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Anion-Peptide Adduct Formation and Decomposition As Studied by Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass Spectrometry

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Anion-Peptide Adduct Formation and Decomposition As Studied by Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass Spectrometry

A Dissertation

Submitted to the Graduate Faculty of the University of New Orleans in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

By

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December, 2013
DEDICATION

This dissertation is dedicated to my father Zhigao Liu and mother Lianmei Xiao.
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ABSTRACT

A new “best match” match model has been developed to account for adduct formation on multiply charged peptides observed in negative ion electrospray mass spectrometry. To obtain a stable adduct, the model necessitates an approximate matching of apparent gas-phase basicity (GB_{app}) of a given proton bearing site on the peptide with the gas-phase basicity (GB) of the anion attaching at that site. Evidence supporting the model is derived from the fact that singly charged adducts were only observed for lower GB anions: HSO_4^-, I^-, CF_3COO-. Ions that have medium GBs (NO_3^-, Br^-, H_2PO_4^-) only form adducts having -2 charge states, whereas Cl^- (higher GB) can form adducts having -3 charge states.

Hydrogen bonds are the main interactions pertinent to the “Best Match” model, however, ion-ion interactions formed between peptides ([Glu]Fibrinopeptide B, Angiotensin I or [Asn^1,Val^5]-Angiotensin II) and low GB anions (ClO_4^- or HSO_4^-) have been established by CID-MS/MS. Evidence for ion-ion interactions comes especially from product ions formed during the first dissociation step, where, in addition to the expected loss of the anion or neutral acid, other product ions that require covalent bond cleavage (i.e., H_2O or NH_3 loss) are also observed.

In this study, the “Best Match” model is further supported by the decomposition behavior of adducts formed when Na^+/H^+ exchange has occurred on peptides. Na^+/H^+ exchanges were found to occur preferentially at higher acidity sites. Without any Na^+/H^+ exchange, F^- and CH_3COO^- can hardly form observable adducts with [Glu]Fibrinopeptide B. However, after multiple Na^+/H^+ exchanges, F^- and CH_3COO^- do form stable adducts. This phenomenon can be rationalized by considering that Na^+ cations serve to “block” the highly acidic sites, thereby forcing them to remain overall neutral. This leaves the less acidic protons available to match with higher GB anions.
According to the "best match" model, high GB anions will match with high GB$_{\text{app}}$ sites on the peptide, whereas low GB anions will match with low GB$_{\text{app}}$ peptide sites. High charge states readily augment GB$_{\text{app}}$ of the peptide (through-space effect). Na$^+$/H$^+$ exchanges substantially decrease GB$_{\text{app}}$ by neutralizing charged sites, while slightly increasing intrinsic GBs by the inductive effect.

Keywords: Anion attachment, New model, Multiple charging, Adduction, Electrospray
1.1 Electrospray ionization mass spectrometry (ESI-MS)

1.1.1 Development of ESIMS

Electrospray ionization (ESI) is a method by which multiply charged gas phase ions can be readily created in a very soft and efficient way from solutions at atmospheric pressure. Compared with other types of ionization techniques, ESI offers three distinguished advantages: Firstly, it is very flexible and can be adapted to liquid-phase based separation techniques, for instance, high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), as well as a variety of mass spectrometry analyzers such as quadrupole, ion-trap and Fourier transform mass spectrometry (FT-MS); Secondly, the ions that are formed by ESI can be multiply charged which compresses the ions into a relatively narrow m/z range that fall in the best performance range of most commercially-available mass spectrometers, Thirdly, ESI produces ions in a very soft way such that thermally-labile molecules, such as proteins, DNA and even non-covalent complexes can be ionized without decomposition or dissociation.

Interfacing ESI to mass spectrometry for the purpose of biomolecule analysis was initially reported by John Fenn in 1984. Because of this significant contribution, he was awarded the 2012 Nobel Prize in Chemistry [1]. The combination between ESI and MS offers a rapid and sensitive way of mass determination for biological molecules. As a
result, the technique has become indispensable in the analysis and sequencing of biopolymers, like proteins and oligonucleotides, as well as in analyzing small molecules, such as drugs, natural products, pesticides, and carbohydrates [2].

ESI-MS actually initially introduced by Malcolm Dole in the 1960s. At that time Malcolm Dole was very interested in the analysis of the polystyrene solutions by mass spectrometry. He believed that mass spectrometric data would provide him invaluable information. But the challenge was how could one convert neutral large polymers into the gas phase ions without decomposition? Inspired by the electrospray technique that was employed for car painting, Dole et al. decided to build an apparatus that integrated ESI with mass spectrometry. Luckily, he was able to observe the formation of gas-phase polystyrene ions with several kDa molecular masses. Although Dole’s instrument and results were not perfect and had some unresolved aspects, he clearly pointed out that electrospray is a very promising ionization method for the mass spectrometry of macromolecules [3].

Dole’s paper caught Fenn’s interest and inspired him to start research on electrospray mass spectrometry when he was working in the field of molecular beams. The ESI source and interface he used were similar to those used by Dole, but he employed nitrogen gas to remove neutral solvent that was produced in the process of droplet evaporation. This modification resulted in much cleaner and simpler mass spectra [4]; this work had a huge influence and brought a revolution to the mass spectrometry world that is continuing to this day.
1.1.2 **Principle of ESIMS**

Figure 1-1 shows a schematic representation of ESI-MS; a solution of analyte is pumped to the tip of a capillary tube [5]. A high voltage is applied between the capillary and the counterelectrode. The counterelectrode typically is a plate with a small hole in the center. The applied voltage can be either negative or positive, depending on the ion that one wants to create or the acidity or basicity of the analyte.

Because of the high electric field gradient which is created by the applied high voltage, the liquid protrudes from the capillary tip and forms a “Taylor cone”. A nebulizer gas flows from outside the capillary to the “Taylor cone” and creates a fine mist of charged droplets with the same polarity as the applied voltage. As these charged particles move toward the mass spectrometer, the solvent continues to evaporate, which

Figure 1-1: Schematic of electrospray process in positive mode under atmospheric pressure. TDC represents total droplet current (I). (Figure adapted from [2])
results in a shrinking droplet with increasing electric field on the droplet surface. When the mutual repulsive force of the charges on the droplet exceeds the solvent surface tension, then fission occurs. Such fission will repeat itself many times until the droplets eventually become small enough that the analyte ions are liberated into the gas phase.

### 1.2 FT-MS

FT-MS, also referred to as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS). Because of its ultra-high mass resolution and accuracy for mass measurement, it has received considerable attention ever since it was invented. The development of FT-MS has experienced three important stages, it started in the 1930s. E. O. Lawrence, who built the first ion cyclotron accelerators for studying nuclear technology, developed the theory of ICR [6]. Later in the 1950s, the theory of ICR was first applied to develop mass spectrometry by Sommer et al [7]. The first FT-MS was built in 1978, when Comisarow and Marshall applied the Fourier transform technique to ion cyclotron resonance spectroscopy [8].

#### 1.2.1 Main components of FT-MS

FT-MS comes with many different kinds of configurations. Regardless of the type of FT-MS instrument, all FT-MS instruments consist of four common components: magnet, analyzer cell, vacuum system and data system. The function of the magnet is to provide a uniform, unidirectional and homogeneous magnetic field for the analyzer cell, it can be a permanent magnet, an electromagnet, or a superconducting magnet. The analyzer cell is the core part of FT-MS instrument, a typical ICR analyzer cell is shown in Figure 1-2. It consists of six plates, the middle cylinder is divided into two pairs of plates, and one of them is used for excitation while the other one is for detection. The
outer plates, called trapping plates, serve to keep the ions within the cell. The third part for FT-MS instrument is an ultra-high vacuum system, needed to achieve high resolution and long trapping times; the pressure within the analyzer must be kept below $10^{-9}$ Torr. The last component of the FT-MS is the data system. The FT-MS data system has many functions, such as, to control the timing of the various events in a measurement, to synthesize all kinds of defined frequencies, to amplify signal for ion isolation and ion detection, and to acquire and process the data.

![Diagram of a typical cylindrical cell for FT-MS.](image)

**Figure 1-2: Diagram of a typical cylindrical cell for FT-MS.**

### 1.2.2 **Ion motion in FT-MS**

There are basically three types of ion motions occur in the ICR cell: cyclotron motion, trapping motion and magnetron motion.

#### 1.2.2.1 **Cyclotron motion**

Cyclotron motion is the most important motion in FT-MS because it is the basis of mass measurement. It arises from interactions of the ions with the magnetic fields. When an ion enters into a magnetic field, a force called the Lorentz force is acting on the ion. This force makes the ion travel in a circular orbit, with a direction perpendicular to
the magnetic field as shown in Figure 1-3. Cyclotron motion is periodic and characterized with its characteristic frequency known as ion cyclotron frequency $\omega_c$: $\omega_c$ can be calculated by equation (1)

$$\omega_c = \frac{qB_0}{m} \quad (1)$$

where $B_0$ is the strength of magnetic field, $q$ is the charge of an ion, and $m$ is the mass of the ion.

Figure 1-3: Diagram showing that in the presence of a uniform, unidirectional, and homogeneous magnetic field, an ion will be subjected to a Lorentz force $F_L$, which forces the ion travel in a circular orbit.

In FT-MS, the magnetic field is held constant, and the cyclotron frequency of an ion is dependent on the mass to charge ratio. Thus, by measuring the cyclotron frequency, one can determine an ion’s mass to charge ratio. According to equation 1, the cyclotron frequency is independent of velocity or kinetic energy. This feature makes ICR especially useful for mass determinations, because kinetic energy exerts a profound deleterious influence in mass determinations for other mass analyzers such as TOF or magnetic sector.
Due to the fact that the cyclotron frequency for an ion of given m/z is constant and \( v = \omega r \), then, increasing the velocity of an ion will lead to enlargement of the radius cyclotron orbit. This feature can be used to excite the ions for detection or dissociation.

1.2.2.2  **Trapping motion.**

A second motion is called trapping motion. As stated above, ion cyclotron motion makes ions travel in a circular orbit which is perpendicular to the axis of the magnetic field, and we denote here as the Z axis. However, the Lorentz force has no effect on the ion motion that is parallel to the magnetic field, so ions can move freely along the Z axis. In order to keep the ion cloud within the analyzer cell, a symmetric DC voltage is applied to the trapping plates. A positive voltage is used to trap positive ions, whereas a negative voltage is employed to trap negative ions. Ions trapped along the Z axis within the potential well will undergo harmonic oscillation between the two trapping plates as shown in Figure 1-4.

![Figure 1-4: trapping motion in the analyzer cell](image-url)
1.2.2.3  **Magnetron motion**

A third fundamental mode of behavior for ions in a FT-MS analyzer cell is magnetron motion. As a result of the symmetric voltage that is applied on the trapping plates, the electric potential in the center of the analyzer cell is not zero, but it is around one-third of the trapping voltage applied to a typical cylindrical cell. This voltage creates an electric field from the center of the analyzer axis point to the excitation and detection plates where the voltage is 0, and thus, a corresponding extra electrical force is acting on the ion. This additional force is the origin of magnetron motion: the center of the cyclotron orbit moves around the central axis of the analyzer cell in an orbit circle. The frequency of this motion is proportional to the trapping potential and is inversely proportional to the magnetic field strength. The frequencies of magnetron are much lower than cyclotron frequencies. Figure 1-5 shows a calculated ion trajectory to illustrate the combination of cyclotron and magnetron motion on an ion within the analyzer cell [9].
Figure 1-5: The combination of both magnetron and cyclotron motion shown in the XY plane perpendicular to the magnetic field. Magnetron motion is the movement with large orbit and has been greatly exaggerated for a clearer view, cyclotron motion is the one with smaller orbit (Figure adapted from [9]).

The smaller radius orbit movement represents the cyclotron motion, while the movement that precesses along an isopotential field line with the larger radius orbit is the magnetron motion. There is no analytical use for magnetron motion. In contrast, it brings several negative effects for mass measurement, such as reducing the observed cyclotron frequency by a value that is equal to the magnetron frequency.
1.3 ESI Combined with FT-MS

The coupling of an ESI source and FT-MS was first reported in 1989 by McLafferty group [10]. It offers many mutual advantages. It allows the ionization and rapid analysis of many labile molecules and the high resolution of FT-MS allows simple identification of ion charge state. As a result, the combination of ESI with FT-MS has led to the worldwide installment of FT-MS, particularly for biomolecule analysis.

Compared to quadrupole, ion trap, or TOF mass spectrometers, one disadvantage of FT-MS instruments is that it takes much longer time for data acquisition. For LC-MS experiments, in order to increase chromatographic resolution, low mass resolution parameters must be used to ensure a short acquisition time (0.5–1 s). However, acquisition time will further increase if performing sustained off-resonance irradiation collision-induced dissociation (SORI-CAD) in the analyzer cell, because collision gas must be introduced into the analyzer region in order for dissociation. The total time required to perform SORI-CAD also must add a significant amount of time (1-10s) to pump out excess gas from the analyzer before detection. Due to the long acquisition time, performing in-cell MS/MS experiments is difficult over the typical length of a chromatographic peak in a LC-MS/MS experiment. In order to increase the duty cycle of FT-MS, hybrid FT-MS instruments have been developed.

1.3.1 Hybrid ESI-FT-MS Instruments

Hybrid FT-MS instrument refers to the modification of placing an additional mass analyzer and collision cell between the ion source and the analyzer cell. For example, Figure 1-6 shows a schematic representation of Qh-FT-MS, where “Q” refers to the mass
filtering quadrupole and the “h” is the RF-only hexapole collision cell. The quadrupole mass filter can either be used as a mass-filter to pass only a specific m/z ion or as an ion guide which passes all ions [11]. Typically, the intensity of mass-selected ions will increase with mass-isolation because more space will be available in the multipole collision cell for storing a single ionic species. The collision multipole cell is operated in the RF-only mode and is used to accumulate, fragment, or transfer ions in a non-mass-selective fashion. The multipole collision cell is kept at a high pressure relative to the surrounding vacuum system and can also be used to dissociate ions simply by accelerating the ions as they enter the collision region. Performing CAD in the hexapole instead of in the analyzer cell eliminates analyzer pump-down time (see below) and decreases the instrument experimental cycle time. As a result, the Q-FT-MS is well-suited for performing LC-MS/MS experiments.

Figure 1-6: schematic representation of hybrid FT-MS (Figure from [2])
1.3.2 **Tandem Mass Spectrometry in hybrid FT-MS**

A wide variety of tandem mass spectrometry (MS/MS) techniques have been applied to hybrid FT-MS. Based on the way that the precursor ions are activated, these techniques can be classified into three types: collisions with neutral molecules, photo-excitation, and electron interactions.

1.3.2.1 **SORI-CAD**

The most simple and robust of collision activated decomposition (CAD) MS/MS applied in analyzer cell is sustained off resonance excitation (SORI) – CAD. SORI has been developed by Jacobson and coworkers in the early 1990s [12]. This technique involves the use of a radio frequency that is 0.3% off of the ion’s resonance frequency to cause a cyclotron orbit of the precursor ion that undergoes periodic expansion and contraction, corresponding to periodically increasing and decreasing the ion’s kinetic energy. For SORI-CAD, collision gas must be introduced into the analyzer cell through a pulsed valve. The pressure will immediately rise to $10^{-4}$ torr level after the introduction, but excess collision gas must be pumped out from the analyzer cell before excitation and detection. The pump-down process can take as long as 1-10s, which significantly increases the experimental duty cycle. The increase of the cyclotron orbit and kinetic energy gain is small during each cycle. However, thousands of collisions could occur and the cumulative effect is sufficient to lead the precursor ion to undergo dissociation. The product ions produced in SORI-CAD are easy to be detected by standard methods.

One must keep in mind when performing SORI-CAD, because the excitation is off-resonance, precursor ions will not be ejected from the analyzer cell. However, product ions that are formed during SORI-CAD which have the same frequency as the
Sori excitation will be removed from the cell, leading to a “blind spot” in the mass spectrum where no ions could be detected. In order to detect all the product ions, two parallel SORI-CAD experiments with different SORI excitation frequency must be performed: In one experiment the SORI excitation is performed at a frequency above the frequency of the precursor ion, whereas in the other experiment the SORI excitation employs a frequency below the frequency of the precursor ion. One advantage of SORI-CAD is that only the precursor is undergoing SORI excitation and the excitation can last for a long time until all precursors have fragmented. Since product ions won’t be excited, there is no need to concern about problems of creating secondary product ions.

1.3.2.2 Light irradiation

In addition to CAD, light irradiation can also be used to activate ions. Of all these techniques, infrared multiphoton dissociation (IRMPD) is the most common for FT-MS. Irradiation typically employs a CO$_2$ laser as the infrared light source. The light is introduced along the central axis of the cell by passing through a hole that is located at the center of the rear trapping plate [13]. To perform an IRMPD experiment, precursor ions must be first isolated, and then irradiated by the IR from a CO$_2$ laser. The time of irradiation depends on the photon density provided by the laser. IRMPD achieves its best efficiency when the IR beam is directly overlapped with the precursor ions.

Product ions that are obtained from IRMPD are similar to those that are produced by SORI-CAD [14, 15], because both of them are threshold methods. IRMPD has a few advantages over SORI-CAD for tandem mass spectrometry in FT-MS. First, the use of a collision gas is not necessary in IRMPD MS/MS; this maintains a low pressure in the analyzer cell and eliminates pressure fluctuations, facilitating its use for on-line HPLC
MS/MS. Unlike SORI-CAD, there is no issue with “blind spots” in the IRMPD product ion spectrum. However, product ions produced on-axis may also absorb the IR radiation to produce secondary fragments. Care must be taken when performing IRMPD to eliminate or minimize the formation of secondary product ions, which are of lower analytical utility than the primary products. This requires that the duration of the event be selected so that no more than half of the precursor ions undergo fragmentation. In contrast with SORI-CAD, 100% of the precursor ions can be converted into products.

1.3.2.3  \textit{ECD and EDD}

The extremely low pressure in the analyzer cell allows the application of a number of electron-ion dissociation methods. Of all these techniques, the most widely used and robust is electron capture dissociation (ECD). ECD was developed by Zubarev et al \cite{16}. The operating procedures of ECD in FT-MS are similar to those for an IRMPD experiment. Precursor ions are first isolated and trapped in the analyzer cell, followed by irradiation with electrons. Finally, the product ions and remaining precursor ions are excited and detected. No collision gas is required to perform ECD, however, excitation can be used to improve the electron capture efficiency.

In ECD, electrons are emitted from the filament or cathode, and then accelerated by a bias voltage. After passing through a hole in the rear trapping plate, they interact with the mass-selected ions in the analyzer cell. ECD produces a different series of product ions from those obtained by CAD or IRMPD. ECD is believed to be nonergodic dissociation process, which means bonds break before internal energy distributes throughout the whole molecule. For peptide and protein analysis, the result is that the C–
N bond is broken along the backbone of the peptide chain, yet labile modifications such as glycosylation, phosphorylation, and sulfation are retained [17-20].

A complementary technique for ECD that is used in negative ion mode is called electron detachment dissociation (EDD) [21]. EDD employs the same set-up as ECD, i.e., electrons are created by the same cathode, but are accelerated with a much higher voltage (about 20 V) than ECD (1.5 V). The higher kinetic energy electron is employed to overcome the repulsive forces arising from the interaction between the electron and the charged negative ion. As a result of the interaction, an electron is lost from the negative ion, thus leaving a radical site that can initiate decomposition. EDD has been successfully applied for the analysis of negative ions, such as oligonucleotides and sulfated carbohydrates [22, 23].

1.3.3  **ESI-FT-MS Applications**

Due to its extremely soft ionization, superb mass accuracy and resolving power, ESI-FT-MS is gaining more and more popularity in the determination of the molecular weights of labile large biomolecules as well as in the analysis of complex mixtures.

One important application field is biomolecular analysis, for example, in “top-down” proteomics, intact gas-phase protein ions are selected and decomposed (e.g., CID, IRMPD, ECD, ETD, etc.) to produce fragment ions for comparison to a database for protein identification, as well as to locate various post-translational modification sites based on the additional mass compared to an unmodified fragment ion. Successful examples of top down studies include a study where 99 proteins were identified by top-down tandem mass spectrometry in *Methanosarcina acetivorans*, and liquid chromatography coupled with FTICR identified 102 endogenous peptides in the
suprachiasmatic nucleus [24, 25]. Recently, FT-ICR-MS has demonstrated unit mass baseline resolution for an intact therapeutic monoclonal antibody of 148 kDa molecular weight [26].

In addition to the measurement of biomolecules, FT-MS can be also used in the fields of food safety, petroleomics and environmental analysis. Trace contaminants that don’t have standards available can be identified by ultra-high resolution mass spectrometry. For example, FT-MS has been successfully used to analyze polyether toxin azaspiracid food contaminants in shellfish [27]. A variety of unreported pesticide transformation products have been identified by targeted and non-targeted screening in wastewater treatment [28]. In addition, FT-MS has also been used to analyze crude oil, more than 8250 components of the total 11,000 species can be identified only by the molecular weight [29]. Furthermore, metabolic changes in an aquatic vascular plant (Myriophyllum spicatum) induced by chemicals have been monitored by high resolution MS [30]. The method is able to provide molecular characterization for dissolved organic matter, such as, from pore water, secondary-treated wastewater, and the Greenland ice sheet, because the FT-MS is capable of resolving compounds in complex molecular mixtures and matrixes, while providing exact elemental composition information [31-33].
1.4 Reference


CHAPTER 2

2.1 Abstract

A new model has been developed to account for adduct formation on multiply charged peptides observed in negative ion electrospray mass spectrometry. To obtain a stable adduct, the model necessitates an approximate matching of apparent gas-phase basicity (GB$_{app}$) of a given proton bearing site on the peptide with the gas-phase basicity (GB) of the anion attaching at that site. Evidence supporting the model is derived from the fact that for [Glu] Fibrinopeptide B, higher GB anions dominated in adducts observed at higher negative charge states, whereas lower GB anions appeared predominately in lower charge state adducts. Singly charged adducts were only observed for lower GB anions: HSO$_4^-$, I$^-$, CF$_3$COO$. Ions that have medium GBs (NO$_3^-$, Br$^-$, H$_2$PO$_4^-$) only form adducts having -2 charge states, whereas Cl$^-$ (higher GB) can form adducts having -3 charge states. The model portends that (1) carboxylate groups are much more basic than available amino groups; (2) apparent GBs of the various carboxylate groups on peptides do not vary substantially from one another; and (3) apparent GBs of the individual carboxylate and amino sites do not behave independently. This model was developed for negative ion attachment but an analogous mechanism is also proposed for the positive ion mode wherein (1) binding of a neutral at an amino site polarizes this amino group, but hardly affects apparent GBs of other sites; (2) proton addition (charge state
augmentation) at one site can decrease the intrinsic GBs of other potential protonation sites and lower their apparent GBs.

2.2 Introduction

In analytical mass spectrometry studies of peptides and other biomolecules, the interpretation of mass spectra often becomes complicated when more than one small molecule species forms adducts with a given analyte. Moreover, the presence of such multiply adducted species disperses the analyte signal over several m/z values, thus raising detection limits. Effects of anions on peptides/proteins have been investigated in solution as far back as the late 19th century, when Hofmeister evaluated the abilities of various anions to precipitate or “salt out” proteins, resulting in the so-called “Hofmeister series” for anions listed in part as: $\text{SO}_4^{2-} > \text{Cl}^- > \text{NO}_3^- > \text{Br}^- > \Gamma > \text{ClO}_4^- > \text{SCN}^-$. Since the introduction of the Hofmeister series, the relative ranking of the anions has been correlated to a wide range of chemical solution parameters [2].

Compared with the number of investigations into solution phase behavior, far fewer reports have investigated the effects of anions on the gas-phase properties of peptides/proteins. Mirza and Chait [3] found that the presence of certain anionic species in initial solutions caused positive ion electrospray mass spectra to undergo shifts in peptide and protein charge states toward lower values compared with solutions devoid of these anions. A two-step mechanism was proposed to account for this charge reduction effect: the first step is a solution phase pairing of an anion with a positively charged basic group on the peptide; the second step occurs during desolvation, or afterwards in the gas phase, where the anion departs with a proton to yield the neutral acid and the peptide with a lowered charge state. The level of charge reduction was found to depend only on the
characteristics of the anionic species and was not dependent on the source of the anion (acid or salt form). The authors noted that the level of charge reduction exerted by the series of anions followed the same trend as the tendency for these same anions to induce folding of globular proteins, and to be retained on an anion exchange column [4].

Our laboratory has been engaged in fundamental studies of anion attachment for quite some time [5-14]. Previous studies have all examined singly charged anionic

---

Table 2-1. Gas-Phase Basicities of Tested Anions and Charge States of Adducts Formed with [Glu] Fibrinopeptide B

<table>
<thead>
<tr>
<th>Anion</th>
<th>Gas-phase basicities (GB) kJ/mol</th>
<th>Adduct charge states observed for [Glu] Fibrinopeptide B</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSO₄⁻</td>
<td>1265.0±10.0ᵃ</td>
<td>-1,-2</td>
</tr>
<tr>
<td>I⁻</td>
<td>1293.7±0.84ᵃ</td>
<td>-1,-2</td>
</tr>
<tr>
<td>CF₃COO⁻</td>
<td>1328.0±8.4ᵃ</td>
<td>-1,-2</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>1329.7±0.84ᵃ</td>
<td>-2</td>
</tr>
<tr>
<td>Br⁻</td>
<td>1331.4±4.6ᵇ</td>
<td>-2</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>1351.0±21.0ᵃ</td>
<td>-2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>1373.6±8.4ᵇ</td>
<td>-2,-3</td>
</tr>
<tr>
<td>CH₃COO⁻</td>
<td>1428.7±8.4ᵇ</td>
<td>No detected signal</td>
</tr>
<tr>
<td>F⁻</td>
<td>1530.5±1.3ᶜ</td>
<td>No detected signal</td>
</tr>
</tbody>
</table>

adducts. One of the major findings was that the gas-phase basicity (GB, Table 1 [15-18]) of the attaching anion moiety vs. that of the deprotonated analyte molecule ([M – H]−) plays an important role in determining the stability of anionic adducts. That is, if we consider that anionic adducts take the form of a proton-bound mixed dimer of anions: [M – H]+···H···[anion]−, then such adducts will have maximum stability when the two anions have approximately equivalent GBs [8]. The present work extends our efforts into the domain of multiply charged, multiply adducted anions. It provides a systematic study of hydrophilic peptide–adduct complexes that aims to (1) elucidate the mechanism of multiple adduct formation in negative ion ES-MS (leading to multiply charged adducts); (2) characterize the sites on the protein to which anions attach; and (3) rationalize the pathways of gas-phase reactions that occur when protein adduct complexes are subjected to sustained off-resonance irradiation collision induced decomposition (SORI-CID).

2.3 Method

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Adduct formation between selected anions introduced as ammonium salts (NH₄X where X = HSO₄−, CF₃COO−, H₂PO₄−, Cl−, Br−, I−, and CH₃COO−), and model peptides ([Glu] Fibrinopeptide B and ACTH 22–39) has been investigated. All experiments were performed in the negative ion mode on a Bruker (Billerica, MA, USA) Apex Qe 7.0 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. To obtain a balance between obtaining reasonably strong signals and maintaining “soft” ionization conditions to promote adduct formation, voltages on the capillary exit and the skimmer were set to −35 and −1.7 V, respectively.
2.4 Results and Discussions

2.4.1 Adduct Formation in Negative Ion Mass Spectra

[Glu] Fibrinopeptide B is a hydrophilic peptide derived from Fibrinopeptide B amino acid residues 1–14. Its sequence is EGVNDNEEGFFSAR, and it consists of two basic sites (one at the arginine (R) side chain and another at the –NH₂ terminal end plus five acidic carboxylic acid sites –COOH, three from side chains of glutamic acid (E), one from aspartic acid (D) and one from the –COOH end group).

Figure 2-1. ES mass spectra of solutions containing 3.2 μM [Glu] Fibrinopeptide B (abbreviated Fbp) and (a) no additive; (b) 64 μM NH₄HSO₄; (c) 64 μM NH₄I; (d) 64 μM NH₄NO₃; (e) 64 μM NH₄Br; and (f) 64 μM NH₄Cl. Insets show blow-ups of adducts of HSO₄⁻ and I⁻ for -1 charge states, plus Cl⁻ at -3 charge states.
Electrospray mass spectra of 3.2 μM [Glu] Fibrinopeptide B in pure methanol solution, with 0 to 64 μM ammonium salts are shown in Figure 2-1. Without addition of ammonium salts, the charge state distribution shows three charge states -1, -2, and -3 corresponding to removal of one, two, or three protons with no adducts from anion attachment observed at any of these three charge states (Error! Reference source not found.a). Addition of 64 μM ammonium bisulfate results in various extents of anion attachment to [Glu] Fibrinopeptide B (Figure 2-1b): HSO₄⁻ had the lowest GB (1265.0±10.0 kJ/mol) of all the anions investigated. HSO₄⁻ adduction occurs for lower charge state ions with the most intense adduct peaks appearing at the -2 charge state; however, [[Glu] Fibrinopeptide B – 2H]²⁻ can hardly be seen. For the -1 charge state, [[Glu] Fibrinopeptide B + HSO₄]⁻ adducts are observed. Moreover, up to two additional H₂SO₄ neutrals can attach to the peptide to form [[Glu] Fibrinopeptide B + HSO₄ + H₂SO₄]⁻ and [[Glu] Fibrinopeptide B + HSO₄ + 2H₂SO₄]⁻. Compared with HSO₄⁻, when I⁻ (GB 1293.7±0.84 kJ/mol) was added (Figure 2-1c) the singly charged adduct ion [[Glu] Fibrinopeptide B + I]⁻ decreased relative to [[Glu] Fibrinopeptide B – H]⁺, and no additional HI neutral attached to the peptide at the -1 charge state. The charge states of anion adducted proteins increase with higher GB anions added. For example, NO₃⁻ and Br⁻ with GBs of 1329.7±0.84 kJ/mol and 1331.4±4.6 kJ/mol respectively (Table 2-1), can only form adducts with [Glu] Fibrinopeptide B at -2 charge state (Figure 2-1d and e); for these two anions, no adducts with [Glu] Fibrinopeptide B were formed at -1, -3, or any charge state other than -2. Cl⁻ has a higher GB (1373.6±8.4 kJ/mol) than all of the anions listed above. It also forms adducts having -2 charge state, however, in contrast to all of the others, Cl⁻ can form adducts having -3 charge states, i.e., [[Glu] Fibrinopeptide B +
3Cl\(^3\)- and [Glu Fibrinopeptide B – H + 2Cl]\(^3\)-, but the [Glu Fibrinopeptide B – 2H + Cl]\(^3\)- peak was not observed (Figure 2-1f). Ions with even higher GBs than Cl\(^-\), e.g., CH\(_3\)COO\(^-\), and F\(^-\), produced no observable adducts at any charge states. Thus, the predominant anion in appearing adducts was observed to change as the charge state of the peptide shifted. The higher GB anions dominated in adducts observed at higher negative charge states, whereas lower GB anions appeared predominately in lower charge state adducts.

Similar trends were observed for the peptide ACTH 22–39 with sequence VYPNGAEDESAEAFPLEF, which consists of one –NH\(_2\) terminal end group and six acidic carboxylic acid sites –COOH, four from side chains of glutamic acid (E), one from aspartic acid (D), and one from the –COOH end group. Figure 2-2 shows electrospray mass spectra of 2 \(\mu\)M ACTH 22–39 in pure methanol solution, with 0 to 64 \(\mu\)M ammonium salts. Without addition of ammonium salts, the charge state distribution displays three charge states -2, -3, and -4 (Figure 2-2a); peaks corresponding to sodium/proton exchange are visible at m/z values just higher than the labeled peaks. Addition of 64 \(\mu\)M ammonium bisulfate resulted in only -2 and -3 charge state ions, and bisulfate adducts only formed at the -2 charge state (Figure 2-2b). Similar results were obtained when 64 \(\mu\)M ammonium bromide was added (Figure 2-2c). Although addition of ammonium chloride doesn’t change the breadth of charge states observed, both -3 and -2 charge states were observed to be comprised of chloride adducts (Figure 2-2d).
2.4.2 The Sites on Peptides Where Anions Attach

In negative ion electrospray, there are two types of electron deficient sites on peptides/proteins to which anions may attach: hydrogens of carboxylic acids and protonated amino (or imino) groups (positively charged). One obvious difference between these two types of attachment is that an anion attached to a carboxylic acid site adds a net negative charge to the peptide whereas anion attachment to a protonated basic site results in a neutralization of that site. For an ion appearing in the mass spectrum of
the form: \([\text{peptide} + A]^-\) (A represents an anion), two types of structures could be envisioned (the dotted lines represent electrostatic attraction of varying strength):

\[
\text{A}^-\cdots\text{H}^+\cdots\text{NH}_2^-\text{Peptide-}\text{COO}^- \quad (1)
\]

\[
\text{NH}_2^-\text{Peptide-}\text{COO}^-\cdots\text{H}^+\cdots\text{A}^- \quad (2)
\]

Prior to electrospray, however, this same peptide is likely to have existed in a neutral solution of moderate salt concentration as:

\[
\text{A}^-\cdots\text{H}^+\cdots\text{NH}_2^-\text{Peptide-}\text{COO}^-\cdots\text{H}^+\cdots\text{A}^- \quad (3)
\]

During desolvation or shortly afterwards in the gas phase, sufficient internal energy is present to cause departure of \(\text{AH}\). The proton attached to the amino group will almost always require less energy to remove relative to the proton on the carboxylate group because the anionic carboxylate sites have far higher GBs than the neutral amino sites. Thus, Structure 1 would be much less favored to survive in the gas phase compared with Structure 2. Furthermore, this same logic implies that, upon desolvation, compounds bearing multiple adducted anions will undergo multiple HA losses from amino sites before any carboxylate sites holding HA lose that HA. An exception to this blanket statement can occur if salt bridge(s) form (see below). This reasoning is supported by experimental evidence given in the following section.

2.4.3 **Adduct Fragmentation: Anion Departure With or Without a Proton?**
A series of adduct ions were selected for MS/MS experiments via SORI-CID. MS/MS spectra of \([\text{[Glu] Fibrinopeptide B} - \text{H} + \text{HSO}_4]^2\) and \([\text{[Glu] Fibrinopeptide B} + 2\text{HSO}_4]^2\) are shown in Figure 2-3a and b, respectively. For \([\text{[Glu] Fibrinopeptide B} - \text{H} + \text{HSO}_4]^2\), most of these ions dissociated to \([\text{[Glu] Fibrinopeptide B} - \text{H}]^2\) and \text{HSO}_4\;^2\); far fewer lose \text{H}_2\text{SO}_4. \([\text{[Glu] Fibrinopeptide B} + 2\text{HSO}_4]^2\), which has two \text{HSO}_4\;^2\ attachment sites, undergoes initial dissociation in two distinct steps: first losing \text{HSO}_4\;^2 followed by loss of \text{H}_2\text{SO}_4, giving rise initially to \text{HSO}_4\;^2 plus \([\text{[Glu] Fibrinopeptide B} + \text{HSO}_4]^2\), and then \([\text{[Glu] Fibrinopeptide B} - \text{H}]^2\) (Figure 2-3b). If, instead, \text{H}_2\text{SO}_4 loss had occurred first, a peak corresponding to \([\text{[Glu] Fibrinopeptide B} - \text{H} + \text{HSO}_4]^2\) would be expected; no such peak was observed. The fact that the first loss is \text{HSO}_4\;^2 can only be rationalized if one considers that this leaving \text{HSO}_4\;^2 is weakly held to the peptide (Figure 2-4, left column). It is highly unlikely that this \text{HSO}_4\;^2 was attached to a protonated amino group because \text{H}_2\text{SO}_4 loss would have been expected from the amino site of low \text{GB}_{\text{app}} (see Appendix for discussion of \text{GB}_{\text{app}}) not the observed \text{HSO}_4\;^2 departure. Instead, this \text{HSO}_4\;^2 was attached to a protonated carboxylate site that had a \text{GB}_{\text{app}} slightly higher than \text{HSO}_4\;^2. Not only is the \text{GB} of the departing \text{HSO}_4\;^2 lower than the \text{GB}_{\text{app}} of the corresponding carboxylate site, but also, compared with the (second) \text{HSO}_4\;^2 that remains, the departing \text{HSO}_4\;^2 is less well matched with the \text{GB}_{\text{app}} of the carboxylate site to which it was bound. If one considers that the remaining \text{HSO}_4\;^2 leaves as \text{H}_2\text{SO}_4 to form \([\text{[Glu] Fibrinopeptide B} - \text{H}]^2\) (Figure 2-3b), then it becomes clear that this second \text{HSO}_4\;^2 was attached to a more acidic carboxylic acid. The data shows that \text{HSO}_4\;^2 only has sufficient basicity to pull off one proton from the more acidic of the two carboxylic acid sites where attachment had occurred. Note that Figure 2-4 omits the possibility that anion attachment occurs at a
protonated amino site. Evidence supporting this omission is given by the fact that HSO$_4^-$ departure is the first loss from [[Glu] Fibrinopeptide B + 2HSO$_4$]$^2^-$. As explained above, had HSO$_4$ been attached to a protonated amino site, H$_2$SO$_4$ loss should have occurred instead. For the other anions that all have GBs higher than HSO$_4^-$, surviving adducts (wherein the number of attached anions is equal to or less than the charge state) are even less likely to exhibit attachment at a protonated amino group because the GB matching of the “proton bound mixed dimer” constituting the adduction site becomes even less favorable.

By contrast, fragmentation of the Br$^-$ adduct is simple; all Br$^-$ in both [[Glu] Fibrinopeptide B – H + Br]$^2^-$ (Figure 2-3c) and [[Glu] Fibrinopeptide B + 2Br]$^3^-$ (Figure 2-3d) will depart as HBr, leading ultimately to [[Glu] Fibrinopeptide B – 2H]$^2^-$, i.e., the GB of Br$^-$ (higher than that of HSO$_4$) is now capable of pulling off both protons from the two sites of attachment. Clearly, when two Br$^-$ anions are attached, the one attached to the more acidic carboxylic acid will be more prone to HBr loss (Figure 2-4, middle column). Notably, the GB of Br$^-$ is not high enough to form a stable adduct at the third most acidic carboxylic acid. However, the higher GB of Cl$^-$ does permit formation of the triply adducted [[Glu] Fibrinopeptide B + 3Cl]$^3^-$. The order of consecutive dissociations of [[Glu] Fibrinopeptide B + 3Cl]$^3^-$ precursors can be deduced from the MS/MS product ion spectrum (Figure 2-3f). The fact that [[Glu] Fibrinopeptide B + 2Cl]$^-$ was hardly observed provides evidence that the preferred first step in decomposition is loss of HCl (not Cl$^-$ departure). Clearly this HCl loss is most favored from the most acidic carboxylic acid (Figure 2-4, right column). The [[Glu] Fibrinopeptide B – H + 2Cl]$^3^-$ ion that is thus formed then decomposes to Cl$^-$ and [[Glu] Fibrinopeptide B – H + Cl]$^2^-$ with no loss of
HCl (no [[Glu] Fibrinopeptide B – 2H + Cl]3− was formed). As was the case for HSO4− above, Cl− loss is most favored at the carboxylate site of highest GBapp, where the proton remains (Figure 2-4, right column). The fact that the [[Glu] Fibrinopeptide B – 2H + Cl]− peak was not observed in either Figure 2-3f or Error! Reference source not found.f can be rationalized if we consider that [[Glu] Fibrinopeptide B – 2H + Cl]3− has a higher GBapp than Cl−. Thus, two protons of the three binding sites on [[Glu] Fibrinopeptide B + 3Cl]3− can be abstracted by Cl−.

Figure 2-3. SORI-CID MS/MS spectra for the following precursors (a) [[Glu] Fibrinopeptide B – H + HSO4]2−; (b) [[Glu] Fibrinopeptide B + 2HSO4]2−; (c) [[Glu] Fibrinopeptide B – H + Br]2−; (d) [[Glu] Fibrinopeptide B + 2Br]2−; (e) [[Glu] Fibrinopeptide B + 2Br + 2HBr]2−; and (f) [[Glu] Fibrinopeptide B + 3Cl]2−. Asterisks (*) mark precursor ions; [Glu] Fibrinopeptide B is abbreviated as Fbp.
Figure 2-4. Fragmentation pathways of HSO₄⁻, Br⁻, and Cl⁻ adducts of [Glu] Fibrinopeptide B. The three most acidic sites on the [Glu] Fibrinopeptide B backbone are shown schematically with attached anions. The gas-phase acidities of these carboxylic acids decrease in descending from top to bottom as marked in top center schematic. The
GBs of the anions increase in the order HSO$_4^- < Br^- < Cl^-$. Whether an anion departs with or without a proton depends upon the relative GBs of the anion versus the carboxylate group to which the proton is attached. At a given carboxylate site HSO$_4^-$ is more prone to depart than Br and much more so than Cl. Cl departure, however, was observed, but only from a higher GB carboxylate site (third site from top in diagram) that was mismatched with the lower GB of HSO$_4^-$, hence no HSO$_4^-$ adduct could form at this site. The rationalization for omitting anion attachment to potentially protonated amino sites is given in the text. The possibility for salt bridge formation exists, but is not shown in this diagram.

2.4.4 A New Model for Anion Attachment to [Glu] Fibrinopeptide B—Negative Ion Model.

Figure 2-5 shows a schematic of a proposed new model for anion attachment to [Glu] Fibrinopeptide B [19]. As solvent evaporates from the droplets, the ammonium salt concentration increases. The anions in solution will be attracted by the neutral acidic sites (−COOH), thus forming R-COO$^-\cdot\cdot\cdot$H$^+\cdot\cdot\cdot$A$^-$ or by the protonated basic sites (−NH$_3^+$) giving rise to R-NH$_2\cdot\cdot\cdot$H$^+\cdot\cdot\cdot$A$^-$. As the last solvent molecules depart from the complex, charged molecules possess a certain amount of rotational-vibrational energy and electrostatic repulsion between negative charges increases. These factors cause low-energy dissociation(s) of charged complexes meaning that anions or protonated anions will depart from these complexes until only proton-bound mixed dimers of anions that have well-matched GBs on the two sides of the central proton remain. The key to our new model is that only stable adducts survive to the detector. An attached anion is only stable when it has a GB close to the GB$_\text{app}$ of the deprotonated site at which it is attaching. In other words, both anions in the proton-bound mixed dimer must exert an approximately equal pull on the central proton. For various carboxylate or amino sites on the peptide/protein, the anion fulfilling this requirement will not be the same.
Figure 2-5. [Glu] Fibrinopeptide B complex formed in a negatively charged electrospray droplet near the end of the droplet’s lifetime. Anions are attracted to the protonated basic sites and acidic sites on [Glu] Fibrinopeptide B before the final solvent departs from the peptide. Capital letters indicate amino acid and $\text{A}^-$ indicates negatively charged anion. Omitted from the diagram for increased visual clarity is the possibility for salt bridge formation. In addition to ammonium-carboxylate interactions, salt bridges can take the form of $\text{A}^-$ linked ammonium and carboxylic acid groups.
2.4.5 Evidence Supporting the Model

In our experiments, low GB anions such as HSO$_4^-$, are best matched with low GB$_{app}$ sites of both analytes, i.e., singly deprotonated or doubly deprotonated peptide forms bearing higher acidity protons. There are two basic amino/imino sites in [Glu] Fibrinopeptide B that are both protonated at near-neutral pH and both sites can interact with HSO$_4^-$. The attachment of HSO$_4^-$ to protonated basic sites is virtually equivalent to a neutral H$_2$SO$_4$ attaching to that vacant basic site. If the GB$_{app}$ of a carboxylate site that is deprotonated is far higher than the GB of HSO$_4^-$, HSO$_4^-$ cannot form a stable adduct at that site on the peptide. From the spectrum of Figure 2-1b, the highest charge state adduct formed by HSO$_4^-$ attachment is $-2$, which indicates that the maximum number of HSO$_4^-$ anions attached to the acidic sites is two. If one adds the two neutral H$_2$SO$_4$ molecules attached at the two most basic amino sites (arginine and N-terminus), the total number of sites for attachment is four, and four adducted bisulfates were indeed observed to be formed at $-2$ charge state. When an anion has attached to every carboxylic acid where there is a reasonably close match of the GBs on the two sides of the proton, and when an anion has attached to every protonated amino group, then, according to our model, the maximum amount of anion attachment has occurred. From this maximally adducted state, as the last solvent molecules depart when the ion enters the gas phase, HSO$_4^-$ or H$_2$SO$_4$ can depart from these sites, especially if there is a significant difference in the GBs of the two sides of the proton-bound mixed dimers, thus producing [[Glu] Fibrinopeptide B – H + HSO$_4$]$^{2-}$ and other [[Glu] Fibrinopeptide B + nHSO$_4$ + mH$_2$SO$_4$]$^n$ species ($n=1, 2; m=0, 1, 2$ where $n + m=3$ or less). Depicted in Figure 2-6a is the rationale explaining the
observed reactivity of the maximally adducted \([\text{[Glu] Fibrinopeptide B + 2HSO}_4 + 2\text{H}_2\text{SO}_4]^{2-}\). \(\text{HSO}_4^-\) loss from the second most acidic carboxylic acid is a favored low-energy pathway. \(\text{H}_2\text{SO}_4\) loss from weak attachment at the second highest basicity amino group, followed by a second \(\text{H}_2\text{SO}_4\) loss from the most basic amino group, also occurs readily. The last remaining site of adduction is at the most acidic carboxylic acid which offers a carboxylate GB most closely matched to \(\text{HSO}_4^-\).

The behavior of anions that are observed to produce only \(-2\) charge state adducts such as \(\text{NO}_3^-\) and \(\text{Br}^-\) can be rationalized by considering that these anions can easily pull off the most acidic proton from neutral \([\text{Glu}]\) Fibrinopeptide B. This means that their GBs are significantly higher than that of \([\text{[Glu] Fibrinopeptide B – H}]^-\), and this is the reason that these anions cannot form stable \(-1\) charge state complexes. Doubly deprotonated peptides are readily formed, however, and because there is a better match with the GB of \([\text{[Glu] Fibrinopeptide B – 2H}]^+\), \([\text{[Glu] Fibrinopeptide B – H + A}]^-\) (where \(A\) represents \(\text{NO}_3^-\) or \(\text{Br}^-\)) ions are also formed. Stable adducts are thus formed as proton-bound mixed dimers with carboxylate anions that are not the least basic of the peptide, i.e., the most acidic carboxylic acid is deprotonated, and the adduct forms at a second carboxylic acid of lower acidity.

An even higher anion GB is required to enable attachment of a third anion. Up to three \(\text{Cl}^-\) can attach to \([\text{Glu}]\) Fibrinopeptide B to form \([\text{[Glu] Fibrinopeptide B + 3Cl}]^{3-}\). For much higher GB anions such as \(\text{F}^-\), no adducts at all were observed. This result is rationalized by considering that the GB of \(\text{F}^-\) is significantly higher than the highest GB of \([\text{[Glu] Fibrinopeptide B – 3H}]^{3-}\) formed by triply deprotonated \([\text{Glu}]\) Fibrinopeptide B. This implies that there is a mismatch between triply deprotonated \([\text{Glu}]\) Fibrinopeptide B.
Fibrinopeptide B and F and that a proton bound mixed dimer of the two would dissociate rapidly to HF and \([\text{[Glu] Fibrinopeptide B} - 3\text{H}]^3\).

\[(a) \quad \text{[Glu] Fibrinopeptide B} + 2\text{HSO}_4^- + 2\text{H}_2\text{SO}_4]^2^- \]

\[
\begin{array}{c}
\text{[Glu] Fibrinopeptide B} \\
\begin{array}{c}
\text{NH}_2 \\
\text{COOH}
\end{array}
\end{array}
\quad (1) \\
\quad (2) \quad \text{H}_2\text{SO}_4 \\
\quad (3) \\
\quad \text{Best matched site} \\
\quad (4) \quad -\text{HSO}_4^- \\
\]

\[(b) \quad \text{[Glu] Fibrinopeptide B} + 2\text{Br}^- + 2\text{HBr}]^2^- \]

\[
\begin{array}{c}
\text{[Glu] Fibrinopeptide B} \\
\begin{array}{c}
\text{NH}_2 \\
\text{COOH}
\end{array}
\end{array}
\quad (1) \\
\quad (2) \quad \text{HBr} \\
\quad (3) \\
\quad \text{Best matched site} \\
\quad (4) \\
\]

\[(c) \quad \text{[Glu] Fibrinopeptide B} + 3\text{Cl}^- + 2\text{HCl}^3^- \]

\[
\begin{array}{c}
\text{[Glu] Fibrinopeptide B} \\
\begin{array}{c}
\text{NH}_2 \\
\text{COOH}
\end{array}
\end{array}
\quad (1) \\
\quad (2) \quad \text{HCl} \\
\quad (3) \\
\quad \text{Best matched site} \\
\quad (4) \quad -\text{Cl}^- \\
\]

Figure 2-6. Maximum number of anions attached and their decomposition pathways for: (a) HSO$_4^-$, (b) Br$^-$, and (c) Cl$^-$ adducts of [Glu] Fibrinopeptide B in the gas-phase. The top amino group is the lower GB amino group of the two that are shown. The gas-phase acidities of the carboxylic acid groups decrease from top to bottom. (1), (2), (3), (4), and (5) indicate the order of decomposition as deduced from Figure 2-3. Dashed arrow indicates the decomposition direction leading to the best matched site. Adducts above the best matched site tend to decompose by losing neutrals; below, they dissociate by losing anions.
2.4.6 Mixed Dimers of Proton-Bound Anions

Additional evidence for the proposed model comes from experiments performed using [Glu] Fibrinopeptide B in combination with mixtures of anions. Shown in Figure 2-7a is an example employing equimolar chloride and iodide simultaneously. Mirroring the single anion results shown in Figure 2-7, iodide (lower GB) forms only singly and doubly charged adducts, whereas chloride (higher GB) forms only doubly and triply charged adducts. This behavior can be rationalized by considering that iodide attaches at the two most acidic carboxylic acids and its GB is adequately matched with those of the corresponding two weakest GB\textsubscript{app} carboxylate groups. In contrast, the GB of chloride is not well matched with the carboxylate corresponding to the highest acidity proton (no [Glu] Fibrinopeptide B + Cl\textsuperscript{−} observed), but instead, it forms stable adducts with the second and third most acidic protons, i.e., [Glu] Fibrinopeptide B – H + 2Cl\textsuperscript{−}. Interestingly, peaks corresponding to [Glu] Fibrinopeptide B + 3Cl\textsuperscript{−}, [Glu] Fibrinopeptide B + 3Cl + HCl\textsuperscript{−}, and [Glu] Fibrinopeptide B + 3Cl + 2HCl\textsuperscript{−} were observed. The presence of these latter peaks indicates that, despite the fact that [Glu] Fibrinopeptide B + Cl\textsuperscript{−} was not formed indicating a mismatch of the GBs of Cl\textsuperscript{−} and the carboxylate bearing the most acidic proton, when the second and third most acidic protons are each holding Cl\textsuperscript{−}, the GB\textsubscript{app} of the site holding the most acidic proton has been altered sufficiently so that it now can accommodate a third Cl\textsuperscript{−}. Thus, attachment of the first two anions apparently alters the balance of GBs at the most acidic carboxylic acid compared with when no other anions are attached to the peptide. We attribute this alteration to a combination of a weak inductive effect, which may slightly raise the intrinsic GB of the implicated carboxylate and Coulomb repulsion that increases GB\textsubscript{app}. 

38
of the anionic peptide. These effects combine to produce a better matching of GBs at this site as compared with the situation when no other Cl anions are attached elsewhere. As depicted in Figure 2-6c, a maximum of five Cl can be carried by a single [Glu] Fibrinopeptide B molecule, with a maximum charge of −3. Those HCl's that are held by amino groups are the ones that depart the most easily, followed by the HCl at the weakest basicity carboxylate. Afterwards, Cl may depart form the third most acidic carboxylic acid, leaving the one remaining Cl attached to the second most acidic carboxylic acid as the most stable site of adduction, meaning that the corresponding carboxylate is best matched with Cl.

Figure 2-7. ES mass spectra of solutions containing 3.2 μM [Glu] Fibrinopeptide B (abbreviated Fbp) along with binary mixtures of anions: (a) 32 μM NH₄Cl and 32 μM NH₄I; (b) 32 μM NH₄HSO₄ and 32 μM NH₄Br.
Figure 2-7b shows the appearance of so-called mixed anionic adducts of [Glu] Fibrinopeptide B from a solution containing an equimolar combination of bromide and bisulfate anions. Mixed adducts are comprised of two amino sites, hence, the former are the sites involved in stable anionic adduct formation; (2) apparent GBs of the carboxylate groups on the peptides are deduced to be close to one another. This is evidenced by the fact that three of the same type of anions can attach simultaneously to one peptide. Stable attachment of three of the same anions at different sites would not be possible if the sites were not of comparable basicity. Moreover, in the few cases where two different anions added to the same peptide (e.g., Figure 2-7), the anions were typically not far apart in GB; (3) apparent GBs of the individual carboxylate and amino sites do not behave independently of one another. Rather, anion attachment or removal of a proton at a particular site can raise the intrinsic GBs of other sites by exerting a weak inductive effect. Furthermore, Coulomb repulsion caused by this prior charged site creation can raise GB\text{app} of other carboxylate sites on the peptide that are holding protons. Attachment of an anion at a protonated amino group (thus forming an overall neutral site), however, does not significantly alter the apparent GBs of other potential binding sites.

2.4.7 **Positive Ion Model**

An analogous model can be constructed for adduct formation in the positive ion mode. For positive mode adduct formation, excess cations, e.g., protons, will be attracted to basic sites on the peptide and form R-NH₂⋯H⁺. For some of these protonated basic sites, nearby anions may form R-NH₂⋯H⁺⋯A⁻. According to our model, the latter type of
adduct will be especially stable when the amino group and the attaching anion have closely matched GBs. This type of attachment is virtually equivalent to a neutral molecule binding to a vacant basic site. Analogous to the situation for negative ions noted in the last paragraph above, compared with charging, the attachment of neutral molecule(s) to a peptide/protein will hardly change $\text{GB}_{\text{app}}$ of other sites on the peptide/protein. This implies that if several sites on the peptide/protein were of comparable basicity, a particular acid could be found that would be able to attach multiple times as a neutral molecule at various basic sites on the peptide/protein. This model is supported by important results from Williams and coworkers [23, 24], who added HClO$_4$ to peptides/proteins in solution in order to determine the number of basic sites in these peptides/proteins. According to our model, the success of this method arises from the advantageous matching of the GB of ClO$_4^-$ with $\text{GB}_{\text{app}}$ of the typical amino site on a peptide/protein. If indeed, all of the amino sites have apparent GBs that are adequately matched with ClO$_4^-$, each basic amino group may hold a stable adducted HClO$_4$ molecule. As recognized by the Williams group [23, 24], this allows the calculation of the number of basic sites from an assessment of the maximum number of HClO$_4$ molecules attached (each one a neutral site) plus the number of protonated sites (equal to the charge state of the protein/peptide). The success of this method hinges upon the ability to either protonate or form a stable HClO$_4$ adduct at every basic site. Our model of adduct attachment contends that progressive addition of neutral molecules does not significantly alter the apparent GBs of other sites on the peptide/protein. However, as stated above for the negative ion model, when the charge state at one site changes, apparent GBs at other sites are affected. In the positive ion case, raising the charge at one
site will lower the intrinsic GBs of other distant sites [via the inductive effect (weak)] and lower GB$_{\text{app}}$ by Coulomb repulsion. This alteration of apparent GBs at amino sites can throw off the balance in GBs that held HClO$_4$ at a particular site, leading to a breakdown of the method enabling calculation of the number of basic sites. This aspect of our model can account for why lower charge states are required to determine the basic sites on peptide/protein using HClO$_4$ adducts. In considering alternatives to ClO$_4^-$, which has an extremely low GB (1180±59 kJ/mol) [15], Stephenson and McLuckey [25, 26] used a higher GB anion, I$^-$, introduced as HI in a quadrupole ion trap to determine the number of basic sites on peptides/proteins. These experiments were successful at this endeavor when the gas-phase acidity of the analyte peptide was low (GB ~1380 kJ mol$^{-1}$), but as can be explained by our model, higher gas-phase acidity peptides could not stabilize I$^-$ adducts, and the result is that not all HI molecules remain attached, hence the ability to determine the number of basic sites is compromised. If even higher GB anions, such as CH$_3$COO$^-$, were used, then virtually no adducts would form in positive mode for peptides/proteins.

The attributes of the positive ion model can be summarized as (1) binding of a neutral at an amino site will polarize this amino group somewhat, but hardly change the apparent GBs of other sites; (2) augmenting the charge state by adding a proton (with no counterion) at one site can decrease the intrinsic GBs of other sites (weak inductive effect) and lower the GB$_{\text{app}}$ of the peptide by Coulomb repulsion.
Reciprocal Stabilization of Opposite Charges and Salt Bridge Formation

Intentionally left out of Figure 2-5 for purposes of increased visual clarity is the possibility of salt bridge formation. Salt bridges, comprised of ionic bonds linking carboxylate sites and protonated amino groups have been reported upon in the literature [27]. They arise when charges opposite to that of the overall charge are stabilized by nearby charge sites corresponding to the excess charges (Figure 2-8a). In larger proteins, this has been referred to as “reciprocal stabilization of opposite charges” [28, 29]. Thus, in the negative-ion mode, protonated amino sites are likely to be stabilized by the negatively charged carboxylate sites, thus preventing proton departure from these basic sites (Figure 2-8a). Similarly, in the positive-ion mode, negatively charged carboxylate groups are likely to be situated near protonated amino sites. The concept of the salt bridge pertains to our negative ion model because there exists the possibility for salt bridge formation between neighboring carboxylic acid and protonated amino sites that are linked by a central attaching anion (Figure 2-8b). Formation of a salt bridge of this type will (1) result in an overall neutral charge at the salt bridge even though the anion has successfully attached; (2) create an exceptional case where the (shared) anion is electrostatically linked to a protonated amino group (not shown in Figure 2-4, Figure 2-5, or Figure 2-6). In the positive ion model, salt bridge formation is also possible and, if present, it will contribute to a lowering of charge states.
Figure 2-8. Schematic of two generic types of salt bridges: (a) direct formation between carboxylate and basic site with bridging proton; (b) involving a bridging anion.
2.5 Conclusion

The observed change in the predominating anion in adducts with shifting peptide charge state appears to be correlated to the extent of matching between the GB of the attaching anion and the GB\textsubscript{app} of the deprotonated peptide at the site of attachment. During the process of negative ion electrospray, lower GB anions match with lower charge state peptide ions, whereas higher GB anions match with higher charge state peptide ions. Adduct stability decreases when there is a mismatch in GBs of the two anions constituting the proton-bound mixed dimer of anions. Mismatched attachment sites are subject to high rates of decomposition. Knowledge of adduct behavior can enable a degree of control over adduct formation at various charge states in ESMS studies.

2.6 Appendix – Thermodynamic Considerations

2.6.1 Trends in Gas-Phase Basicities of Carboxylic Acids

For straight-chain mono-carboxylic acids of the form CH\textsubscript{3}(CH\textsubscript{2})\textit{n}COOH (n=0, 1, 2, 3, 4), the GB of the deprotonated acid falls in the range of 1418.5±9.6 kJ/mol to 1428.7±8.4 kJ/mol [15]. However, if the terminal CH\textsubscript{3} group is transformed into a second COOH group, the corresponding deprotonated dicarboxylic acids have substantially lower GBs that range from 1318.0±0.12 kJ/mol to 1342.28±0.12 kJ/mol [30]. Glutamic acid and aspartic acid are the two amino acids that have two –COOH groups in their free amino acid structures and the GBs of the corresponding singly deprotonated anions are 1348±21 kJ/ mol and 1345±14 kJ/mol, respectively [31]. Extending the trend, we can expect that more glutamic/aspartic amino acids included in a peptide sequence will
further lower the GB of the corresponding singly deprotonated form. This reasoning can rationalize why it is possible to observe stable bisulfate (1265.0±10.0 kJ/mol) attachment to the neutral or singly charged peptides employed in this study, whereas acetate (1428.7±8.4 kJ/mol) could not form a stable adduct.

2.6.2  **Apparent Gas-Phase Basicity Versus Intrinsic Gas-Phase Basicity**

Within this paper in negative ion descriptions, anion GB represents -ΔG° of the reaction B⁻ + H⁺ → BH. Furthermore, the term “apparent gas-phase basicity” abbreviated as “GB_app” has been employed to describe the gas-phase basicities of multiply charged peptides. An apparent GB differs from an intrinsic (Brønsted-Lowry) GB because the accumulated electrostatic attraction (Coulomb energy) of like-charged anionic sites toward protons must be factored in. Thus, if a given carboxylic acid site is deprotonated, a second carboxylic acid site on the same molecule holds more tightly its proton. The apparent GB of this latter site is thus the intrinsic GB plus the additional Coulomb energy, δ [32]:

\[
\text{GB}_{\text{app}}([M - n\text{H}^{\text{n}^-}) = \text{GB}([M - n\text{H}^{\text{m}^-}) + \delta \quad (4)
\]

For a generic proton-bound anionic adduct, typically, two low-energy decomposition pathways are often competing:
In the case of a singly charged proton-bound dimer (i.e., where $n = 1$), the so-called “reverse activation energy barrier” is often considered to be close to zero. Even for a multiply charged peptide that is undergoing a neutral loss (Pathway 5a), the reverse activation energy barrier can still be considered to be negligible. By contrast, when a multiply charged peptide decomposes to form two charged product ions (Pathway 5b) electrostatic repulsion between the formed negatively charged peptide and the departing anion would create a significant reverse activation energy barrier, $\varepsilon_{or}$, which is equal to the Coulomb repulsion between the two charged productions. The presence of such a reverse activation barrier makes the activation energy required to pass through this exit channel appear greater than what would be anticipated by solely considering the intrinsic GB at the site in question. Hence, it is more correct to say that “matching of apparent GBs” rather than “matching of GBs” leads to increased stability of the proton-bound mixed dimer of anions.
2.7 References


CHAPTER 3

3.1 Abstract

The existence of gas-phase ion-ion interactions between peptides ([Glu] Fibrinopeptide B, Angiotensin I and [Asn¹, Val⁵]-Angiotensin II) and attaching anions (ClO₄⁻ and HSO₄⁻) derived from high-acidity acids has been confirmed by CID MS/MS. Evidence for ion-ion interactions comes especially from the product ions formed during the first dissociation step, where, in addition to the expected loss of the anion or neutral acid, other product ions that require covalent bond cleavage (i.e., H₂O or NH₃ loss) are also observed. For [[Glu] Fibrinopeptide B + HSO₄⁻], under CID, H₂O water loss was found to require less energy than H₂SO₄ departure. This indicates that the interaction between HSO₄⁻ and the peptide is stronger than the covalent bond holding the hydroxyl group, and must be an ion-ion interaction. The strength and stability of this type of ion-ion interaction are highly dependent on the accessibility of additional mobilized charges to the site. Positive mobilized charges such as protons from the peptide can be transferred to the attaching anion to possibly form a neutral that may depart from the complex. Alternatively, an ion-ion interaction can be disrupted by a competing proximal additional negatively charged site of the peptide that can potentially form a salt bridge with the positively charged site and thereby facilitate the attaching anion's departure.
### 3.2 Introduction

There are multiple motivating factors driving the study of interactions between proteins/peptides and anions in the gas-phase. First of all, anions derived from dissociation of high gas-phase acidity acids, such as ClO$_4^-$, Br$^-$ and I$^-$ can form stable adduct ions with multiply protonated peptides/proteins, and the total number of these attached anions may related to the number of basic sites (R, K, H side chains and the N-terminus) on the peptides/proteins [1-3]. Secondly, certain anions, such as tartrate, citrate, chloride, and nitrate have been reported to stabilize protein structures in the gas phase [4]. Thirdly, the choice of buffer solution (often added to maintain native protein conformation) has been shown to influence the level of dispersion of the ion signal observed in ESI-MS due especially to proton/sodium ion exchange [5, 6], thus affecting detection limits.

Various interactions between proteins/peptides and anions in the gas phase fall outside the category of covalent interactions. These include dipole-dipole, ion-dipole and ion-ion electrostatic interactions. Compared to covalent bonds, dipole-dipole or ion-dipole interactions in the gas phase are typically far weaker interactions that can be disrupted easily under collision induced dissociation (CID) at very low energy in the collision cell of a mass spectrometer. However, experimental evidence has been offered that supports the notion that stronger electrostatic interactions may also exist. For example, a recent report shows that ClO$_4^-$, unlike 12 other anions, can form strong interactions with proteins, as no HClO$_4$ dissociated from the protein under 22 eV collisions [4]. Similar conclusions were reached in another recent report that showed that attachment of ClO$_4^-$, I$^-$ or HSO$_4^-$ to multiply protonated proteins can result in compact gas-phase protein conformations, which suggested the presence of ion-ion or salt-bridge interactions for these gas-phase protein ions [7].
Ion-ion interactions have been reported to exist in gas-phase non-covalent complexes where backbone bond cleavages of proton-bearing peptides were observed to be highly competitive with the break-up of the non-covalent interaction with negatively charged DNA [8]. A more recent example shows a non-covalent complex formed between linear polysulfated glycosaminoglycan oligosaccharides and dibutylamine. MS/MS of this latter complex showed SO₃ loss in competition with neutral dibutylamine loss in the first decomposition step. This competition in dissociation pathways revealed that the non–covalent interaction between glycosaminoglycan and dibutylamine was as strong as the covalent bond that linked SO₃ to the glycosaminoglycan [9], which indicated that the non–covalent interaction was a gas phase ion-ion interaction. Ion-ion interactions were also reported for complexes of singly protonated angiotensin II cations with doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid anions. Evidence came from CID of the complex in an ion trap that first underwent dehydration, followed by cleavage of amide bonds in competition with departure of deprotonated 4-formyl-1,3-benzenedisulfonic acid. This fragmentation pathway is most reasonably explained by an ion-ion interaction in the initial complex. In the absence of such an ion-ion complex, water loss is not expected as the first decomposition step [10].

Our previously proposed “best match” model for multiply charged adduct ion formation between charged peptides and anions was introduced to explain the particular stability of specific anions towards acidic sites and protonated amino groups on peptides. The initially presented “best match” model especially considered hydrogen bond formation between anions and peptides. However, the possibility of ion-ion interactions in the form of salt-bridges was also raised [11], and is directly expanded upon in the current paper.
3.3 Methods

All experiments were performed in the negative ion mode on a Bruker (Bremen, Germany) Solarix 7.0 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Parameters were set to obtain a balance between obtaining reasonably strong signals and maintaining “soft” ionization conditions to promote adduct formation. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Adduct formation occurred between selected anions introduced as sodium salts (NaX, where X = HSO₄⁻ and ClO₄⁻), and model peptides ([Glu] Fibrinopeptide B, Angiotensin I and [Asn¹, Val⁵]-Angiotensin II).
3.4 Results

3.4.1 MS/MS of [Glu] Fibrinopeptide B + HSO₄⁻

In our previous study of anionic adducts of [Glu] Fibrinopeptide B formed in negative mode ESI-MS [11], we examined the behavior of a wide variety of anions: HSO₄⁻, Cl⁻, Br⁻, NO₃⁻, H₂PO₄⁻, CF₃COO⁻ or I⁻. Of all the adduct ions tested, only those with HSO₄⁻ (i.e., [Glu] Fibrinopeptide B + 2HSO₄⁻ and [Glu] Fibrinopeptide B - H + HSO₄⁻) exhibited the following unique decomposition pathway. In addition to either anion departure or loss of the neutral acid
that had been observed for all other anionic adducts, the loss of H₂O was observed to be competitive in the first dissociation step [11]. Shown in Figure 3-1 are new CID experiments obtained using precursor ions comprised of singly charged negative ion adducts formed between bases derived from dissociation of high gas-phase acidity acids (i.e., HSO₄⁻ or ClO₄⁻ anions) and [Glu] Fibrinopeptide B. These two anionic adducts display similar decomposition pathways. For HSO₄⁻ adducts (Figure 3-1a), two principal product ions [[Glu] Fibrinopeptide B + HSO₄ - H₂O]⁻ or [[Glu] Fibrinopeptide B - H - H₂O]⁻ were observed, corresponding to loss of H₂O or [H₂O + H₂SO₄], respectively. In a sequential MS³ experiment (hexapole CID of [[Glu] Fibrinopeptide B + HSO₄]⁻, followed by SORI-CID of [[Glu] Fibrinopeptide B + HSO₄ - H₂O]⁻), consecutive dissociation of the latter results in H₂SO₄ loss leading to the formation of [[Glu] Fibrinopeptide B - H - H₂O]⁻ (Figure 3-1b).

Notably, H₂O loss requires the break-up of covalent bonds. Because H₂O loss occurred in preference to H₂SO₄ (or HSO₄⁻) departure (Figure 3-1a), this indicates that the interaction between the HSO₄⁻ anion and the peptide is stronger than the covalent bond holding the hydroxyl group. Additionally, because [[Glu] Fibrinopeptide B + HSO₄ - H₂O]⁻ decomposition yielded H₂SO₄, it is clear that the bisulfate hydroxyl group did not participate in the initial H₂O loss. HSO₄⁻ is an anion derived from an acid of high gas-phase acidity (1265 ± 23 kJ/mol) [12]. We next sought to test another anion, ClO₄⁻, derived from an acid of even higher gas-phase acidity (1200 ± 50 kJ/mol) [13], to further assess the role of gas-phase proton donor/acceptor properties in the establishment of ion-ion interactions. Analogous results were obtained for ClO₄⁻ attachment (Figure 3-1c) where H₂O loss occurred in preference to HClO₄ or (or ClO₄⁻) departure. In general, ion-dipole or dipole-dipole interactions are expected to be far weaker than covalent bonds, thus, mass spectral evidence strongly suggests suggests that the interaction
between these anions derived from high gas-phase acidity acids and the peptide in both cases is an ion–ion interaction.

### 3.4.2 Energy resolving experiment

![Figure 3-2](image)

Figure 3-2. Breakdown curves for the dissociation of \([\text{[Glu] Fibrinopeptide B} + \text{HSO}_4^-]\) as precursor ion.

In order to get a more complete picture of the competition involved in the dissociation of singly charged anionic adduct precursors, CID experiments were performed with an extended collision energy range in the hexapole collision cell of the hybrid Qq-FT-MS. Product ions formed from the decomposing \([\text{[Glu] Fibrinopeptide B} + \text{HSO}_4^-]\) precursor were monitored from m/z 75 to 2500. There are three main product ions formed within the employed experimental conditions; however, the expected m/z 97 (HSO$_4^-$) was not observed. The percentage yield for
each ion (calculated as the abundance of the selected ion divided by the sum of the abundances of all ions x 100) vs. collision energy ($E_{\text{Lab}}$) is plotted in Figure 3-2. The singly charged precursor adduct ion begins to decline at $E_{\text{Lab}} = 6$ eV as CID energy. At this same energy, the [[Glu] Fibrinopeptide B + HSO$_4^-$ – H$_2$O]⁻ product ion appears (H$_2$O loss) and steadily rises in abundance until $E_{\text{Lab}} = 20$ eV, whereupon its abundance starts to decrease with rising energy. In striking contrast, the [[Glu] Fibrinopeptide B - H]⁻ product ion (corresponding to H$_2$SO$_4$ loss) appears only at a CID energy of 16 eV ($E_{\text{Lab}}$), and its relative abundance keeps increasing. The [[Glu] Fibrinopeptide B - H - H$_2$O]⁻ product ion appears at about the same energy (20 eV ($E_{\text{Lab}}$)), and it increases steadily in abundance, although the signal is always weaker than that of [[Glu] Fibrinopeptide B - H]⁻.

The energy resolved CID experiment clearly indicates that the interaction between HSO$_4^-$ and [Glu] Fibrinopeptide B is stronger than the covalent bonds that link the hydroxyl group to the peptide, because the energy required for the loss of H$_2$SO$_4$ (16 eV ($E_{\text{Lab}}$)) is significantly higher than that required to observe loss of H$_2$O (6 eV ($E_{\text{Lab}}$)). Nonetheless, between 20 and 30 eV as CID energy ($E_{\text{Lab}}$), an increasing portion of H$_2$SO$_4$ is lost, with no backbone bone cleavage occurring. The decrease in relative abundance of [[Glu] Fibrinopeptide B + HSO$_4$ - H$_2$O]⁻ above 20 eV ($E_{\text{Lab}}$) can be explained by considering two contributing factors. First, beyond a critical energy, the [[Glu] Fibrinopeptide B + HSO$_4$ - H$_2$O]⁻ product ion can undergo consecutive decomposition to form [[Glu] Fibrinopeptide B - H - H$_2$O]⁻. Secondly, because the [[Glu] Fibrinopeptide B - H]⁻ product ion rises sharply above 20 eV, it appears that the precursor ion begins to favor initial H$_2$SO$_4$ loss over H$_2$O loss at higher CID energies. This may be rationalized if one considers that the frequency factor [12] for H$_2$SO$_4$ loss is greater than that for H$_2$O loss, even though the latter has a lower threshold energy for decomposition.
3.4.3 MS/MS of [Angiotensin I + HSO₄]⁻

![Image of Angiotensin I structure with MS/MS spectrum]

Figure 3-3. Hexapole-CID (E_{lab} = 20 eV) MS/MS spectrum for [Angiotensin I + HSO₄]⁻. Asterisks (*) mark precursor ions. Angiotensin I structure is shown at top.

In an effort to examine whether similar ion-ion interactions could be formed between HSO₄⁻ and other arginine-containing peptides, bisulfate adducts of Angiotensin I (DRVYIHPFHL) were selected for tandem mass spectrometry studies. Figure 3-3 shows the hexapole CID (E_{lab} = 20 eV) MS/MS spectrum of [Angiotensin I + HSO₄]⁻. In this CID spectrum, two distinct pathways were observed during the first dissociation step. Similar to the results
obtained for [[Glu] Fibrinopeptide B + HSO₄]⁻, the departure of H₂SO₄ is in competition with the loss of H₂O. The fact that this competition exists between neutral acid departure and covalent bond cleavage to give water loss indicates once again that ion-ion interactions exist between Angiotensin I and HSO₄⁻.

3.4.4 **MS/MS of [[Asn¹, Val⁵]-Angiotensin II + HSO₄]⁻**

In order to further investigate the mechanism of water loss that is in competition with H₂SO₄ departure, [Asn¹, Val⁵]-Angiotensin II (NRVYVHPF) was chosen as a test compound for MS/MS experiments. Similar to [Glu] Fibrinopeptide and Angiotensin I, [Asn¹, Val⁵]-Angiotensin II contains a strong basic amino acid (arginine, R). However, Angiotensin II contains only one carboxylic acid group (C-terminus) whereas Angiotensin I has two carboxylic groups, one from the aspartic acid side-chain in addition to the C-terminus, whereas [Glu] Fibrinopeptide has five such acidic groups. The CID spectrum shown in Figure 3-4a was acquired using the same CID energy (E_{lab} = 20 eV) as employed during the acquisition of the spectrum shown in Figure 3-3. A small peak corresponding to loss of NH₃ is observable (Figure 3-4a) indicating a competition between H₂SO₄ loss and NH₃ loss in the first step of dissociation. This competition again suggests the presence of ion-ion interactions in [[Asn¹, Val⁵]-Angiotensin II + HSO₄]⁻ adducts.
Figure 3-4. Hexapole CID (E<sub>lab</sub> = 20eV) MS/MS spectra for (a) [[Asn<sup>1</sup>, Val<sup>5</sup>]-Angiotensin II + HSO<sub>4</sub>]<sup>−</sup> and (b) [[Asn<sup>1</sup>, Val<sup>5</sup>]-Angiotensin II + NaSO<sub>4</sub>]<sup>−</sup> as precursor ions. Asterisks (*) mark precursor ions. The structure of [[Asn<sup>1</sup>, Val<sup>5</sup>]-Angiotensin II appears at the top.
3.4.5 Comparison of [Angiotensin I + HSO₄⁻] vs. [[Asn¹, Val⁵]-Angiotensin II + HSO₄⁻]

Notably, instead of H₂O loss observed for bisulfate adducts of Angiotensin I, NH₃ departure was observed for [[Asn¹, Val⁵]-Angiotensin II + HSO₄⁻]. This difference can be rationalized by considering that Angiotensin I has two carboxylic acid groups. While one carboxylic acid group became deprotonated to create the -1 charge, the second carboxylic acid site can participate in H₂O neutral formation. By comparison, [[Asn¹, Val⁵]-Angiotensin II + HSO₄⁻] contains only one carboxylic acid group which, upon deprotonation, leaves no –OH group available to form H₂O (a phenolic group does exist on the side chain of tyrosine residue, but it is not a good leaving group for water loss because the carbon-oxygen bond is strengthened by partial double bond character). Thus, other low-energy dissociation pathways such as NH₃ loss become competitive. Based on these considerations, one can implicate the neutral carboxylic acid of Angiotensin I in the water loss pathway. The nitrogen-bound hydrogens on either histidine of Angiotensin I are the most acidic protons available for this transfer. Similarly, the single nitrogen-bound hydrogen is the most acidic proton on Asn¹, Val⁵]-Angiotensin II.

Of the three tested peptide adducts with HSO₄⁻, only [Angiotensin I + HSO₄⁻] and [[Asn¹, Val⁵]-Angiotensin II + HSO₄⁻] yield H₂SO₄ loss (under low-energy CID, [[Glu] Fibrinopeptide B + HSO₄⁻] did not). The presence of an ion-ion interaction implies a high barrier for decomposition; the rate constant of such a decomposition will also depend upon how readily the anion can abstract a proton from the peptide. If a cation-anion interaction exists, it is not necessarily a proton that is bound to the cationic portion that will leave as a neutral with the anion. The most acidic groups on the peptides that can serve as proton sources are the carboxylic
acids (Figure 3-5a). For [Glu] Fibrinopeptide B and Angiotensin I only, there are additional
carboxylic acid sites beyond the one providing the charge. The presence of these acidic groups
on the peptide anion, and thus the availability of protons for transfer, can explain the relatively
low activation energy needed to promote H$_2$SO$_4$ loss from the corresponding [peptide + HSO$_4$]$^-$
complexes exhibiting ion-ion interactions.

3.4.6 *Weakening of ion-ion or salt-bridge interaction*

Of the three tested peptide adducts with HSO$_4^-$, only [Angiotensin I + HSO$_4$]$^-$ and [[Asn$^1$,
Val$^5$]-Angiotensin II + HSO$_4$]$^-$ yield H$_2$SO$_4$ loss (under low-energy CID, [[Glu] Fibrinopeptide B
+ HSO$_4$]$^-$ did not). The presence of an ion-ion interaction implies a high barrier for
decomposition; the rate constant of such a decomposition will also depend upon how readily the
anion can abstract a proton from the peptide. If a cation-anion interaction exists, it is not
necessarily a proton that is bound to the cationic portion that will leave as a neutral with the
anion (Figure 3-5a, pathway 1). The most acidic groups on the peptides that can serve as proton
sources are the carboxylic acids. For [Glu] Fibrinopeptide B and Angiotensin I only, there are
additional carboxylic acid sites beyond the one providing the charge. The presence of these
acidic groups on the peptide anion, and thus the availability of protons for transfer (Figure 3-5a,
pathway 2), can explain the relatively low activation energy needed to promote H$_2$SO$_4$ loss from
the corresponding [peptide + HSO$_4$]$^-$ complexes exhibiting ion-ion interactions.
Figure 3-5. Weakening of salt bridge by (a) proton transfer, (b) negative charge mobility for an ion-ion interaction formed between protonated Arg and HSO₄⁻.

Figure 3-4b shows the product ion spectrum of the [[Asn¹, Val⁵]-Angiotensin II + NaHSO₄]⁺ precursor. Similar to [[Asn¹, Val⁵]-Angiotensin II + HSO₄⁻], two product ions are observed during the first dissociation step, i.e., the loss of NH₃ or the loss of NaHSO₄. The appearance of the loss of NaHSO₄ instead of the loss of H₂SO₄ strongly suggests that the proton of HSO₄⁻ has been exchanged with a sodium cation, thus forming NaSO₄⁻. Had the sodium
exchanged instead with a proton of the peptide, then sodium would not be expected to depart with HSO$_4^\cdot$. When comparing Figure 3-4b with Figure 3-4a, the lower affinity of the proton for NaSO$_4^\cdot$ vs. HSO$_4^\cdot$ in the gas phase results in the formation of a lower abundance of [[Asn$^1$, Val$^5$]-Angiotensin II – H$^\cdot$]. In other words, NaSO$_4^\cdot$ is less effective than HSO$_4^\cdot$ at abstracting a proton from the peptide. This can be rationalized by considering that NaSO$_4^\cdot$ has a structure wherein the sodium cation is chelated between the two negatively charged oxygen atoms. Furthermore, compared with the proton, the presence of the sodium cation augments the facility for NH$_3$ departure as evidenced by the relatively high abundance of [[Asn$^1$, Val$^5$]-Angiotensin II + NaSO$_4$ – NH$_3$]$^\cdot$ in Figure 3-4b vs. Figure 3-4a. We postulate that the departing NH$_3$ originates from the N-terminus (Figure 3-4). If the attaching anion is engaged in an ion-ion interaction with the protonated arginine group, then NaSO$_4^\cdot$ would have a higher mobility than HSO$_4^\cdot$, leading to increased coulombic repulsion between the neighboring protonated N-terminus and protonated arginine. This electrostatic repulsion will promote NH$_3$ loss that will be more favorable for the NaSO$_4^\cdot$ adducts (Figure 3-4b) than for the HSO$_4^\cdot$ adducts (Figure 3-4a).

Notably, in our experience, NaSO$_4^\cdot$ rarely attaches to proteins/peptides to form adducts. Interestingly, addition of Na$_2$SO$_4$ in protein solution can result in HSO$_4^\cdot$ attachment in the positive ion mode. Evidence for this comes from the fact that H$_2$SO$_4$ loss is observed when CID is used to dissociate the adduct. We propose that formation of the NaSO$_4^\cdot$ adducts observed in Figure 3-4b is promoted because NaSO$_4^\cdot$ is forming an ion-ion interaction with [Asn$^1$, Val$^5$]-Angiotensin II.

Just as the ion-ion interaction can be destabilized by the presence of a mobilized positive charge, such as a proton (Figure 3-5a), similarly it can also be destabilized by a mobilized negative charge. Figure 3-6 shows the CID spectrum of [[Glu] Fibrinopeptide B - H + HSO$_4$]$^{2-}$;
the $\text{H}_2\text{O}$ loss in the first step of dissociation indicates the existence of an ion-ion interaction. However, rather than losing $\text{H}_2\text{SO}_4$ in competition with $\text{H}_2\text{O}$ loss, $\text{HSO}_4^-$ departure occurred instead. Because of the strength of the attraction, it is impossible to directly separate the two ions that constitute the ion-ion interaction by SORI-CID. However, the loss of $\text{HSO}_4^-$ becomes possible if there is assistance from the extra negative charge. As shown in Figure 3-5b, the negative charge of the carboxylate group can compete with the bisulfate anion to form an interaction with the positively charged arginine site. The presence of the second anionic (carboxylate) site will weaken the ion-ion interaction between protonated arginine and bisulfate anion, thus leading to the departure of the bisulfate anion.

Figure 3-6. SORI-CID MS/MS spectrum of $[[\text{Glu}] \text{Fibrinopeptide B - H + HSO}_4]^2^-$. Asterisks (*) mark precursor ions; $[\text{Glu}] \text{Fibrinopeptide B}$ is abbreviated as Fib.
3.5 Conclusions

The existence of ion-ion interactions in the gas phase between peptides ([Glu] Fibrinopeptide B, Angiotensin I or [Asn$^1$, Val$^5$]-Angiotensin II) and anions (ClO$_4^-$ or HSO$_4^-$) derived from high gas-phase acidity acids have been established by CID MS/MS. Evidence for ion-ion interactions comes especially from the product ions formed during the first dissociation step, where, in addition to the expected loss of the anion or neutral acid, other product ions that require covalent bond cleavage (i.e., H$_2$O or NH$_3$ loss) are also observed.

H$_2$O loss in the first step of dissociation appears to be promoted by the presence of an intact carboxylic acid group on the peptide that provides the proton for transfer. This conclusion is supported by the fact that for both [[Glu] Fibrinopeptide B + HSO$_4^-$] and [Angiotensin I + HSO$_4^-$], which have neutral carboxylic acids available, H$_2$O loss is observed in the initial dissociation step. However, for [[Asn$^1$, Val$^5$]-Angiotensin II + HSO$_4^-$], which contains no intact carboxylic acid site at the -1 charge state, no H$_2$O loss is obtained; instead, NH$_3$ departure is observed.

A strong ion-ion interaction can be destabilized by a mobilized charge. Of the three tested peptide adducts with HSO$_4^-$: [Angiotensin I + HSO$_4^-$], [[Asn$^1$, Val$^5$]-Angiotensin II + HSO$_4^-$] and [[Glu] Fibrinopeptide B + HSO$_4^-$], all can yield H$_2$SO$_4$ after passing the required threshold collision energy. This indicates that a proton originating from the peptide has been transferred to the bisulfate anion, and it is the arrival of the mobile charge that weakens the ion-ion interaction. Alternatively, ion-ion interactions can also be disrupted by the excess charge site. The additional negative charge site(s) in the complex can compete with the attaching anion to interact with a positively charged site in these zwitterionic species, and thereby weaken the interaction holding the attaching anion at that site, thus freeing this anion to depart.
3.6 Reference

CHAPTER 4

4.1 Abstract

The "Best Match" model has been extended to account for the role that Na$^+$/H$^+$ exchange plays on anion attachment in negative ion electrospray. Without any Na$^+$/H$^+$ exchange on [Glu]Fibrinopeptide B, the higher basicity anions F$^-$ and CH$_3$COO$^-$ can hardly form observable adducts; however, after multiple Na$^+$/H$^+$ exchanges, adduct formation is enabled. Moreover, dissociation pathways of CF$_3$COO$^-$ adducts with singly deprotonated peptides that have undergone 0 to 3 Na$^+$/H$^+$ exchanges exhibit a shift in CID product ions from losing predominately CF$_3$COOH (case of 0 Na$^+$/H$^+$ exchanges) to losing predominately CF$_3$COO$^-$ (case of 3 Na$^+$/H$^+$ exchanges). These phenomena can be rationalized by considering that Na$^+$ cations exchange at, and serve to “block”, the most acidic sites, thereby forcing implicated anions to attach to lower acidity protons. In addition to forming ion pairs with carboxylate groups, Na$^+$ also participates in formation of tri-atomic ions of the form ANaA$^-$ during adduct dissociation. The fact that low GB anions preferentially form ANaA$^-$ species, even though high GB anions form more stable tri-atomic species, indicates that the monatomic ions were not in close contact in the initial adduct. The propensity for formation of stable anionic adducts is dependent on the degree of matching between anion GBs and GB$_{app}$ of deprotonated sites on the peptide. The GB$_{app}$ is raised dramatically as the charge state of the peptide increases via a through-space
effect. The presence of \( \text{Na}^+ \) on carboxylate sites substantially decreases the \( \text{GB}_{\text{app}} \) by neutralizing these sites, while slightly increasing the intrinsic GBs by an inductive effect.

**4.2 Introduction**

The ability to produce multiply charged ions in an extremely soft and efficient manner from solution phase analytes has established electrospray ionization (ESI) mass spectrometry (MS) as a key technology for characterization and sequencing of biological macromolecules, such as DNA and proteins [1-3]. However, the performance of ESI-MS can be significantly influenced by the presence of certain cations (such as \( \text{Na}^+ \), \( \text{K}^+ \)) as well as certain anions (such as \( \text{HSO}_4^- \), \( \text{H}_2\text{PO}_4^- \)) [4-6]. In addition to the signal suppression that often accompanies ESI-MS analysis of high salt solutions, these types of cations and anions can attach to multiply charged ions to form stable adducts which results in the dispersion of analyte signals over various m/z signals corresponding to the varying masses of the different adducts. Compared to attached cations, typically anions can be removed more readily from proteins in collision induced dissociation (CID) experiments by increasing the collision energy [6-8]. A recent study in positive mode ESI-MS [9] reports that the extents of acid molecule attachment and \( \text{Na}^+/\text{H}^+ \) exchange are inversely related.

Our group has recently described a “Best Match” model [10] to explain adduct formation between peptides and attaching anions in the negative ion mode. According to our model, the stability of anionic adducts that we consider to exist as proton bound mixed dimers of anions depends on the degree of matching between the gas-phase basicity (GB) of the attaching anion (conjugate base of the acid form) and the apparent GB (\( \text{GB}_{\text{app}} \)) of the deprotonated site on the
peptide. Anions derived from low gas-phase acidity acids such as Cl⁻ can attach to low acidity protons of the peptide to form stable anionic adducts of high charge state (the high acidity sites of the peptide are readily deprotonated), whereas low GB anions, like HSO₄⁻, prefer to attach to high acidity protons and thus tend to appear in low charge state anions. However, with even higher GB anions such as F⁻ and CH₃COO⁻, no observable adducts were formed with peptides. This can be rationalized by considering that these high GB anions can readily pull off multiple protons to form high charge state, multiply deprotonated peptide anions. Multiple anionic sites will significantly increase the GB_{app} of the attaching site, in contrast to the (opposite) effect observed upon multiple protonation in the positive ion mode [11]. Here we report on the effects of Na⁺/H⁺ exchange, occurring at the highest acidity peptide sites, on observed anion attachment and deduced stabilities of adducts in negative ion ESI-MS and ESI-MS/MS.

### 4.3 Experimental

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Employed selected anions were introduced as sodium salts (NaX, where X = F⁻, CH₃COO⁻, Cl⁻, CF₃COO⁻ and I⁻). All experiments were performed in the negative ion mode on a Bruker (Bremen, Germany) Solarix 7.0 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Employed parameters (capillary exit (-230 V), skimmer 1 (-10 V), hexapole collision energy (-2.5 V) and trap time (1 S), hexapole CID gas control (100%)) were set to strike a balance between obtaining reasonably strong signals and maintaining “soft” ionization conditions to promote adduct formation.
4.4 Results

4.4.1 Effect of Na⁺/H⁺ exchange on adduct stability and dissociation pathway

([Glu] Fibrinopeptide B) was used as a model peptide to study the effect of Na⁺/H⁺ exchange on anion attachment. This 14-residue peptide (Figure 1), with the sequence EGVNDNEEGFFSAR contains five carboxylic acid groups. Figures 1a-d show the SORI-CID MS/MS spectra of four adducts that formed between CF₃COO⁻ and singly deprotonated [Glu] Fibrinopeptide B molecules that have undergone 0, 1, 2, or 3 Na⁺/H⁺ exchanges, respectively. Attachment of CF₃COO⁻ on these peptides leads to a shift in CID product ions from losing predominately CF₃COOH (case of 0 Na⁺/H⁺ exchanges) to losing predominately CF₃COO⁻ (case of 3 Na⁺/H⁺ exchanges). Based on relative peak intensities, without Na⁺/H⁺ exchange, 86% of [Fib – H + CF₃COO]⁻ will dissociate by losing CF₃COOH, whereas the other 14% of these precursor ions will decompose by departure of CF₃COO⁻. After one Na⁺/H⁺ exchange, the ratio of losing CF₃COOH versus CF₃COO⁻ departure is slightly decreased to: 84% versus 16%. A more substantial change was observed when two Na⁺/H⁺ exchanges occurred, as the ratio is decreased to 63% CF₃COOH loss versus 37% CF₃COO⁻ departure. Surprisingly, after three Na⁺/H⁺ exchanges occurred, CF₃COOH loss barely can be observed and almost all of the [[Glu] Fibrinopeptide B – 4H + 3Na + CF₃COO] decomposition occurs by CF₃COO⁻ departure.
Figure 4-1. Structure of [Glu] Fibrinopeptide B (top) and SORI-CID MS/MS spectra for the following precursors: (a) \([\text{[Glu] Fibrinopeptide B - H + CF}_3\text{COO]}^-\), (b) \([\text{[Glu] Fibrinopeptide B - 2H + Na + CF}_3\text{COO]}^-\), (c) \([\text{[Glu] Fibrinopeptide B - 3H + 2Na + CF}_3\text{COO]}^-\), (d) \([\text{[Glu] Fibrinopeptide B - 4H + 3Na + CF}_3\text{COO]}^-\). Asterisks (*) mark precursor ions; [Glu] Fibrinopeptide B is abbreviated as "Fib".

The fact that departure of CF3COO- is competing with loss of CF3COOH under SORI-CID in Figure 4-1a, 1b, and 1c clearly indicates that CF3COO- is attaching to a proton instead of Na+. Had the CF3COO- attached to Na+, then, a competition between CF3COO- departure or CF3COONa loss would be expected. According to the "Best Match" model, the shift of CID
product ion spectra of these four adduct indicates that the sites to which CF3COO- are attaching are becoming more and more basic as the number of Na+/H+ exchanges increases. This finding suggests that Na+/H+ exchanges are occurring preferentially at the higher acidity sites and that these exchanges have served to “block” the higher acidity sites. For [[Glu] Fibrinopeptide B – H + CH3COO]2-, the most acidic proton has been removed to form a negative charge, thus, CF3COO- is attaching to the second most acidic proton. When Na+/H+ exchange has occurred at the single most acidic site, the second most acidic proton will be pulled off to form one negative charge and CF3COO- will be forced to attach to the third most acidic proton to create a doubly charged anion. Following this logic, the higher the number of Na+/H+ that are exchanged at the most acidic sites on the peptide, the lower is the acidity of the hydrogen to which CF3COO- is attached. The structures of the four doubly charged adduct precursors from Figure 4-1 are represented in Figure 4-2a~2d
Figure 4-2. Schematic structure of [Glu] Fibrinopeptide B shown at top in neutral form with all five carboxylate sites bearing protons. In moving from the top to the bottom of any peptide representation, the acidity of the carboxylic acid group is progressively decreasing. (a) [[Glu] Fibrinopeptide B - H + CF₃COO]²⁻, (b) [[Glu] Fibrinopeptide B - 2H + Na + CF₃COO]²⁻, (c) [[Glu] Fibrinopeptide B - 3H + 2Na + CF₃COO]²⁻, (d) [[Glu] Fibrinopeptide B - 4H + 3Na + CF₃COO]²⁻.

Based on Figure 4-2, one can infer that if an attaching anion cannot bind to the sodium that exchanged with the most acidic proton on the peptide, then this site is actually “blocked” by the exchanged sodium. As a result, any anion attaching to the peptide will be forced to bind to a hydrogen of lower acidity that is available on the peptide. In other words, to form an ion of -2
charge state from a deprotonated peptide with 0, 1, 2 or 3 Na⁺/H⁺ exchanged, the CF₃COO⁻ are "forced" to attach to the second, third, fourth, or fifth most acidic proton, respectively. As the acidity of the site of attachment decreases, the proton at the attaching sites is held more tightly by the peptide and it becomes more difficult for CF₃COO⁻ to pull off this proton. This is supported by the fact that, as the number of Na⁺/H⁺ exchanges increases from zero to three, the percentage of CF₃COO⁻ loss is increasing from 14% to 100%, while the percentage of CF₃COOH loss is decreasing from 86% to 0.

According to the “best match” model, the stability of the proton bound adduct that is formed between anion and peptide is dependent on the matching of the GB of the attaching anion with the GB_app of the corresponding deprotonated site on the peptide. If an anion has been forced to attach to a higher GB_app site due to Na⁺/H⁺ exchange, the stability of the adduct will likely be altered. SORI – CID MS/MS spectra of these four adducts shown in Figure 4-1 were acquired under similar activation energies (0.7% SORI power, pressure reservoir 5.0 mbar and 0.3% frequency offset), the dissociation percentages for 0, 1, 2, and 3 Na⁺/H⁺ exchange precursor ions are 97%, 79%, 46%, and 33% respectively (calculation based on (Σ abundances of decomposition products/Σ abundances of decomposition products plus abundance of surviving precursor) x 100), which indicates that the stability of these adducts is increasing as the number of Na⁺/H⁺ exchanges is increased.

In all three Na⁺/H⁺ adduct product ion spectra shown in Figure 4-1b, 1c and 1d, no CF₃COO⁻Na loss was observed, even from the most acidic site whose deprotonated form has a low GB_app compared to CF₃COO⁻. This indicates that a Na⁺ that has exchanged with the most acidic proton is much harder to be removed compared to the lower acidity protons. An elevated degree of solvation of Na⁺ by both oxygens of the carboxylate group to which it is attached on
the peptide may be contributing to the increased stability of the sodium cation relative to protons at the same site.

4.4.2 Na⁺/H⁺ exchange at low charge states and effect upon analyte ion abundance

As noted by other researchers [12, 13], multiple Na⁺/H⁺ exchanges on peptide/protein ions are always prevalent at lower charge states, but the underlying reason behind this observation is not well established. Based on the effect of Na⁺/H⁺ exchange on anion attachment to peptides observed in negative mode ESI, one can deduce a reasonable explanation for this phenomenon. As a specific number of Na⁺/H⁺ exchanges occur on the peptide/protein, that same number of the most acidic sites on the peptide/protein become "blocked" from anion attachment. In order to form an ion of the same charge state as an "unblocked" peptide, the peptide/protein now must remove protons that are much more strongly attached at less acidic sites. For this reason, it becomes very hard to form ions of high charge state in negative ESI for peptides/proteins that have undergone multiple Na⁺/H⁺ exchanges. In fact, multiple Na⁺/H⁺ exchanges on a peptide could go so far as to render a peptide or protein quite difficult to ionize, and thus cause an overall diminution of signal while favoring the lowest charge state ions.
4.4.3 Interaction between attached anions and exchanged Na$^+$

Figure 4-3. Hexapole-CID MS/MS spectra for the following precursors: (a) [[Glu] Fibrinopeptide B – H + Na + 2Cl]$^2$-, (b) [[Glu] Fibrinopeptide B – H + Na + 2CF$_3$COO]$^2$-, (c) [[Glu] Fibrinopeptide B – H + Na + 2I]$^2$-. Asterisks (*) mark precursor ions; [Glu] Fibrinopeptide B is abbreviated as "Fib".

In order to examine whether the above Na$^+/H^+$ effect on anion attachment is also applicable to multiple anion attachment adducts, [[Glu] Fibrinopeptide B – H + Na + 2A]$^2$- (A = Cl, CF$_3$COO, I) were selected as precursor ions for tandem mass spectrometry experiments. Figure 4-1 shows the hexapole CID MS/MS spectra of adducts of the form [[Glu] Fibrinopeptide B – H + Na + 2A]$^2$-. In keeping with the discussion above, for [[Glu] Fibrinopeptide B – H + Na + 2A]$^2$-, one Na$^+$ has exchanged with the most acidic proton and the two anions will attach to the
remaining best matched acidic protons. The product ion spectra of all these adducts clearly show
two-step fragmentation pathways wherein either A\textsuperscript{−} or HA departs in each step depending on the
GB of A\textsuperscript{−}. According to the "Best Match" model, if one of the two anions is dissociated by losing
HA, then the more acidic proton of these two attaching sites should be the easier one to be
removed. Therefore, the site that the remaining anion is binding to on the peptide is less acidic
than the deprotonated site and the Na\textsuperscript{+}/H\textsuperscript{+} exchanged site. For Cl\textsuperscript{−} ([[Glu] Fibrinopeptide B – H +
Na + 2Cl]\textsuperscript{2−} precursors, Figure 4-3a), the highest GB anion among the three tested anions, two
chloride anions can consecutively pull off two protons from the second and third most acidic
sites of [Glu] Fibrinopeptide B. Thus, [[[Glu] Fibrinopeptide B – 2H + Na + Cl]\textsuperscript{2−} is formed in the
first step, with [[[Glu] Fibrinopeptide B – 3H + Na]\textsuperscript{2−} formed in the second step; no product ion
corresponding to Cl\textsuperscript{−} departure is observed. For CF\textsubscript{3}COO\textsuperscript{−} ([[Glu] Fibrinopeptide B – H + Na + CF\textsubscript{3}COO]\textsuperscript{2−} precursor, Figure 4-3b), with an intermediate GB, the second most acidic proton is
readily removed from the peptide, producing [[[Glu] Fibrinopeptide B – 2H + Na + CF\textsubscript{3}COO]\textsuperscript{2−};
further dissociation of this product ion will result in either departure of CF\textsubscript{3}COO\textsuperscript{−} or loss of
CF\textsubscript{3}COOH as shown in Figure 4-1b (but not in Figure 4-3b due to insufficient CID energy).
Iodide ([[Glu] Fibrinopeptide B – H + Na + 2I]\textsuperscript{2−} precursor, Figure 4-3c), with the lowest GB of
the three anions, yields a wider variety of product ions compared to either chloride or
trifluoroacetate. The first dissociation step leads to either I\textsuperscript{−} departure or loss of HI, thereby
producing [[[Glu] Fibrinopeptide B – H + Na + I]\textsuperscript{−} or [[[Glu] Fibrinopeptide B – 2H + Na + I]\textsuperscript{2−},
respectively. In considering consecutive decompositions of these latter two ions, [[[Glu]
Fibrinopeptide B – 2H + Na]\textsuperscript{−} was the only product ion observed indicating that the attached I\textsuperscript{−} in
both cases are well-matched to their respective protonated carboxylate sites and that the energy
required for HI departure from the more acidic attachment site on [[Glu] Fibrinopeptide B – H +
Na + I] is approximately the same as that required for I departure from the less acidic attachment site of [[Glu] Fibrinopeptide B – 2H + Na + I]^{2-}.

In addition to the product ions that formed by A− departure or HA loss from [[Glu] Fibrinopeptide B – Na + H + 2A]^{2−}, the ion [[Glu] Fibrinopeptide B - H]− was also formed in all three product ion spectra (Figure 4-3). The appearance of ANaA− ions in Figure 4-3b and Figure 4-3c confirms that these ions can be formed by departure of intact ANaA−, as opposed to a two-step process. As one may notice, sodium ions are removed in these dissociation pathways, and this seems to be the only pathway permitting Na+ release from the peptide. Interestingly, when comparing for example, INaI− with ClNaCl−, even though the latter is much more stable [14], the abundance of ANaA− is much higher for the former. For Cl−, CF3COO−, and I−, listed in order of decreasing GB, the percentage of ANaA− is 0.0%, 0.4%, and 3.0%, respectively (calculated as abundance of ANaA−/Σ abundances of all ions in the spectrum). For each instance of ANaA− departure, [[Glu] Fibrinopeptide B – H]− is simultaneously produced, and the same trend is manifested in the percentage of [[Glu] Fibrinopeptide B – H]− observed in Figure 4-3 for Cl−, CF3COO−, and I−, i.e., 3.5%, 15.7%, and 24.0%, respectively (calculated as intensity of [[Glu] Fibrinopeptide B – H]−/Σ intensities of all peptides ions in the spectrum)).

The fact that in each case where ANaA− formation appears, HA loss is also occurring in competition, suggests that ANaA− is unlikely to have existed as an electrostatically-linked, close contact, tri-atomic entity in the original doubly charged precursor adduct. Rather, we propose that the sodium cation is initially engaged in an ion-ion interaction with a low basicity carboxylate group. We know that this ion-ion interaction is strong because no sodium ion can be removed during CID of any of the precursor adducts shown in Figure 4-1b~d. Release of ANaA− is proposed to be favored from a conformation where a first anion attached to a carboxylic acid is
initially in close proximity to the sodium cation (Figure 4-4, right). Notably, the close proximity of this anion is insufficient to cause NaA loss as the corresponding product ion was never observed in any CID spectrum. While still in the form of a doubly charged ionic complex, CID may cause release of the second A\(^-\) (Figure 4-4, left) that occurs most efficiently for doubly charged adducts of iodide (Figure 4-3c) and progressively less efficiently for trifluoroacetate, and lastly chloride anions. This released anion can be attracted to the sodium cation, and the arrival of this second A\(^-\) can apparently sufficiently weaken the carboxylate-sodium interaction to allow departure of ANaA\(^-\) (Figure 4-4). The relatively high abundance of the INaI\(^-\) ion (characterized by the lowest stability among the three ANaA\(^-\) ions) can be rationalized by the fact that I\(^-\) departure (the second anion in the scenario above) occurs most readily for this doubly charged adduct. In contrast, even though ClNaCl\(^-\) has the highest stability, Cl\(^-\) does not depart without a proton in tow.
Figure 4-4. Schematic structure of \([\text{[Glu]} \text{ Fibrinopeptide B - H + Na + 2A}]^2\). Two \(A^-\) are in close proximity to the exchanged Na cation. Release of the anion \(A^-\) adducted to the carboxylic acid on the left, can destabilize the sodium cation and anion \(A^-\) located on the right, leading to formation of \(A\text{NaA}^-\).

### 4.4.4 High GB anions attach to peptides exhibiting multiple \(\text{Na}^+/\text{H}^+\) exchanges

#### 4.4.4.1 NaF

Further evidence that \(\text{Na}^+\) can serve to "block" highly acidic sites on peptides comes from experiments investigating adducts formed between higher GB anions and [Glu] Fibrinopeptide B. The ESI mass spectrum produced from aqueous solutions containing 5 µM [Glu] Fibrinopeptide B and 0.5 mM NaF is shown in Figure 4-5a. With NaF, the number of sodium cations and fluoride anions that adduct to the peptide can be as high as 16 and 13 respectively. The first four major peaks are separated by 22 mass units, which is caused by 0, 1, 2, or 3 sequential \(\text{Na}^+/\text{H}^+\) exchanges. The following peaks that have higher m/z are separated by 42
mass units, which corresponds to the mass of NaF. The mass spectrum shown in Figure 4-5b was obtained under the same conditions except that a 60 eV in-source CID (nozzle-skimmer) potential was employed. The high in-source CID potential causes a relatively high amount of energy uptake for all ESI-desorbed adduct species and leads to drastic changes in the mass spectrum: upon collision, two or three HFs can be lost from each adduct if adducts have at least three fluorides attached; up to six Na⁺/H⁺ exchanged adducts have been obtained for the NaF-containing sample. The proton that leaves with fluoride must have been provided by the peptide. The loss of HF for all the fluorides indicates that fluorides are binding to protons.

![Mass Spectra](image)

Figure 4-5, (a) ESI-MS of solution containing 5 µM [Glu] Fibrinopeptide B and 0.5 mM NaF; (b) Same condition as (a) except “in-source” CID collision energy = 60 eV (E_{lab}). [Glu] Fibrinopeptide B is abbreviated as "Fib". For "(x, y)" nomenclature, x represents the number of Na⁺ and y is the number of F⁻.

In our previous study [10], one conclusion regarding fluoride attachment was that without any Na⁺/H⁺ exchange on the peptide, fluoride cannot form any observable adducts with [Glu] Fibrinopeptide B. However, with multiple Na⁺/H⁺ exchanges, fluoride does form stable adducts.
For example, the first fluoride attaches to the peptide after four Na\(^+/H^+\) exchanges. Clearly, the attachment of fluoride on the peptide is facilitated by the Na\(^+/H^+\) exchanges. [Glu] Fibrinopeptide B contains 5 carboxylic acid groups, which are the five most acidic sites on the peptide. Figure 4-5 shows that, at -2 charge state, none of the GB\(_{app}\)s of the remaining protonated carboxylate sites could match the GB of a fluoride (the GB\(_{app}\)s were all too low). For [[Glu] Fibrinopeptide B – 5H + 4Na + F]\(^{-2}\), according to the best “match model” the first fluoride is attaching to the sixth most acidic sites when four of these five carboxylic sites have been blocked by Na\(^+/H^+\) exchange and the last one has been deprotonated to form a charge. Moreover, for [[Glu] Fibrinopeptide B – 5H + 5Na + 2F]\(^{-2}\), (marked (5,2) in Figure 4-5a) attachment of two fluorides would occur at the sixth and seventh most acidic sites, while the five most acidic sites have all been Na\(^+/H^+\) exchanged.

In considering the structure of [Glu] Fibrinopeptide B (Figure 4-1) in the context of typical pKa values of the remaining substituents, it is clear that once the five carboxylic acids have all been deprotonated (pKa's in the range of 3.2-4.8 for most organic carboxylic acids [15]), the next most labile protons are far less acidic. In attempting to gauge which specific functionalities constitute the sixth and seventh most acidic sites, we note that there are amide functions on the asparagine side chains as well as on the peptide backbone, whereas the serine side chain holds a primary alcohol. The N-H bond of an amide function has been reported as having a pKa of approximately 15.1[15], whereas the pKa of an alcohol of the form RCH\(_2\)OH has been given as approximately 16 [16].

The above observations concerning the conditions permitting F\(^-\) attachment to the peptide can be rationalized by considering that Na\(^+\) exchanges with, and thus serves to “block”, the highly acidic sites, thereby forcing them to remain overall neutral. This leaves the less acidic
protons available to match with higher GB anions. The reason that no given carboxylic acid site will form a stable adduct with fluoride is because the highly basic fluoride anions will always pull off these rather acidic protons and depart as HF, thus leaving behind charged carboxylate anions.

The presence of sodium in place of protons, however, raises the *intrinsic* basicity of nearby proton-bearing potential sites of anion attachment. This increase in intrinsic basicity is brought about because the electron density increases at the oxygen in closest contact with the sodium cation relative to the case of a proton. This higher electron density exerts an inductive effect on nearby atoms thereby lowering the acidity of nearby protons; this inductive effect, however, weakens rapidly with distance away from the sodium atom. Upon multiple Na⁺/H⁺ exchanges, the slight increase in the intrinsic basicity of the peptide resulting from this inductive effect can contribute to a better matching of remaining potential sites of anion attachment with F⁻.

In addition to the two fluorides that are attaching to the peptide to form adducts of -2 charge state, Figure 4-5 shows that many more fluorides are capable of attaching to the peptide. Because the adduct maintains an overall “-2” charge state, each extra fluoride must be neutralized by an additional sodium ion. This neutralization suggests the preservation of a rather strong interaction between the attached fluoride and the sodium resulting from the added NaF.

4.4.4.2 \( \text{CH}_3\text{COONa} \)

In an effort to confirm the results that high GB anions can attach to the peptide under multiple Na⁺/H⁺ exchange, CH₃COONa was selected as a second test salt to conduct experiments
analogous to those shown in Figure 4-5. Similar to F\textsuperscript{−}, because of its high GB, CH\textsubscript{3}COO\textsuperscript{−} cannot form stable adducts with the deprotonated peptide in the absence of Na\textsuperscript{+}/H\textsuperscript{+} exchange. Figure 4-6a shows negative ion spectra obtained from a solution containing 0.5 mM CH\textsubscript{3}COONa and 5 µM [Glu] Fibrinopeptide B; both Na\textsuperscript{+} and CH\textsubscript{3}COO\textsuperscript{−} readily attach to the peptide. However, the first CH\textsubscript{3}COO\textsuperscript{−} anions start to attach to the peptide only after 3 Na\textsuperscript{+}/H\textsuperscript{+} exchanges, which suggests that CH\textsubscript{3}COO\textsuperscript{−} can match to the fifth most acidic site on the peptide to form a doubly charged anion. The results correlate to the GB trends, i.e., CH\textsubscript{3}COO\textsuperscript{−} (1427 ± 8.4 kJ/mol) [17] has a substantially lower GB than fluoride (1530 ± 0.75 kJ/mol) [18], thus, CH\textsubscript{3}COO\textsuperscript{−} can match with the fifth most acidic site whereas F\textsuperscript{−} matches with the sixth. In addition, for sodium acetate, the maximum number of sodium and anions that may attach is far less compared to NaF; the maximum number of CH\textsubscript{3}COO\textsuperscript{−} and Na\textsuperscript{+} attached are 6 and 9, respectively, compared to 13 and 16 for NaF. It appears that F\textsuperscript{−} is more successful than CH\textsubscript{3}COO\textsuperscript{−} at forming stable adducts with low acidity protons not located on carboxylic acids. That is to say, the remaining protons that are candidates for anion attachment are located on anionic sites characterized by GB\textsubscript{app}s that are too far above the GB of CH\textsubscript{3}COO\textsuperscript{−} to allow formation of many stable adducts (they are better matched with F\textsuperscript{−}). Upon in-source CID (E\textsubscript{lab} = 60 eV), one (or at most two) anion(s) will depart from the peptide as CH\textsubscript{3}COOH and the maximum number of Na\textsuperscript{+}/H\textsuperscript{+} exchanges is five in this case (shown in Figure 4-6b). The formation of stable CH\textsubscript{3}COO\textsuperscript{−} adducts only after multiple Na\textsuperscript{+}/H\textsuperscript{+} exchanges further affirms the finding that high GB anions can attach to the peptide only when the most acidic sites are blocked by multiple Na\textsuperscript{+}/H\textsuperscript{+} exchanges in accordance with the “Best Match” model. Owing to the larger size of the acetate anion relative to fluoride and chloride anions, its interaction with the peptide may be further stabilized, for example by additional hydrogen bonding [19] involving the carbonyl oxygen.
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Figure 4-6, (a) ESI-MS of solution containing 5 µM [Glu] Fibrinopeptide B and 0.5 mM CH₃COONa; (b) Same condition as (a) except “in-source” CID collision energy = 60 eV (E_{lab}). [Glu] Fibrinopeptide B is abbreviated as “Fib”. For the ”(x, y)” nomenclature, x represents the number of Na⁺ and y is the number of CH₃COO⁻.

4.4.4.3 **NaCl**

Another outcome of the Best Match model is that attachment of a low GB anion to a proton located at a high GB_{app} site will result in anion departure. Chloride anion was selected to examine the dissociation pathway of a relatively low GB anion attached to higher GB_{app} sites. Cl⁻ has a lower GB (1374. ± 8.4 kJ/mol) than CH₃COO⁻[17], in fact, unlike CH₃COO⁻ or F, chloride can attach to [Glu] Fibrinopeptide B at -2 charge state without any Na⁺/H⁺ exchange [10]. Figure 4-7a shows the mass spectrum obtained upon addition of 0.5 mM NaCl to 5 µM [Glu]
Fibrinopeptide B solution. The resulting spectrum exhibits similar adduct formation tendencies as CH$_3$COONa, as evidenced by the similar numbers of Na$^+$/H$^+$ exchanges and A$^-$ adducts. However, after two Na$^+$/H$^+$ exchanges, Cl$^-$ attachment is observed, whereas for acetate or fluoride 3 and 4 are needed, respectively. In contrast to the result obtained for CH$_3$COONa, for NaCl after 60 eV ($E_{\text{lab}}$) in-source CID, four Na$^+$ is the maximum number attached to the peptide, whereas for acetate the maximum is five, and for F$^-$ it is six. This capacity for Na$^+$/H$^+$ exchange correlates with increasing GB of the anion. Furthermore, the appearance of a very small peak corresponding to [[Glu] Fibrinopeptide B – 6H + 4Na]$^{2-}$ (Figure 4-7b), indicates that chloride can barely pull off the sixth most acidic proton from the peptide at -2 charge state.

Figure 4-7. (a) ESI-MS of solution containing 5 µM [Glu] Fibrinopeptide B and 0.5 mM NaCl; (b) Same condition as (a) except “in-source” CID collision energy = 60 eV ($E_{\text{lab}}$). [Glu] Fibrinopeptide B is abbreviated as "Fib". For "(x, y)" nomenclature, x represents the number of Na$^+$ and y is the number of Cl$^-$.
In order to get a more complete picture of the chloride adduct dissociation behavior, $[[\text{Glu}] \text{ Fibrinopeptide B} - 4H + 4Na + 2Cl]^2-$ was selected for SORI-CID. For this adduct, 4 $Na^+/H^+$ exchanges will occur at the four most acidic carboxylic acid sites, and two chloride anions will attach to the fifth and sixth most acidic sites. Upon CID, the precursor ion can either lose $HCl$ or $ClNaCl^-$ (Figure 4-8). This result indicates that chloride is able to pull off the fifth most acidic proton resulting in $HCl$ loss and formation of $[[[\text{Glu}] \text{ Fibrinopeptide B} - 5H + 4Na + Cl]^2-$, but it can barely pull off the proton from the sixth most acidic site forming only a small peak corresponding to $[[\text{Glu}] \text{ Fibrinopeptide B} - 6H + 4Na]^2$. $HCl$ loss, however, is in competition with $ClNaCl^-$ formation. Based on these observations, and the fact that $Cl^-$ was never observed, one can conclude that the GB of chloride anion is higher than the $GB_{app}$ of the fifth most acidic site and very close to that of (best matched with) the $GB_{app}$ of the sixth most acidic site at -2 charge state. Therefore, any anion that was attached to sites having higher acidity than the fifth site or lower acidity than the sixth would not provide an adduct of high stability.

In our previous study [10], we showed that with no sodium cations present, for the 2-charge state, chloride anions were best matched with the second most acidic proton of $[[\text{Glu}] \text{ Fibrinopeptide B}$. To rationalize why the best matched sites for $Cl^-$ have shifted to the fifth and sixth most acidic sites when 4 $Na^+/H^+$ exchanges have taken place (2- charge state), we first consider what would happen if the 4 sodium cations were not present and the corresponding carboxylate sites held no counterion. The resulting 4 negative charges would raise the $GB_{app}$ of the last remaining carboxylate site of the peptide substantially, rendering the GB of $Cl^-$ insufficient to create a stable adduct. However, if the 4 sodium cations are present to neutralize the 4 most acidic sites, the absence of the 4 corresponding negative charges substantially lowers the overall $GB_{app}$ of the adduct, thereby enabling $Cl^-$ to match with sites of higher intrinsic GB.
(e.g., the fifth and sixth most acidic sites). This through-space reduction in GB_{app} enabled by the diminution of coulombic repulsion caused by the presence of Na^{+} cations at otherwise anionic sites is a large effect compared to the previously mentioned inductive effect.

4.4.5 Adduction of sodium verse anion Sodium/proton exchange vs. anion attachment

In negative ion mode, the extent of sodium / proton exchange is strongly dependent on the GB of the anions that are present. In comparing the three tested anions, F^{−}, CH_{3}COO^{−} and Cl^{−} at the 2- charge state, because fluoride has the highest GB, it thus has the highest ability to abstract protons from the peptide, thereby enabling 6 Na^{+}/H^{+} exchanges (Figure 4-5a). CH_{3}COO^{−}
, with an intermediate GB, is less capable of abstracting protons, and can only enable 5 Na\(^+\)/H\(^+\) exchanges (Figure 4-6a). Chloride, the lowest GB anion, can enable only 4 Na\(^+\)/H\(^+\) exchanges (Figure 4-7a). The trend can be rationalized by considering that the more deprotonated sites that are created, the higher the probability that Na\(^+\)/H\(^+\) exchange will occur.

4.5 Conclusions

This study investigates the effects of Na\(^+\)/H\(^+\) exchange on anion attachment. The results obtained provide additional important evidence to support the “Best Match” model for anion attachment on peptides. Without any Na\(^+\)/H\(^+\) exchange, neither F\(^-\) nor CH\(_3\)COO\(^-\) can attach to the peptide. Na\(^+\)/H\(^+\) exchange occurs preferentially at the most acidic sites where protons are most easily removed. Anion attachment is not favorable at a site that is occupied by a sodium cation. This deduction is based on the observation that attached anions are lost as neutral acids (and not as sodium salts) when the GB of the anion is higher than the GB\(_{\text{app}}\) of the attachment site bearing a proton. At a given charge state, as the number of Na\(^+\)/H\(^+\) exchange increases, a shift occurs in CID mass spectra from predominately neutral loss to anion departure, because anions attaching to the peptide are forced to interact with the remaining less acidic sites on the peptide.

In addition to “blocking” highly acidic sites by forming ion pairs with carboxylate groups, sodium cations can also interact with the attached anions. This is revealed by the dissociation pathway of multiply attached anion adducts of the form \([\text{[Glu] Fibrinopeptide B – H + Na + 2A]}^2-\), where tri-atomic ions, ANaA\(^-\), appear in the product ion mass spectra. Importantly, during CID of the investigated adducts, ions of the type ANaA\(^-\) are preferentially formed for low GB anions, even though high GB anions form more stable tri-atomic species.
According to the "Best Match" model, the extent of anion attachment observed in the mass spectrum is dependent on the degree of matching between anion GBs and GB_{app} of deprotonated sites on the peptide. High GB anions will match with high GB_{app} peptide sites, whereas low GB anions will match with low GB_{app} peptide sites. High negative charge states readily augment GB_{app} of the peptide (through-space effect). Sodium / proton exchange substantially decreases GB_{app} by neutralizing charged sites, and slightly increases intrinsic GBs by the inductive effect.

4.6 References

VITA

The author was born in Jiangxi, China. He obtained his Bachelor’s degree in pharmaceutical engineering from Beijing institute of Technology in 2005. And later he went Beijing Municipal institute of Labour Protection and got his master degree in environmental and occupational health in 2008. He joined University of New Orleans chemistry graduate program to pursue a PhD in analytical chemistry, and became a member of Professor Richard B. Cole’s research groups in Jan 2009.