitor with the enzyme surface is attributed to the inhibitor’s side chain residues.
Trypsin and chymotrypsin catalyze peptide bond cleavage by identical mechanisms\(^{219}\) and possess very similar tertiary structures.\(^{172}\) However there are significant differences in size, shape, and hydrophilicity of the trypsin and chymotrypsin binding pockets.\(^{220}\) Only two of the five binding sites have the character of a real pocket. The rest of the subsites are formed from relatively shallow surface openings. Chymotrypsin has a preference for large hydrophobic residues in its binding sites, whereas trypsin prefers generally hydrophilic residues with a significant percentage of them being small.\(^{220}\)

The structure of trypsinogen does not exclude the possibility of substrate binding in a mode similar to that found for trypsin.\(^{178}\) Bovine trypsinogen has a large and accessible cavity at the site where the native enzyme binds specific side chains of a
substrate. Surprisingly, even though the structural appearance of trypsin-BPTI and trypsinogen-BPTI interfaces are almost identical, their stabilities differ drastically from each other. Conformational changes and alterations in the binding site induced in trypsinogen upon binding of the inhibitor are prime candidates for explaining the relative instability of trypsinogen compared to trypsin. The substrate binding site of trypsinogen is organized quite differently from that of the activated proteinase and is disordered. The reorganization of the activation domain upon inhibitor binding is similar to that found upon activation cleavage and follows an induced-fit mechanism. The free energy needed to force ordering of the disordered trypsinogen segments of the activation domain is provided in part by the free energy of binding, with a reduction in affinity.

5.4.2. The α-amylase - tendamistat system

Fig. 21. α-Amylase-Tendamistat noncovalent complex. The drawings is made from coordinates in the Brookhaven Protein Data Bank file 1 BVN.
α-Amylases hydrolyze the α-(1,4) glycosidic linkage in starch, amyllose, glycogen and other polysaccharides. The ability of α-amylase to hydrolyze glycosidic bonds is widely utilized in the food and starch processing industry and has made it one of the most used enzymes in modern biotechnology.\textsuperscript{222} This enzyme is widely distributed in microorganisms, plants and animal secretions. Porcine pancreatic α-amylase (PPA) consists of 496 amino acid residues and has a MW 55,377. It has a high degree of homology with human, mouse and rat amylases in the amino acid sequences.\textsuperscript{223} Specific inhibitors of α-amylases are of interest in a number of different applications. These include mechanistic studies of the enzymes themselves, studies of glycoprotein processing, and potential therapeutic uses. Several naturally occurring non-covalent inhibitors of amylases are known, including the microbial inhibitor, tendamistat (MW 7,952).\textsuperscript{224} In contrast to BPTI, it has only two disulphide bridges, which are non-overlapping. Loops created by these bridges are of significantly different size. The tertiary structure is characterized by two β-pleated sheets each consisting of three antiparallel strands.\textsuperscript{225} Recently the structure of porcine pancreatic α-amylase in complex with the microbial inhibitor tendamistat (74 aminoacid residues) has been reported.\textsuperscript{226} The complex has a very low dissociation constant $10^{-12}$ M, consists of 567 aminoacid residues and has a MW of 63,329. The inhibitor tendamistat binds in an extended groove in the α-amylase. The interaction is mediated by four segments.\textsuperscript{226} The low value of dissociation constant and high inhibitory activity can be attributed to interactions involving these four different regions. The head of tendamistat binds directly to the active site region, blocking the central five sugar binding subsites.\textsuperscript{227} Tendamistat used in this study was genetically engineered and modified in the laboratory of Steven Withers by addition of 15 His aminoacid residues at the end of the
sequence for expression purposes, which brings the MW of tendamistat to 9495 and MW of the complex to 64,872.

The binding of tendamistat to α-amylase was first described in detail by Wiegand\textsuperscript{226} in 1995. Slow progressive inhibition of α-amylase upon tendamistat binding was demonstrated by Vertesy \textit{et al.} whose work implied the cooperative character of binding of different segments.\textsuperscript{224} The pronounced specificity towards mammalian α-amylases and the unusually high binding constants is due to the complex binding behaviour. Binding of tendamistat to α-amylase can be attributed to a remarkable ability of α-amylase to accommodate tendamistat, which does not exhibit any major conformational changes upon its binding to α-amylase. Interactions involving four different regions of α-amylase are believed to cause the very low dissociation constant of 10^{-12} M.

Crystallography data showed that the first segment of tendamistat (Trp18, Arg19, Tyr20) along with three additional regions that are in tight contact with the α-amylase molecule are essential for the strong inhibitor interaction.\textsuperscript{226} The area that makes contact with the α-amylase molecule consist of all four binding segments and forms a substructure on the surface of the rigid β-barrel. The bulky head containing the six amino acid residues Trp18, Arg19, Tyr20, Thr55, Gly59 and Tyr60 is formed by the binding segments 1 and 4 of tendamistat. It mediates the hydrophobic contacts to the extended hydrophobic regions in PPA. The active site region of PPA consisting of five central sugar binding subsites is blocked by the first segment of tendamistat. Subsites 5 and 6 of PPA interact with the second and third segments of tendamistat while subsites 1 and 2 of PPA are interfacing with 4-th segment of the inhibitor. Residues in α-amylase that are further away from the active site participate in additional interactions. Molecules of α-amylase experience a number of conformational changes upon tendamistat binding.
5.5. Detection of noncovalent protein-protein complexes

5.5.1. α-Amylase - tendamistat noncovalent complexes

Fig. 22 shows ESI mass spectra of α-amylase (M.W. 55kDa) and its microbial inhibitor tendamistat (M.W. 9495)

![Mass Spectra of α-amylase and Tendamistat](image)

Fig. 22. ESI mass spectra of α-amylase and tendamistat. Proteins were dissolved in aqueous solution at pH 7 to a final concentrations of 10 μM. Numbers denote the charge states of the protein ions.

At physiological conditions α-amylase produces charges states ranging from +11 up to +21 with the dominance of the +19 charge state. Tendamistat produces charge states starting from +4 to +9 with the maximum intensity at the +7 charge state. Gradual addition of acetic acid (from 0.1% to 2%) leads to a bimodal charge state distribution for α-amylase with an eventual shift towards higher charge states with a maximum at +35 (data not shown).

ESI mass spectra of the noncovalent complex between α-amylase and tendamistat (Fig. 23) produced charge states from +3 to +8 with a maximum intensity at +5 for tendamistat. There is shift of the maximum, towards a lower charge state in comparison with its individual mass spectrum. A similar phenomenon was observed for α-amylase. A shift towards lower charge states was observed with a maximum at +11 in comparison with +19 for the isolated protein. The noncovalent complex showed
the appearance of charge states ranging from +12 up to +18 with a maximum at the +16 charge state which is interestingly, equal to the sum of the maxima for α-amylase and tendamistat, +11 and +5 respectively.

Fig. 23. ESI mass spectra of noncovalent complex between α-amylase and tendamistat. Tendamistat and α-amylase were mixed to final concentrations of 50 μM and 10 μM respectively at pH 7 and allowed to incubate overnight at room temperature to form a complex. Tendamistat-T, α-amylase-A, complex - (*).

ESI mass spectra of the mixture showed the appearance of all three species in spite of the quite low value of the dissociation constant (10^{-12} M) for the noncovalent complex and the excess of inhibitor (5 times) which was used. Equilibrium calculations show that the final concentration of the complex in solution should be around 10 μM with virtually no α-amylase. This issue will be explored in detail further in the discussion.

A deconvolution of the mass spectrum of Fig. 23 is shown in Fig. 24, and demonstrates the formation of the noncovalent complex between α-amylase and tendamistat with MW of 64,946. The difference in molecular weight between the noncovalent complex and α-amylase was 9496 amu. The M.W. of tendamistat

89
calculated from deconvolution of the same mass spectra was 9485 ± 3 (theoretical M.W. 9495) which corresponds to a 0.03% uncertainty in determination.

Fig. 24. Deconvoluted mass spectra of the noncovalent complex between α-amylase and tendamistat.
5.5.2. BPTI-tryptic proteins noncovalent complexes.

ESI mass spectra of the individual proteins at physiological conditions at pH 7 showed that BPTI forms charge states from +3 to +7 with the most intense signal from the +5 charge state while tryptic proteins have very similar charge state distributions, producing charge states from +7 to +15 with the maximum intensity at the +11 and +12 charge states (Fig.25).

![Fig. 25. ESI mass spectra of BPTI, trypsin, trypsinogen and chymotrypsin samples. Proteins were dissolved in aqueous solution to a 10 μM concentration. Numbers indicate the charge states of the protein ions.](image)

ESI mass spectra of mixtures of BPTI and trypsin, chymotrypsin and trypsinogen (Fig.26) produced charge states ranging from +12 to +14 for noncovalent complexes while the individual tryptic proteins produced charge states ranging from +9 to +12.
Fig. 26. ESI mass spectra of protein-protein complexes. Trypsin/BPTI (a), Chymotrypsin/BPTI (b), and Trypsinogen/BPTI (c), at pH 7. T-trypsin, C-chymotrypsin, Tg-trypsinogen, *-complex, numbers indicate the charge state.

A slight shift in the position of the maximum for trypsin, chymotrypsin and trypsinogen in comparison with Fig.25 can be explained by a change of parameters of the mass spectrometer. To detect noncovalent complexes the difference between the orifice and skimmer potentials was maintained at a minimal level of 10 volts and other experimental parameters were optimized to get the maximum signal from the complexes. In comparison, when individual proteins were detected, the difference between orifice and skimmer potentials was 70 volts with slight differences of other experimental settings.

Figure 27 shows nanospray ionization mass spectra of these three complexes at pH 7. Charge states from +13 up to +15 and in case of trypsin up to +16 are observed for the noncovalent complexes with the maximum at +14. In comparison to ESI there is a shift towards higher charge states in the charge state distribution and also in the position of the major peaks. ESI mass spectra for the noncovalent complexes produced charge states ranging from +12 to +14 with the maximum intensity at +13 and +14.
Discussion

**Charge state distribution**

ESI mass spectra of the mixture between α-amylase and tendamistat showed a shift in the charge state distributions of the proteins towards lower charge states than those for the free proteins. The free proteins showed the dominance of charge states +19 and +7 for α-amylase and tendamistat respectively (Fig. 22 and 23). However ESI mass spectra of the mixture showed the maximum intensity at the +5 charge state for tendamistat and at +11 for α-amylase (Fig. 24). This phenomenon along with the narrowing of charge state distributions has been observed for nearly all studies of noncovalent complexes involving proteins so far. 

For biopolymer complexes, complexes with low molecular weight ligands and synthetic supramolecular complexes this was explained by corresponding “neutralization” effects as the remaining higher order structure restricts the number of available protonation sites. Coulombic restraints on the proximity of charge sites in a more compact structure also contribute to
the decrease in charge numbers for ions of protein complexes relative to the complex components. Surprisingly ESI mass spectra of BPTI and its target enzymes for both free proteins and noncovalent complexes showed no significant shift in charge state distributions between them (Fig.26). The maximum intensity remained at the +11 charge state for the trypsin-like proteins and +5 charge state for BPTI. However detection of the individual target enzymes and BPTI was conducted with different spraying conditions than that of the mixtures. Higher Or/Sk difference was used for individual proteins in order to achieve a higher signal intensity.

Relative abundance of the species in mass spectra

Another interesting aspect is the presence of all three species i.e. inhibitor, protein and non-covalent complex in the ESI mass spectra in all four cases studied (Fig.23). While this might be rationalized in the case of trypsinogen-BPTI, which has a relatively high $K_D$ ($10^6$ M) and possibly in the case of chymotrypsin-BPTI ($K_D \sim 10^9$ M) it is unexpected for the other two systems, i.e. $\alpha$-amylase-tendamistat and trypsin-BPTI which have dissociation constants at $10^{-12}$ M and $10^{-14}$ M respectively.

Taking into account their low dissociation constants, the low value of orifice-skimmer potential (10 volts) and the initial concentrations of proteins (five times excess inhibitor over the proteins), one can expect the appearance of only inhibitor and the non-covalent complex itself.

\[
K_D = [\alpha\text{-Amylase}][\text{Tendamistat}]/[\alpha\text{-Amylase} \cdot \text{Tendamistat}] = 10^{-12} \text{ M}
\]

$[\text{Tendamistat}]_0 = 50 \mu\text{M}$

$[\alpha\text{-Amylase}]_0 = 10 \mu\text{M}$

$[\alpha\text{-Amylase}]_f = n\text{M}$

$[\text{Tendamistat} \cdot \alpha\text{-Amylase}]_f = 10 \mu\text{M}$

94
One possible explanation is that the spraying process by itself is not gentle enough to preserve solution equilibria. Counterflow of curtain gas, activation voltages (difference between orifice and skimmer) and the relatively high flow rate can change the equilibrium in solution as solvent molecules need to be removed from the ion prior to its detection. Despite a carefully maintained level of activation, noncovalent protein-protein complexes are fragile and the presence of water molecules in most cases is essential to maintain existing noncovalent interactions. However it is possible that even five times excess of inhibitor over protein was not enough for ESI conditions and that further increase of the inhibitors' excess will shift equilibrium towards complexes resulting in a situation resembling solution behaviour. Observations of dissociation of multimeric proteins led Light-Wahl et al. to suggest that "under some conditions these noncovalent complexes may dissociate prior to complete desolvation. Dissociation of these multimers can occur at any stage of the ion formation/ion desolvation process; for instance within the droplet before the charge state has been determined, or after the desolvation process and charge state determination." It is quite possible that observed charge state distribution of the inhibitor in fact is overlap between charges states corresponding to a "free" inhibitor and charges states of inhibitor coming from a complex dissociated during ESI sampling process. Further attempts to clarify this particular issue can be explored using titration experiments. Calibration curves corresponding to the intensities and charge state distributions of individual proteins can be used to evaluate exact concentration of protein in the gas phase. Overall the above results provide further support for the use of ESI in the study and detection of noncovalent complexes in the broad range of $K_D$ values from $10^5$ to $10^{14}$ M.
5.7. Summary

- The results described in this section confirmed the capability of Nano- and ESI-MS to detect noncovalent protein-protein complexes consisting of two different proteins having a wide range of dissociation constants.

- Generally, detection of the noncovalent complexes involves a compromise between providing adequate ion desolvation while trying to minimize the effects of complex dissociation i.e. there is a fine line between sufficient desolvation and dissociation of the gas-phase complex.

- Observation of noncovalent complexes in the gas-phase establishes grounds for the potential broad application of mass spectrometry in biological research. Initial screening of compounds with unknown binding affinities to specific targets suggest an important application in the pharmaceutical industry, if conditions can be found where the gas phase ion binding energy correlates with solution binding energy.

- Detection of noncovalent complexes in the gas-phase suggests further conventional biochemical approaches. Variations in ligand concentrations and use of Scatchard plots can be used to determine binding constants in solution by means of MS. In addition relative binding affinities can be established using competitive binding experiments. Thus data obtained using ESI-MS can be used to provide a qualitative, and in some cases even quantitative, characterization of equilibria of noncovalent complexes in solution.
6. Noncovalent interactions studied by Nano- and ESI Tandem Mass Spectrometry

The results presented in Chapter 5 conclusively demonstrated the ability of Nano- and ESI-MS to detect noncovalent protein-protein complexes having a wide range of dissociation constants. However, one crucial question remained unanswered. How well do the binding energies of gas phase noncovalent complexes resemble the solution energies? Attempts to answer this question are made in this Chapter using tandem mass spectrometry, collision cross section measurements and calculations of the relative internal energy required to induce dissociation of the complexes. Section 6.1 reviews different ways to investigate binding energies of noncovalent complexes. Section 6.2 describes ESI-MS/MS studies of noncovalent complexes followed by calculations of relative internal energies required to induce dissociation. Work described in Section 6.3 uses NanoESI-MS/MS for the same purposes.
6.1. Brief survey of the literature

Noncovalent binding of small molecules (organic inhibitors, antibiotics) and biomolecules (peptides, proteins) to their target ligands, represents a form of molecular recognition which is of considerable interest for the pharmaceutical industry. Recent work in a number of laboratories has demonstrated the ability of ESI-MS and MS/MS to estimate qualitatively and in some cases even quantitatively the affinity constants in solution. However a number of published reports appeared to contradict this particular application of ESI-MS. These studies revolve around the more general question of whether ESI-MS may be used to probe binding affinities in solution and in particular whether binding characteristics of the complexes in solution are retained in vacuo. A brief review of work which has been done on this subject is given here.

Highly charged holoMb ions were studied by Chen et al. They were formed with a continuous-flow mixing apparatus and examined by MS/MS and ion trapping experiments. A comparison of the relative internal energies required to dissociate heme from different charge states was made possible by interpretation of tandem MS experiments with the new collision model described in section 2.5.5. of this thesis. The trapping experiments showed that the heme binding energies in the gas phase are comparable to energies required to dissociate heme in solution. Based on the gas-phase and solution similarities for Mb ions in the heme loss mechanism (unfolding of holoMb followed by heme loss), and similarities of binding energies and persistence of individual hydrogen bonds between the protein and heme, it was concluded that "there is increasing evidence that gas phase hMb appears to retain at least some of its solution properties".

Another interesting study on interactions between heme and Mb was conducted by E. Williams' group using the BIRD technique. Ions trapped in a cell of a Fourier-
transform mass spectrometer are activated by absorption of photons emitted from heated walls of the vacuum chamber and are eventually dissociated. Arrhenius activation parameters were determined from the temperature dependence of the unimolecular dissociation rate constants. For large ions energy exchange with the walls of the chamber is much faster than dissociation of the ions, resulting in a Boltzmann distribution of internal energy. Thus information about relative thermal stability of the ions can be obtained from dissociation rates at a given temperature. The authors concluded that their results "demonstrated that the relative gas-phase thermal dissociation rates of heme from two proteins...are indicative of their relative thermal stabilities in solution."

Stabilities of noncovalent complexes between bovine carbonic anhydrase II and series of organic inhibitors were estimated in another investigation. A Fourier transform ICR mass spectrometer and SORI-CAD were used to assess the relative stabilities of these complexes in the gas-phase. It was concluded that thermodynamic stabilities of the complexes in solution and in the gas-phase "are not directly related". It had already been demonstrated that the dissociation rates for complexes in solution were correlated with the hydrophobic interactions between the protein and the ligand, thus emphasizing the importance of hydrophobic interactions in solution. Another interesting conclusion of this work was a "clear correlation" between the total polar surface area of the inhibitor and the amplitude of irradiation. It was found that polar surface area increased steadily with the number of inhibitors' amino acid residues. Based on these findings it was suggested that electrostatic or hydrogen bonding interactions between polar surfaces play a major role in the stability of the complexes in the gas-phase. At the same time, for the inhibitors having the same polar surface, the presence of aromatic amino acid side chains resulted in a stronger binding interaction in the gas-phase with the protein compared to a inhibitors with aliphatic side chains.
Obviously interactions involving aromatic amino acid residues contribute to a greater extent to stability of the gas-phase complexes than interactions involving aliphatic side chains. It was concluded that "the dissociation of these complexes in solution is mainly affected by hydrophobic interactions between the inhibitor and the enzyme, while their corresponding gas phase stabilities appear to be primarily determined by polar surface interactions". It was pointed out that great caution should be exercised when making conclusions about stability in solution based on gas phase measurements.

The gas phase stability of noncovalent complexes between glycopeptide antibiotics and peptides, mimicking cell wall receptors, was recently probed by CAD triple quadrupole mass spectrometry. It was found that the relative stabilities of these noncovalent complexes in the gas-phase are different from those found in solution. In addition, specific solution stereoselectivity of these complexes was not observed in the gas phase. It was concluded "that the solution binding properties of these noncovalent complexes can not be determined by CAD-MS, and indicate that in general the applicability of CAD-MS for quantitative characterization of the stability of noncovalent complexes in solution is probably limited".

H/D exchange in a Fourier transform ICR ion trap was also used to probe gas phase reactivity of the same complexes. H/D exchange studies surprisingly showed increases in deuterium incorporation upon complex formation with the ligand. Based on the results presented, it was concluded in both cases that the gas-phase stabilities of antibiotic-ligand noncovalent complexes may be different from the corresponding solution-phase stabilities.

Noncovalent complexes between vancomycin antibiotics and peptide ligand stereoisomers were a subject of investigation by another research group. They also used CAD in a triple quadrupole mass spectrometer to get information on molecular recognition in the gas phase. Surprisingly, measurements of gas phase stabilities using
positive and negative ion modes demonstrated pronounced differences. The order of relative gas phase stabilities of the negatively charged complexes was in agreement with the solution behaviour. In contrast, measurements in the positive ion mode did not display this correlation. It was concluded "that in some cases the known solution behaviour of noncovalent complexes ... is directly reflected in the stereoselective fragmentation of ... ions".\textsuperscript{236}

A recent study by Rogniaux\textsuperscript{237} et al. provided another example of the evaluation of gas-phase binding of noncovalent complexes by CAD. The authors used a series of aldose reductase inhibitors with MW in the range from 200-400 Da. Relative stabilities of noncovalent complexes in the gas phase were evaluated using CAD - MS in the interface region of the mass spectrometer. The accelerating voltage of the ions in the interface of ESI-MS needed to induce 50% dissociation of noncovalent complex was used as a measure of the gas-phase stability of the enzyme-inhibitor complex. It was shown that the gas-phase stabilities measured by mass spectrometry for these complexes were not correlated with the binding energies measured in solution. However calculations of electrostatic and H-bond binding energies computed from a crystallographic model showed that the halfway dissociation voltages of the complex were related to the energy of electrostatic and H-bond interactions involved in the contact areas. It was concluded that, "in the general case, a direct correlation between the gas-phase stabilities and the binding affinities in solution should not be expected".\textsuperscript{237}

A survey of the literature revealed two major opposite trends in this field. However the majority of experiments reviewed here were performed on relatively simple noncovalent systems between protein and small organic molecules with a low spread in their affinity. None of the results were concerned with evaluation of the gas phase binding of protein complexes formed between two different proteins. In order to fill this gap it was decided to perform evaluation of the gas phase binding of protein
complexes formed between BPTI and its target enzymes. These complexes have quite different values of the binding free energy in solution and thus provide a good model to study noncovalent interactions in the gas phase. The following methods were used to evaluate gas phase binding of protein complexes. Formation of gas phase ions using Nano- and ESI sources, dissociation of the ions of protein complexes in the collision cell of a triple quadrupole mass spectrometer, measurements of the collision cross section for gas phase ions of protein complexes, and calculation of the relative internal energies required to dissociate gas phase ions of protein complexes using a collision model for ion activation. Information obtained using this approach can be used further to test the hypothesis about the relative contributions of electrostatic and hydrophobic components to the binding of the protein complexes in the gas phase.
6.2. ESI-MS/MS of protein-protein complexes

6.2.1. Dissociation pathways of the complexes

Examination of the dissociation pathways for the +12 charge states of the noncovalent complexes (Fig. 28) showed the appearance of charge states from +3 to +6 for BPTI with the maximum intensity at the +4 and 5 charge state. Ions of proteases produced charge states generally ranging from +7 to 9 with the exception of ChT/BPTI complex showing the appearance of +10 charge state.

Fig. 28. Mass spectrum of the +12 charge states (∗,+12) for protein-protein complexes.
(a) Trypsin/BPTI, (b) Chymotrypsin/BPTI, and (c) Trypsinogen/BPTI. B-BPTI, T-trypsin, C-chymotrypsin, Tg-Trypsinogen, *-noncovalent complex. Numbers indicate the charge states of fragment/precursor ions. The collision gas was 1 mtorr of argon and Q0-Q2 was 120 volts.

There is slight difference in the position of the most abundant peak. It is +8 for trypsin- and Tgen-BPTI complexes and +9 for ChT/BPTI complex. Corresponding pairs of product ions were formed as a result of the fragmentation of the precursor ion.

Increases in the collision energy resulted in increases of the signal corresponding to fragment ions while their relative abundances remained the same (data not shown).
Fig. 29. Mass spectrum of the +13 charge states (*,+13) for protein-protein complexes.
(a) Trypsin/BPTI, (b) Chymotrypsin/BPTI, and (c) Trypsinogen/BPTI. B-BPTI, T-trypsin, C-chymotrypsin, Tg-Trypsinogen. Numbers indicate the charge states of fragment/precursor ions. 1 mtorr of argon at the value of Q0-Q2 equal 90 volts.

The dissociation pathways for +13 charge state of the complexes are shown in Figure 29. There is shift toward +5 charge state in the position of the most abundant peaks for BPTI product ions. Product ions corresponding to trypsinogen and chymotrypsin show shift in the position of the maximum towards +10 and 9 charge state respectively, which is in accord with the presence of an extra charge on the BPTI. However the maximum remained at +8 charge state in the case of trypsin product ion.

6.2.2. Factors influencing complex dissociation in the gas-phase

There are number of factors influencing the dissociation of noncovalent complexes in the gas-phase. These include the difference between orifice and skimmer (Or/Sk) potentials, the charge state, nature of the collision gas and its pressure. Dissociation curves were obtained by subtraction of normalized areas of the product
ions from 1 as previously described in Section 3.9 of this thesis. The halfway
dissociation voltage (D50%), i.e. the voltage differences (Q0 - Q2) corresponding to 50%
dissociation of the precursor ion, was calculated from dissociation curves and used in
later computations. The following experiments were done once. No tests of statistical
significance were performed.

Figures 30 & 31 show that a decrease in the Or/Sk difference from 50 to 10 for
trypsin- and trypsinogen-BPTI complexes results in a steeper dissociation curve
probably due to a more narrow distribution of internal energies.

Further decrease in Or/Sk difference below 10 volts resulted in drastic loss of a signal.
Thus, all experiments were done at an Or/Sk difference of 10 volts.

Charge state also plays a significant role (see p. 116 for further discussion).
Addition of only one proton significantly decreases the D50% of the complexes
observed. For a given $E_{lab}$ an increase in charge state leads to a decrease in the required
Q0-Q2 voltage difference (Fig. 32&33).

Fig. 30. Effect of Or/Sk potential difference on
D50% of Trypsin/BPTI complex. 0.5 mtorr of Kr,
+12 c.s. Legends indicate differences in volts.

Fig. 31. Effect of Or/Sk potential difference on
D50% of Trypsinogen/BPTI complex. 0.5 mtorr
of Kr, +12 c.s. Legends indicate differences in
volts.
The nature of the collision gas affects the D50% of the complexes as well (Fig. 34 and 35). In both cases it is almost halved when going from neon to argon, and from argon to krypton. Experiments with Trypsin-BPTI complexes show the same trend (data not shown).

Fig. 36 & 37 show that increases in the pressure of the collision gas lead to a rapid decrease of the D50% of the trypsin-BPTI and chymotrypsin-BPTI complexes.
An increase in collision gas pressure for the Trypsinogen/BPTI complex shows the same trend (data not shown).

6.2.3. Relative stability of the noncovalent protein-protein complexes

Figures 38 & 39 show dissociation curves of the noncovalent complexes between BPTI and its target enzymes (trypsin, chymotrypsin and trypsinogen) evaluated with two collision gases (krypton and argon). Experiments were done once. In both cases the
chymotrypsin/BPTI complex has the highest dissociation voltage, trypsin/BPTI the lowest and trypsinogen/BPTI is intermediate. Dissociation curves of noncovalent complexes with Ne as collision gas show the same order (data not shown).

Halfway dissociation voltages can not be used directly to assess relative binding energies of noncovalent complexes in the gas phase. It is necessary to account for the different cross sections resulting in different number of collisions. Collision cross-section measurements are necessary to calculate the change in the internal energy as described in Section 2.5.5.2. These measurements can also provide information about protein ion conformation. The averages of six separate cross section experiments done on different days were calculated as described in Section 4.6 along with respective standard errors. The results are shown in the Table 8. In all three cases the collision cross sections for the +12 charge state are generally smaller than that for the +13 charge state and, for a given charge state, collision cross sections are almost identical among different noncovalent complexes.

**Table 8. Collision cross-sections for protein complexes**

<table>
<thead>
<tr>
<th>Complexes</th>
<th>+12</th>
<th>+13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-BPTI</td>
<td>1866 - 26</td>
<td>1928 - 21</td>
</tr>
<tr>
<td>Chymotrypsin-BPTI</td>
<td>1887 - 18</td>
<td>1912 - 17</td>
</tr>
<tr>
<td>Trypsinogen-BPTI</td>
<td>1899 - 14</td>
<td>1896 - 18</td>
</tr>
</tbody>
</table>

The change in the internal energy needed to induce 50% loss of complex ions in the gas-phase was used as a measure of the relative binding energies. The changes in the internal energy were calculated for the three noncovalent complexes at five different pressures of three collision gases and are shown in the Table 9.
Table 9. Internal energy required to cause dissociation calculated for noncovalent protein-protein complexes at different pressures of collision gas.

<table>
<thead>
<tr>
<th>Gas</th>
<th>P mtorr</th>
<th>Trypsin-BPTI complex</th>
<th>Chymotrypsin-BPTI complex</th>
<th>Trypsinogen-BPTI complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+12</td>
<td>+13</td>
<td>+12</td>
</tr>
<tr>
<td>Ne</td>
<td>1</td>
<td>135.23</td>
<td>119.5</td>
<td>173.79</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>136.36</td>
<td>120.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>138.43</td>
<td>121.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>138.82</td>
<td>121.53</td>
<td>198.84</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>136.55</td>
<td>119.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
<td>203.92</td>
<td>176.17</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td></td>
<td>208.99</td>
<td>182.14</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td></td>
<td></td>
<td>174.25</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td></td>
<td>198.43</td>
<td>165.29</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>193.18</td>
<td>172.39</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>197.87</td>
<td>168.92</td>
</tr>
<tr>
<td>Ar</td>
<td>0.4</td>
<td>115.69</td>
<td>107.76</td>
<td>150.61</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>109.67</td>
<td>97.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>113.66</td>
<td>99.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>115.26</td>
<td>101.81</td>
<td>168.37</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>110.42</td>
<td>96.80</td>
<td>162.44</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td></td>
<td></td>
<td>116.25</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td></td>
<td>165.07</td>
<td>136.63</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td>156.67</td>
<td>124.92</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td></td>
<td>156.17</td>
<td>118.27</td>
</tr>
<tr>
<td>Kr</td>
<td>0.2</td>
<td>107.97</td>
<td>96.44</td>
<td>143.56</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>109.75</td>
<td>91.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>107.41</td>
<td>91.48</td>
<td>155.44</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td></td>
<td>159.33</td>
<td>129.92</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>107.76</td>
<td>93.66</td>
<td>152.24</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>110.24</td>
<td>93.31</td>
<td>156.41</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td></td>
<td>158.16</td>
<td>125.42</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td></td>
<td>153.87</td>
<td>120.39</td>
</tr>
</tbody>
</table>

The data show that in all cases results obtained at different collision cell pressures do not show large variances of relative internal energy which provides additional evidence for the validity of the collision model. In all cases less energy is needed to dissociate the +13 charge state compared to the +12 charge state. The internal
energies calculated at 5 different pressures for a given charge state agree within a range from 1 to 9%. Increasing cell pressures generally resulted in a slight decrease of the calculated energies. This is because higher pressure of the collision gas required lower injection energies resulted in a longer reaction time for an ion. Thus less internal energy is required to induce dissociation of the ion in collision cell. The average value of change in the internal energy for a given collision gas was calculated for every complex with respective standard errors are shown in Table 10. Comparison of relative internal energies for the same complex between different collision gases for trypsin and chymotrypsin complexes shows a decrease in the order Ne > Ar > Kr i.e. higher mass of the targets resulted in lower relative internal energies. This can be rationalized as follows: heavier targets require lower $E_{\text{lab}}$ to give the same relative internal energy to an ion. Thus heavier targets do not require as big a relative internal energy, because an ion has more time to react in the cell due to its lower speed. For the trypsinogen-BPTI complex this order was not observed. However there is a large uncertainty in the measurements for this particular complex. Calculations for both charge states for all three collision gases showed that the chymotrypsin - BPTI complex is the most stable in the gas phase, the trypsin/BPTI complex is the most unstable and trypsinogen/BPTI complex is intermediate. However in solution trypsin/BPTI complex is the most strongly bound ($\Delta G^0 = 0.82$ eV), trypsinogen/BPTI complex is the most weakly bound ($\Delta G^0 = 0.35$ eV) and chymotrypsin/BPTI is intermediate ($\Delta G^0 = 0.53$ eV).

Internal energies for gas phase ions, are in the 100 eV range, while dissociation free energies in solution, are in the 1 eV range. However it is necessary to keep in mind that comparison is made in relative rather than in absolute terms. In addition values for the binding free energy in solution are obtained for a whole molecule rather than individual charge states. Conversion of the values for relative internal energy from a
given charge state in the gas phase to the values of binding energy in solution is unavailable at this time.

*Table 10. Internal energy required to cause dissociation calculated for noncovalent protein-protein complexes*

<table>
<thead>
<tr>
<th>Target gas</th>
<th>Trypsin-BPTI complex</th>
<th>Chymotrypsin-BPTI complex</th>
<th>Trypsinogen-BPTI complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+12</td>
<td>+13</td>
<td>+12</td>
</tr>
<tr>
<td></td>
<td>+13</td>
<td>+13</td>
<td>+13</td>
</tr>
<tr>
<td>Neon</td>
<td>137.1 - 0.7</td>
<td>120.6 - 0.5</td>
<td>200.2 - 2.2</td>
</tr>
<tr>
<td>Argon</td>
<td>112.9 - 1.2</td>
<td>100.7 - 2.0</td>
<td>161.7 - 2.4</td>
</tr>
<tr>
<td>Krypton</td>
<td>108.6 - 0.6</td>
<td>93.4 - 0.9</td>
<td>155.9 - 1.1</td>
</tr>
</tbody>
</table>

Calculation of the "kinetic shift" can also explain the large difference in absolute values between binding free energies in solution and $E_{\text{int}}$ values for the gas phase complexes. A kinetic shift is the additional energy required to increase the decomposition rate of the ions so that they dissociate within the residence time (~1 msec) in the collision cell. As discussed in section 2.5.3. (p. 27) the internal energy required for ion dissociation can be roughly estimated from the expression for the unimolecular rate constant for dissociation (eq.15). Taking $v = 10^{12} \text{s}^{-1}$, $E^0 = 1 \text{eV}$, $p = 6243$ (for trypsin-BPTI complex) and $k = 10^3 \text{s}^{-1}$ results in the value of $E_{\text{int}}$ equal to $301.8 \text{eV}$. The thermal energy at 295 K accounts for $158.7 \text{eV}$ (1kT per harmonic oscillator). Thus an additional $143.1 \text{eV}$ of internal energy through collisional activation is needed. This value of relative internal energy, corresponds to an ion temperature of $561 \text{K}$. This is similar to the temperatures used in BIRD experiments (400-500 K).232
6.3. NanoESI-MS/MS of protein-protein complexes

6.3.1. Dissociation pathways for the +13 charge state of the noncovalent complexes.

With nanospray only the +13 charge state was chosen for analysis as it was used in the previous analysis using ESI. The intensity of the signal from the +12 charge was too low to conduct any experiments. Krypton was chosen as the collision gas due to its maximum efficiency in dissociation among the three collision gases which were used during ESI-MS/MS experiments. Examination of the dissociation pathways of the +13 charge states revealed a pattern similar to that obtained with ESI. The +13 charge states of the noncovalent complex produced charge states ranging from +3 to +6 for BPTI fragment ions with the maximum intensity at the +5 charge state while fragment ions corresponding to tryptic proteins produced charge states ranging from +7 to +12 with prevailing +8, +9 and +10 charge states.

Fig. 40. Mass spectrum of the +13 charge states for protein-protein complexes using nanospray ionization. (a) Trypsin/BPTI, (b) Chymotrypsin/BPTI and (c) Trypsinogen/BPTI. B-BPTI, T-trypsin, C-chymotrypsin, Tg-Trypsinogen. *-complex. The charge states of ions are indicated by the numbers. 0.3 mtorr of krypton at the value of Q0-Q2 equal 80 volts.
6.3.2. Dissociation curves of the complexes at different collision gas (Kr) pressures.

Figures 41 & 42 show the effects of the collision gas pressure on the half way dissociation voltages. As in the case of ESI, an increase in the pressure of the collision gas leads to a decrease in halfway dissociation voltages for chymotrypsin-BPTI and trypsin-BPTI noncovalent complexes. Experiments were done once.

![Fig. 41. Effect of pressure on dissociation of ChT-BPTI complex. +13 charge state, collision gas Krypton. Legends indicate pressure in mtorr.](image1)

![Fig. 42. Effect of pressure on dissociation of Trypsin-BPTI complex. +13 charge state, collision gas Krypton. Legends indicate pressure in mtorr.](image2)

Increasing the collision gas pressure for Trypsinogen/BPTI complex showed the same trend (Data not shown).

6.3.3. Relative stability of the noncovalent protein-protein complexes using NanoESI.

Figures 43 & 44 compare the dissociation curves of the +13 charge state of the noncovalent protein-protein complexes measured with Kr as the collision gas. Dissociation curves, as in the case with ESI, show that the chymotrypsin/BPTI complex has the highest dissociation voltage. However there is a shift in the position of the dissociation curves corresponding to Trypsinogen/BPTI and Trypsin/BPTI complexes. Their 50% dissociation voltages become comparable.
Measurement of the collision cross sections using nanospray ionization (Table 11) showed greater differences in values among the complexes and these values were generally smaller compared to ESI. The averages of five separate cross section experiments done on different days were calculated as described in section 4.6 along with respective standard errors.

Table 11. Collision cross-sections for protein-protein complexes formed by Nanospray Ionization MS

<table>
<thead>
<tr>
<th>+13 charge state</th>
<th>Cross section, ( \text{m}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-BPTI</td>
<td>1812 – 10</td>
</tr>
<tr>
<td>Chymotrypsin-BPTI</td>
<td>1643 – 25</td>
</tr>
<tr>
<td>Trypsinogen-BPTI</td>
<td>1760 – 20</td>
</tr>
</tbody>
</table>

Calculations of relative internal energies required to induce dissociation at different pressures of collision gas are shown in Table 12. As in the case with ESI, different collision cell pressures do not result in big variances of relative internal energies.
Table 12. Change of internal energy required to cause dissociation calculated for the +13 charge state of noncovalent complexes using different pressure of Kr

<table>
<thead>
<tr>
<th>Gas</th>
<th>P (mTorr)</th>
<th>Trypsin-BPTI complex</th>
<th>Chymotrypsin-BPTI complex</th>
<th>Trypsinogen-BPTI complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kr</td>
<td>0.15</td>
<td>105.05</td>
<td></td>
<td>102.68</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>108.09</td>
<td></td>
<td>100.95</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>108.41</td>
<td></td>
<td>99.21</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>106.17</td>
<td>134.61</td>
<td>97.61</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td></td>
<td>130.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>102.08</td>
<td>119.43</td>
<td>90.81</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>112.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td></td>
<td>113.37</td>
<td></td>
</tr>
</tbody>
</table>

The average values of changes in the internal energy were calculated for each complex with respective standard errors and are shown in Table 13. These results show that the chymotrypsin/BPTI complex is the most stable and show that it takes approximately the same amount of energy to dissociate the noncovalent complexes of trypsin and trypsinogen with BPTI.

Table 13. Change of internal energy required to cause dissociation calculated for the +13 charge state of the noncovalent protein-protein complexes

<table>
<thead>
<tr>
<th>Target gas</th>
<th>Trypsin-BPTI complex</th>
<th>Chymotrypsin-BPTI complex</th>
<th>Trypsinogen-BPTI complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krypton</td>
<td>106.0 – 1.2</td>
<td>122.1 – 4.4</td>
<td>98.2 – 2.0</td>
</tr>
</tbody>
</table>
6.4. Discussion

6.4.1. Factors influencing halfway dissociation voltage

The results presented in this thesis show that the halfway dissociation voltage of the complexes can be affected by a change in the Or/Sk potential, charge state of the complexes, variations in the pressure of the collision gas, and the mass of the collision gas.

A decrease in Or/Sk potential from 50V to 10V resulted in a steeper dissociation curve (Fig.30). As was previously pointed out, this can probably be attributed to a narrower distribution of internal energies at an Or/Sk potential of 10 volts. Generally, increasing the collisional excitation in the interface region by increasing the Or/Sk potential beyond a certain level (80 Volts in the case of protease - BPTI protein complexes) dissociated noncovalent complexes and they were not observed in the mass spectra. Greater Or/Sk potentials will generally decrease solvation of the molecular ions, but also result in dissociation of the noncovalent complex. Dependence of dissociation voltage on Or/Sk potential indicative that thermalization in Q1 region is not complete, however similar excitation is expected for ions of protein complexes, which are of similar sizes.

The decrease of halfway dissociation voltage with an increase in charge state is not surprising (Fig.32). It may be attributed to the combination of two causes: an increase in cross section and an increase in translational energy. Data on cross-sections presented in this thesis show that there is little difference in cross sections for the +12 and +13 charge states for all three noncovalent complexes. Thus the decrease in voltage can be attributed to an increase in translational energy of the gas-phase ions. Higher charge states are injected at higher absolute energies and acquire a given internal energy at proportionally lower voltages.
The probability of multiple collisions increases with increased pressure of the collision gas (Fig.34). Therefore for a given collision energy the yield of fragment ions also increases with increased pressure.

The rapid decrease of the halfway dissociation voltage with an increase in mass of the collision gas shown in the experiments can be most easily explained using the center-of-mass coordinate system (Fig.36). The maximum energy for conversion into internal energy of the ion is the energy of the collision partners in center-of-mass coordinates, $E_{cm}$ given by eq.14. This is greater for a collision of a ion with a heavier target than the $E_{cm}$ of the same ion with a lighter target.\textsuperscript{238,239}

### 6.4.2. Nanospray vs. Electrospray

It is tempting to compare measurements of the collision cross sections and calculations of the relative internal energy obtained using Nano- and ESI sources. However it should be noted that these data should be treated with caution as noncovalent complexes hardly can be considered the best system for comparison of these two ionization sources due to the relatively low signal-to-noise ratio.

#### Collision cross sections

Comparisons of the collision cross sections (Table 14) of complexes formed by NanoESI showed somewhat smaller values than those obtained using ESI.

**Table 14. Comparison of collision cross sections for noncovalent complexes using ESI and NanoESI**

<table>
<thead>
<tr>
<th>+13 charge state</th>
<th>Cross section, ( \text{m}^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ESI</td>
</tr>
<tr>
<td>Trypsin-BPTI</td>
<td>1928 – 21</td>
</tr>
<tr>
<td>Chymotrypsin-BPTI</td>
<td>1912 – 17</td>
</tr>
<tr>
<td>Trypsinogen-BPTI</td>
<td>1896 – 18</td>
</tr>
</tbody>
</table>
Collision cross sections of ions produced by NanoESI showed greater variance. Interestingly the most stable among the complexes, i.e. chymotrypsin, has the lowest cross-section. The observed difference in collision cross sections between ESI and NanoESI remained unexplained at this moment.

Relative internal energies

Comparison of the relative internal energy required to induce dissociation between NanoSpray and ESI for the +13 charge state for a given complex using Kr as a collision gas reveals very similar values (Table 15).

Table 15. Comparison of the changes in the internal energies using ESI and NanoESI sources for noncovalent complexes

<table>
<thead>
<tr>
<th>Internal Energy, eV</th>
<th>Target gas</th>
<th>Trypsin-BPTI complex</th>
<th>Chymotrypsin-BPTI complex</th>
<th>Trypsinogen-BPTI complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NanoESI</td>
<td>ESI</td>
<td>NanoESI</td>
<td>ESI</td>
</tr>
<tr>
<td>Krypton</td>
<td>106.2 ± 1.1</td>
<td>94.3 ± 0.9</td>
<td>122.3 ± 4.3</td>
<td>128.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>98.7 ± 1.9</td>
<td>109.5 ± 4.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It is an intriguing question whether changes in the internal energy should be similar or different for the same complex. Consider the chymotrypsin-BPTI complex as a model for comparison. The pressure of the collision gas and collision cross sections are two major factors contributing to the value of the change in the internal energy. Comparison of halfway dissociation voltages between ESI and NanoESI for the +13 charge state of Chymotrypsin-BPTI complex showed generally smaller values for ESI especially at lower pressures (Table 16). Though collision cross sections were larger for ESI than for NanoESI. The combination of these factors cancel each other, resulting in a very close value for changes in the internal energy.
Table 16. Comparison of halfway dissociation voltages for Chymotrypsin-BPTI complex using ESI and NanoESI sources

<table>
<thead>
<tr>
<th>Halfway dissociation voltages, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>+13</td>
</tr>
<tr>
<td>Pressure of Kr, mtorr</td>
</tr>
<tr>
<td>c.s.</td>
</tr>
<tr>
<td>ESI</td>
</tr>
<tr>
<td>NanoESI</td>
</tr>
</tbody>
</table>

However it is possible to conceive a different situation when these factors will not counteract each other but act together resulting in different values for changes in the internal energy. This is the case for the trypsinogen-BPTI complex. Table 17 shows that the halfway dissociation voltages were smaller for NanoESI compared to ESI. The collision cross section for the +13 charge state was also smaller resulting in a still smaller value of the relative internal energy (98.7 eV) compared to ESI (109.5 eV).

Table 17. Comparison of halfway dissociation voltages for +13 charge state of Trypsinogen-BPTI complex using ESI and NanoESI sources

<table>
<thead>
<tr>
<th>Halfway dissociation voltages, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>+13</td>
</tr>
<tr>
<td>Pressure of Kr, mtorr</td>
</tr>
<tr>
<td>c.s.</td>
</tr>
<tr>
<td>ESI</td>
</tr>
<tr>
<td>NanoESI</td>
</tr>
</tbody>
</table>

Thus variations in both the halfway dissociation voltages and collision cross sections for NanoESI compared to ESI may cancel each other or act cumulatively producing similar or different values of relative internal energies respectively.
Charge state distribution

In the case of BPTI and its target enzymes complexes, a shift of charge state distributions towards higher charge states was observed using NanoESI compared to ESI. This result may appear surprising especially considering that the appearance of higher charge states quite often indicates unfolding of a protein. However this situation may be looked upon from another angle. Fenn proposed that the observed charge state distribution is affected by the evaporation rate of the charged droplets. According to his model excessive charges are located on the outer surface of the droplet. During the course of droplet evaporation, analyte species approach the droplet surface and come into contact with this excessively charged area. It was proposed by Fenn that the specific surface-charge density of the droplet during ion evaporation influences the charge state of the desorbed ion. Consequently ions evaporated at the early stages of the droplet shrinking will carry fewer charges than those desorbing at later stages. A relatively large primary droplet size, as in the case of ESI, leads to a longer lifetime of the droplet in the low surface-charge regime which in turn increases the proportion of lower charge state ions. A decrease in the size of the primary droplet as in the case with NanoESI will lead to an increase in surface-charge density of the droplet. As a consequence desorbed ions of analyte molecules will be exposed to the greater influence of the highly charged surface area and accept more charges. It has also been reported that the larger distances between spray capillary and the sampling orifice led to discrimination against higher charge states.

6.4.3. Binding energies of noncovalent complexes: solution vs. gas phase

A comparison of solution and gas-phase binding for noncovalent protein-protein complexes has not been described previously. The noncovalent complexes between BPTI and tryptic proteins described in this thesis and the cytochrome c - cytochrome b₅
(cyt c - cyt b₅) complexes investigated in the work of Y.L. Chen²⁴¹ are the first reports on the subject.

Role of hydrophobic, electrostatic forces to the gas-phase binding

The noncovalent complexes between BPTI and its target enzymes trypsin, chymotrypsin and trypsinogen exhibited a wide range of dissociation constants, from $10^{-6}$ to $10^{-12}$ M, making them good models for studying protein-protein interactions both in solution and in the gas phase (Fig. 20). Calculations of the internal energy required to induce 50% dissociation showed significant differences in absolute values for the noncovalent complexes and, what is most important, these values did not correlate well with their solution analogs (Table 10). More energy is needed to dissociate the chymotrypsin - BPTI complex than trypsin - and trypsinogen - BPTI complexes in the gas phase. Comparison of the latter two complexes shows that a greater value of relative internal energy is required to dissociate the trypsinogen - BPTI complex (Table 10). Calculations of the relative internal energies using nanospray ionization showed that it takes approximately the same amount of relative internal energy to dissociate trypsin - and trypsinogen - BPTI complexes and this value was again less than the amount of internal energy required for dissociation of the chymotrypsin - BPTI complex (Table 13). It is clear that residues participating in the active site of the complexes, conformational changes of the proteins occurring during formation of noncovalent complex and the implications of these structural changes on the thermodynamics of association are among factors affecting the stability of the complex. It is quite possible that the nature of interactions in the interface region changes while undergoing the "jump" from solution into the gas phase, resulting in the differences in the behaviour between two phases. Understanding the thermodynamic components of the binding in solution and in the gas-phase can provide a clue to the observed differences. Partial loss of hydrophobic interactions between protein and ligand in the
gas-phase and preservation of the electrostatic interactions and hydrogen bonds have been already reported in the literature.\(^{244,242}\) Rogniaux et al. argued that "the binding energy in solution may be divided in two components: an enthalpic part, in which are included the electrostatic and H-bond interactions, and an entropic part, mainly produced by hydrophobicity, while the gas-phase stabilities only depend on the enthalpic part".\(^{237}\) The assumption was made "that gas-phase stabilities reflect the electrostatic and H-bond contacts, to the exclusion of hydrophobic interactions".\(^{237}\) However the calculations of the binding energy in the cited work also revealed that the greatest contribution comes from the electrostatic interactions, while contributions from H-bonds were approximately the same for all inhibitors, ranging from -7 to -10 kcal/mol. Based on the data presented and using the halfway dissociation voltage as a measure of stability in the gas-phase (which is arguable, see sec.6.2.3) the authors of the paper concluded that "in the general case, a direct correlation between gas-phase stabilities and the binding affinities in solution should not be expected".\(^{237}\)

Exact calculations for the contributions of H-bond and electrostatic contacts for complexes between BPTI and its target enzymes are unavailable at this time. However data presented in the literature show approximately the same number of Van der Waals' contacts between trypsin-BPTI and chymotrypsin-BPTI complexes.\(^{243}\) Authors of the cited work pointed out that the number of hydrogen bonds between enzyme and inhibitor are "remarkably small".\(^{243}\) It had been noted by Janin and Chotia\(^{165}\) that the stability of protein-protein interactions in solution is "an effect of solvent entropy rather than of intermolecular interactions". In other words it is hydrophobicity which maintains two proteins in association. Their data indicate that hydrophobicity is a major component in free binding energy of the trypsin-BPTI complex in solution. In view of this data the observed relatively low value of gas-phase binding for this complex is not surprising. It is quite possible that detailed calculations of translational and rotational
energy along with contributions to a free energy from hydrophobicity and polar
interactions will provide an answer to the correlation between solution and gas-phase
behaviour for noncovalent complexes between BPTI and its target enzymes. The ratio
between hydrophobic and polar interactions for a given complex might be a
determining factor. Evaluation of the contributions from hydrophobic, electrostatic and
conformational-entropic components of binding free energy for a number of other
noncovalent systems can provide additional tests of this theory.

Entropic-enthalpic balance

Solution and gas phase reactions are considerably different. There are several
important ways in which the dissociation of a noncovalent complex in solution differs
from that in the gas phase. Thermodynamic considerations show that enthalpy change
reflects the difference in the magnitude of noncovalent interactions between molecules
that occur in the two phases. Entropy change reflects change in disorder. Endothermic
dissociation and exothermic resolvation of the surfaces are major factors associated
with the enthalpy of the solution reaction. Formation of the complex is associated with
the exothermic resolvation of the surfaces, while breaking of the bonds between ligand
and host is responsible for the endothermic dissociation process. From the entropic
point of view there is a balance between the increase in the translational degrees of
freedom and the decrease of the vibrational degrees of freedom, attributed to the loss of
intermolecular bonds in the complex. The former is entropically favourable while the
latter is unfavourable. Measurements of enthalpic and entropic components in the free
binding energy for noncovalent complexes can reveal driving force of the gas phase
stability.

The nature of the host and ligand can also play a considerable role in the
complex enthalpic-entropic compensation relationship contributing to the solution
dissociation constant. However a very different situation exists in the gas phase. Taking
into account the complex nature of relations between entropic and enthalpic terms in solution and the increased importance of the electrostatic interactions between the host and the ligand in the gas phase it is possible to speculate that any correlation between the dissociation constant in solution and the stability in the gas phase can be a matter of pure luck.\textsuperscript{236}

*Effect of the Or/Sk potential*

It has been argued in the literature that "the stronger the binding energy of the non-covalent complex, the less is the effect on the stability of the complex produced by increasing the orifice potential"\textsuperscript{192}. However data on dissociation curves vs. variation in Or/Sk potential presented in this thesis show that this statement should include mention of the phase (gas or solution) where these complexes are being studied.

Experiments on altering orifice voltages (Fig. 30 and 31) showed no difference in dissociation curves for the most stable complex in the gas-phase (i.e. for chymotrypsin-BPTI complex $\Delta D50\% = 1$ volt) and the biggest difference was observed for the least stable complex in the gas-phase (for trypsin-BPTI complex $\Delta D50\% = 8$ volts). In contrast it is the trypsin-BPTI complex that is the most stable in solution. So the above statement should be corrected to read as follows: "the stronger the binding energy of the non-covalent complex," in the gas-phase "the less is the effect on the stability of the complex produced by increasing the orifice potential".

*Comparison with cyt c - cyt b, noncovalent complex*

In comparison, the noncovalent complex of cyt c - cyt b\textsubscript{5} is characterized by a relatively weak dissociation constant $\sim10^{-6}$ M.\textsuperscript{241} A series of mutants of cyt c have also been used to form complexes. Unfortunately information about association constants of these mutants with cyt b\textsubscript{5} is not available. However dissociation constants reported in experiments on the mutational analysis of the proteins did not show large variations in the orders of magnitude of dissociation constant values.\textsuperscript{185,244} It was found that
mutations produced no substantial perturbation for the formation and cross sections of ions of these complexes in the gas phase. Likewise different mutant complexes required similar values of $E_{\text{int}}$ (within 1%) for dissociation, indicating that these mutations do not cause a substantial difference in the stability of the complexes in the gas phase.

**Correlation between solution and gas-phase properties of noncovalent complexes**

The data presented here contribute to the comparison of binding of non-covalent complexes in the gas phase with their behaviour in solution. A resemblance of structures between solution and gas-phase noncovalent protein-ligand complexes has been implied based on calculated dissociation constants for these complexes, specificity of non-covalent interactions between components and observed stoichiometry, which is consistent with solution-based observations. Observation of the relatively labile intact tertiary structure of multimeric proteins in the gas-phase also showed good correlation with the known solution behaviour of these species. However unspecific associations of proteins with buffers, surfactants and counterions and ligands have also been frequently observed in ESI mass spectra. These associations were attributed to non-specific aggregation in the ESI process. However it has not been demonstrated conclusively that such properties as activity, binding strength, or conformation are correlated for noncovalent complexes in the gas-phase and in solution. Examples with noncovalent complexes between BPTI and its target enzymes, presented in this thesis, also demonstrated that correlation between stability of noncovalent complexes in solution and in the gas-phase is not straightforward, raising questions about applications of ESI-MS to investigate the solution binding properties.

Table 18 shows selected examples of positive and negative correlation between solution and gas phase behaviour of noncovalent complexes.
Table 18. Examples of positive and negative correlations based on comparison in affinities for noncovalent complexes in solution and in the gas-phase

<table>
<thead>
<tr>
<th>System</th>
<th>Host</th>
<th>Ligand</th>
<th>Correlation</th>
<th>Method employed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Mb</td>
<td>heme</td>
<td></td>
<td>X</td>
<td>X(^{185})</td>
</tr>
<tr>
<td>cyt b(_5)</td>
<td>heme</td>
<td></td>
<td>X(^{185})</td>
<td></td>
</tr>
<tr>
<td>Mb</td>
<td>heme</td>
<td></td>
<td>X(^{232})</td>
<td>BIRD</td>
</tr>
<tr>
<td>Mb</td>
<td>heme</td>
<td></td>
<td>X(^{73})</td>
<td>trapping</td>
</tr>
<tr>
<td>v-Src SH2</td>
<td>peptide</td>
<td></td>
<td>X(^{208})</td>
<td>titration</td>
</tr>
<tr>
<td>peptide</td>
<td>antibiotics</td>
<td></td>
<td>X(^{236})</td>
<td>X</td>
</tr>
<tr>
<td>peptide</td>
<td>antibiotics</td>
<td></td>
<td>X(^{211})</td>
<td>X</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>organic inhibitors</td>
<td></td>
<td>X(^{244})</td>
<td>SORI-CAD</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>organic inhibitors</td>
<td></td>
<td>X(^{257})</td>
<td>CAD</td>
</tr>
<tr>
<td>tryptic proteins</td>
<td>BPTI</td>
<td></td>
<td>X(^{this \ work})</td>
<td>CAD</td>
</tr>
</tbody>
</table>

There are a number of trends in this table which are interesting to point out. First of all, most of the examples of positive correlation between solution and gas phase behaviour of noncovalent complexes were observed with the heme-Mb and heme - cyt b\(_5\) complexes. A positive correlation for the heme-Mb system is not surprising in lieu of the theory proposed by Rogniaux.\(^{237}\) As was pointed out by Hunter \textit{et al.}, heme interacts with Mb by the axial coordination of heme to protein ligands, van der Waals contacts and hydrogen-bonding interactions.\(^{185}\) The absence of the hydrophobic interactions between the heme group and Mb may be the reason for the observed positive correlation. This concept found support in the conclusions of the work by Henion \textit{et al.}\(^{210}\) It was pointed out " that in cases where hydrophobic interactions play a prominent role in complex formation in aqueous solution, the abundances of derived, gas phase complex ions do not accurately reflect solution binding affinities".\(^{210}\) At the same time noncovalent interactions involving strong,
intermolecular hydrogen bonds and Coulombic attractions are highly favourable in the
gas phase and similarity between the gas phase binding energetics and solution
behaviour is expected. Interestingly, the positive correlations between gas-phase and
solution affinities were found for peptide-antibiotics and protein-peptides system based
on the use of the relative abundances of the protein complex gas-phase ions. These
experiments employed titration and competitive binding techniques. It has already
been demonstrated that in some cases data from titration ESI-MS experiments can be
used to construct Scatchard plots for measuring binding constants. Calculated
binding constants were in reasonable agreement with previously reported solution
characteristics. However it was pointed out that the comparative relative
abundances between components of the complex and complexes themselves "are not
likely to be quantitative". Moreover these measurements are not indicative about the
binding in the gas phase. A large discrimination effect between complex components of
larger and smaller MW (proteins and ligands respectively) was observed. In addition
ionization efficiencies are certainly different for all studied species and dependent on
their structure. However an increase in complexity and MW of the ligand i.e. use of
organic inhibitors, and proteins showed that the positive correlation between solution
and gas-phase behaviour was not preserved. Interestingly the application of CAD-
MS to the study of noncovalent complexes mostly indicated that noncovalent
complexes in the gas phase did not retain their structurally specific solution
interactions. One of the major concerns in application of ESI source to the study of
biomolecules is its intolerance to the relatively high concentrations of salt present in
biological fluids, which are about 150 mM. The low level of salts presented in solution
applicable for ESI creates a highly unnatural ionic environment for proteins in solution,
thus raising important question about relevance of measuring properties of noncovalent
complexes using ESI source.
This survey on the possible correlation between solution and gas phase characteristics of noncovalent complexes shows positive and negative correlations. Joseph Loo pointed out that "a survey of the published literature does not always reflect the true situation in this field of research. Negative results, experiments that "failed" to observe a noncovalent complex that was either "expected" or unknown, are rarely reported. Nonetheless, the numerous "successful" reports suggest that ESI-MS has utility for studying noncovalent complexes." Over 100 reports have been published on the subject of MS of noncovalent complexes; yet, it is probably safe to say that each biomolecular system has its unique experimental features. Taking into consideration all the above mentioned results and arguments it is possible to conclude that despite numerous and persistent reports that some features of the solution structure may be preserved and reflected by gas-phase ions, their stability may or may not be correlated with the solution-phase dissociation constant and should be considered on a case-to-case basis.

6.5. Summary

- Noncovalent complexes of BPTI and its target enzymes, trypsin, chymotrypsin and trypsinogen were characterized by Nano & ESI-MS/MS. Tandem mass spectrometry, cross sections measurements, and corresponding internal energy calculations were used to evaluate relative gas phase binding of the noncovalent complexes. MS/MS revealed the dominant fragmentation pathways for the +12 and +13 charge states of the noncovalent complexes.

- Collision cross section measurements using ESI-MS showed similar values for a given charge state among three noncovalent complexes. These values were slightly greater for the +13 charge state compared to the +12 charge state for all three complexes.
• The energies required to induce dissociation of these complexes have been estimated using tandem mass spectrometry and a collision model. The calculations showed the following order of the binding energies of noncovalent complexes in the gas phase: **Chymotrypsin-BPTI > Trypsinogen-BPTI > Trypsin-BPTI.** The results show that the charge state +12 is more stable than +13.

• Collision cross section measurements using nanoESI-MS resulted in greater variance of the values. The relative order of the binding energies in the gas phase with NanoESI-MS/MS remains unchanged: **Chymotrypsin-BPTI > Trypsinogen-BPTI = Trypsin-BPTI.**

• The relative order of the binding energies in the gas phase do not correlate well with the relative order of the binding energies in solution for these complexes.
7. Summary, Conclusions, and Future work

The following summary surveys completed work on the study of the individual proteins and their noncovalent protein-protein complexes by Nano- and ESI MS and MS/MS.

Protein conformations of two relatively small globular proteins (BPTI, ubiquitin) were probed by changes in the solution pH and the addition of organic solvents. Their charge state distributions were not significantly affected by changes in the pH of the solution from 7 up to 3. Surprisingly at pH 2 a slight shift of the charge states distribution towards lower charge states was observed. A narrowing of the charge state distribution, decrease in the signal intensity and a slight shift in the position of the maximum were observed for BPTI in the presence of urea. Unlike BPTI, ubiquitin showed drastic shifts in the charge state distribution and the position of the maximum in the presence of methanol. These results provide additional evidence that ESI-MS in the positive mode can be used to detect and study changes of solution protein conformations by changes in charge state distribution.

The conformation of BPTI in solution and the gas phase upon reduction of its disulphide bonds was also investigated. BPTI unfolding in solution was detected by a shift in the charge state distributions. BPTI unfolding in the gas phase was detected by measuring collision cross sections for native, reduced and mutant [5-55]AlaBPTI. An increase in mass was observed following reduction of all three disulphide residues of BPTI. Reduced BPTI produced higher charge states than both mutant and native BPTI. For a given charge state, reduced BPTI gives greater collision cross sections than native BPTI while mutant BPTI gives cross sections intermediate between those of native and reduced BPTI. Collision cross sections show that removing the disulphide bonds causes BPTI to unfold in the gas phase but the increase in size is rather small.
Thus the disulphide bonds of BPTI contribute to the folding of the gas phase ion but even in the absence of disulphide bonds, the protein maintains a compact structure.

An investigation of the correlation between stability of noncovalent complexes in solution and in the gas phase was conducted using BPTI and its target enzymes as a model system to study noncovalent protein-protein interactions. Complexes of BPTI with tryptic enzymes were observed by electro- and nanospray ionization mass spectrometry. The abundances of the complex ions in the mass spectra are less than expected from the values of the dissociation constants of the complexes in solution. The internal energy required to induce dissociation was measured over a wide range of collision gas pressures and collision energies for Ne, Ar, Kr collision gases using an electrospray ionization source. A recently developed collision model was used to estimate the energy required to induce dissociation of the gas phase complex ions. These results were repeated with a nanospray ionization source. The order of binding energies in the gas phase ions for both Nano- and ESI appears to differ from that of the solution complexes. The implication is that solution structure is not preserved in these complexes. Comparison of the collision cross sections measured with these two ionization sources showed generally smaller values for the NanoESI source while the measurements of relative internal energy produced similar values for both sources.

One of the interesting results produced by this work is direct comparison of the Nano- and ESI source properties using collision cross section measurements and calculation of relative internal energies for the ions produced by these two sources. Unfortunately this comparison was hampered by the relatively low intensity of the signal for noncovalent complexes. However comparison of the collision cross sections of the ions produced by these two ion sources can be greatly improved by using individual proteins having quite a high intensity of the signal. While it will not be an easy task to make a comparison between Nano- and ESI for a change in internal energy
for noncovalent protein-protein complexes due to the low intensity of the ESI signal one can either use smaller ligands to improve the signal or employ such a well studied system as myoglobin for more exact measurements. These studies can provide additional insight on the electrospray ionization process itself, which still remains a matter of scientific dispute.

While this work presents one of the first reports on the stability of noncovalent protein-protein complexes in the gas-phase using a recently proposed collision model, there are other methods which can be used to evaluate the stability of these complexes. Use of mutants with different known solution dissociation constants gives a method of testing whether the relative order of solution binding survives in the gas phase. The application of this method implies utilization of the system which can be genetically modified and readily expressed in sufficient quantities. The noncovalent complex formed between α-amylase and its microbial inhibitor tendamistat provides such a system. In fact this system was originally proposed for this thesis, however unexpected problems with expression systems forced the termination of the research on the stage of the detection of noncovalent complexes. Future work could include study of the α-amylase-tendamistat system using additional mutants that are becoming available. However work on the noncovalent complexes formed by the mutants of cyt c and cyt b5 showed that mutations had no substantial effect on the cross sections of these complexes. Similar values of energy were required to induce dissociation of cyt c - cyt b5 complexes. In view of this result and the known inability of CAD-MS to estimate even qualitatively the solution binding constants the application of titration and competitive binding experiments looks preferential for estimation of solution characteristics. Calculations of hydrophobic, electrostatic and conformational-entropic components to the free binding energy in solution could help explain the observed lack of correlation between solution and gas phase binding.
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