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More than Mass to Charge: Innovative Applications of Mass Spectrometry in Protein Studies

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by

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Acknowledgments

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Chapter 2: Hamdy, O. M.; Lam, S.; Julian, R. R., Identification of Inherently Antioxidant Regions in Proteins with Radical-Directed Dissociation Mass Spectrometry. Anal Chem 2014, 86 (7), 3653-3658.

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Chapter 5: Moore, B. N.; Hamdy, O.; Julian, R. R., Protein structure evolution in liquid DESI as revealed by selective noncovalent adduct protein probing. Int J Mass Spectrom 2012, 330, 220-225.

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Dedication

To my brother Hamdy,

for giving me the motivation to make you proud,

I wish you were here to see this.

ABSTRACT OF THE DISSERTATION

More than Mass to Charge: Innovative Applications of Mass Spectrometry in Protein Studies

by

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Doctor of Philosophy, Graduate Program in Chemistry
University of California, Riverside, June 2015
Dr. Ryan R. Julian, Chairperson

Over the past two decades, Mass Spectrometry (MS) has advanced to become an indispensable analytical tool in protein analysis. While the role of proteins in critical biological pathways is well established, their potential as powerful therapeutics has only begun to be realized. This discrepancy motivates the development of novel analytical techniques for protein characterization. The focus of this dissertation is the development of novel MS-based techniques with application in protein studies. The identification of antioxidant peptides/proteins and ionization of proteins under native conditions are the two principle foci of this dissertation.

Maintaining redox homeostasis, or the balance of oxidant and antioxidant forces, is essential for proper cellular functioning in biology. The identification of all contributing antioxidant species is necessary to understand this critically sensitive balance of redox active species. Solution-phase methods used to identify antioxidant peptides require a large

amount of sample and are very time-consuming. An MS-based method for the identification of antioxidant peptides is thus desirable and is explored herein. Radical directed dissociation (RDD) is a method developed in the Julian laboratory that utilizes radical chemistry to direct site-specific fragmentation of peptides. RDD is employed to compare the different fragmentation patterns for peptides with known and unknown antioxidant capacities. Antioxidant peptides displayed limited to no evidence of radical directed fragmentation, suggesting that these peptides have a unique ability to sequester and limit migration of the radical. A good correlation between gas phase and solution phase experiments is observed. However, gas-phase experiments are approximately tenfold faster and require significantly less sample.

Extending RDD to the investigation of the antioxidant capacity of peptides from an enzymatic digest of Human Serum Albumin (HSA) revealed four new antioxidants. These findings sparked the work in chapter 3 which sought to identify antioxidant peptides in different proteins with distinct localizations. The presence of antioxidant peptides in all five proteins investigated suggests that the ability to sequester radicals may be common to many proteins as a preservation mechanism to survive exposure to radical containing reactive species (RS). In addition, a detailed analysis of antioxidant peptides reveals interesting findings. Perhaps most surprisingly, is the lack of methionine and cysteine (sulfur containing) residues in many of the antioxidant peptides identified.

The second focus of this dissertation discusses our current understanding of different ionization sources (namely, ESI and liquid-DESI) with an emphasis on achieving “native” conditions for the analysis of proteins via MS. Selective non-covalent adduct protein

probing (SNAPP) is utilized to examine protein structural evolution in both liquid DESI and standard ESI under a variety of conditions. Experiments with Cytochrome C (Cytc) demonstrated that methanol induced conformational shifts previously observed with ESI are also readily observed with liquid DESI. The effects of ammonium acetate buffer on liquid DESI SNAPP experiments were examined by monitoring structural changes in myoglobin. Heme retention and SNAPP distributions were both preserved better in liquid DESI than traditional ESI, suggesting superior performance for liquid DESI in buffered conditions. Collectively, the work presented herein contributes to and expands the toolbox of mass spectrometry in protein characterization and has demonstrated considerable potential that merits further exploration utilizing these techniques.

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Chapter 1

Mass Spectrometry: an Invaluable Tool for Protein Characterization

1.1 Introduction

Proteins have been, and continue to be, one of the most intriguing macromolecules in biological systems. Proteins are essential in the daily functioning of living cells and are involved in a myriad of biological functions contributing to their complexity. Different protein functions include acting as molecular chaperones[1,2] and involvement in cell signaling pathways.[3,4] Most proteins have well defined primary functions in biology that can only be carried out if structural fidelity is maintained. Proteins are a diverse set of linear polymers and are composed of 20 amino acids in many different sequences. The connectivity of amino acids in a specific order makes up the primary structure of proteins, and is unique to each protein. Amino acid sequence of the proteins is only one of the features that requires elucidation to understand function in biological processes. Additionally, proteins adopt secondary, tertiary and in some cases quaternary structures. Extended polypeptide chains can form regular structures such as an α-helix or a β-pleated sheet (secondary structure). Tertiary structure is defined as the three-dimensional folding of the protein. Folding patterns are largely affected by the side chain chemistry of the amino acids present. Finally, quaternary structure occurs when more than one polypeptide chain combines to form an oligomer.[5] For example, Hemoglobin, is a tetramer composed of two alpha and two beta chains (Figure 1.1). Any type of disruption to protein structure (all levels) can result in hindrance of protein function and subsequently lead to different

biological disorders and diseases. As a result, gaining insight into protein function through characterization is invaluable to many different industries, specifically drug development/pharmaceutical industries.

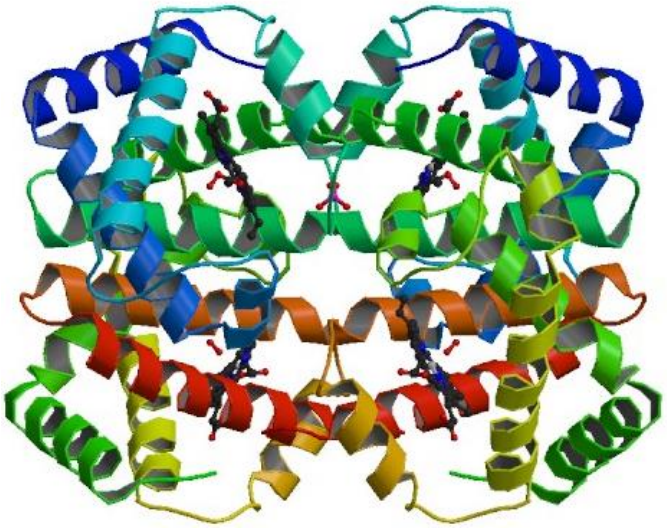

Figure 1.1 Human Hemoglobin Structure (PDB 1hho).

1.2 Utility of Mass Spectrometry in Proteomics

The toolbox for protein characterization includes many different modern analytical techniques such as Nuclear Magnetic Resonance (NMR)[6], surface plasmon resonance (SPR)[7], and fluorescence just to name a few. Mass Spectrometry (MS) emerged and has been solidified as an indispensable technique for protein characterization.[8,9] The wide use of MS for protein studies came with the advent of soft ionizations techniques; electrospray ionization (ESI)[10] and matrix assisted laser desorption/ionization (MALDI).[11] Soft ionization methods revolutionized gas phase characterization of proteins while maintaining biological relevance. The power of mass spectrometry in proteomics arises from the ability to rapidly determine the primary sequence of proteins and identify post-translational

modifications. Advances in mass analyzers have made mass spectrometry the gold standard for protein sequencing.

Protein examination using mass spectrometry is typically done by one of two methods; a top-down or bottom-up approach.[12] In top-down proteomics, proteins are introduced into the mass spectrometer and fragmented (dissociation techniques are discussed below) without prior solution phase sample preparation. Different fragments resulting from dissociation are determined by mass allowing the identification of the protein. The bottom-up technique, however, requires solution phase sample preparation prior to analysis with mass spectrometry. Enzymatic digestion of a protein is done in solution resulting in smaller peptide fragments. These peptide fragments are then separated using liquid chromatography (LC), introduced to the mass spectrometer one at a time and sequenced by dissociation in the gas phase. Coupling liquid chromatography with mass spectrometry (LC-MS) is capable of the identification and quantitation of proteins in complex biological samples.[13-15]

Protein sequencing can be done using multiple dissociation techniques; each with unique advantages and distinguishing features. In past years, the most common technique used was collision induced dissociation (CID).[16] In CID, the isolated molecule of interest is exposed to multiple collisions with bath gas (Helium). The molecules are thus heated which results in breaking the weakest bond in the molecule. In peptides/proteins, this is typically the peptide bond in positively charged ions in the gas phase.[17] This type of cleavage results in "b" and "y" ions (see Figure 1.2). A drawback of CID is the common dissociation of post-translational modifications (eg: phosphorylation, glycosylation).

Different dissociation techniques; electron capture dissociation (ECD)[18], electron transfer dissociation (ETD)[19], and radical directed dissociation (RDD)[20] are capable of retaining PTMs during the dissociation process.[21] The radical-based dissociation methods above result in more selective peptide cleavages that result in a/x and c/z ions. Sequencing is just one of the many powerful applications of mass spectrometry in protein characterization.

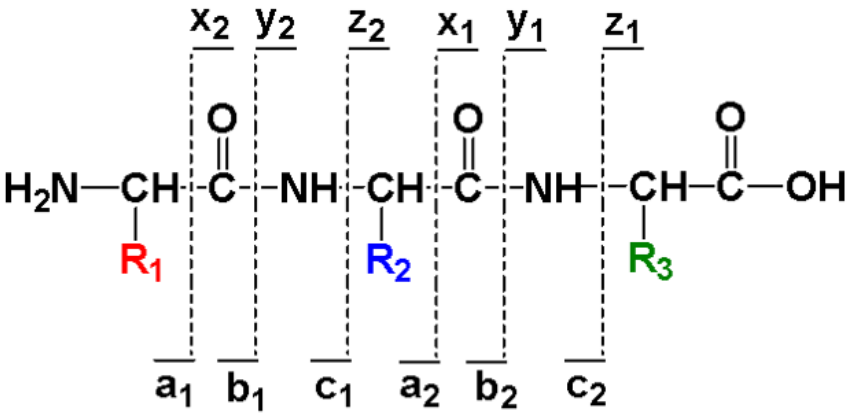

Figure 1.2 Peptide Backbone Fragmentation Nomenclature. b/y ions are commonly see in CID. Radical dissociation pathways result in a/x and c/z ions.

1.3 More than Sequencing

1.3.1 Radical Chemistry of Proteins

Free radicals are defined as molecules or atoms with free unpaired electrons. The production of free radicals (a type of reactive species) is inevitable in biological systems. These highly reactive species are two-faced, playing critical roles in biological processes necessary for survival and the development of different diseases/disorders. On the positive end of the spectrum, free radicals mediate crucial processes in cell survival. For example, radical chemistry is utilized by the enzyme, ribonucleotide reductase, for DNA replication.[22] Nitric oxide ($^{\bullet}$NO) and other radicals have roles in cell signaling and

regulating vascular tone.[23] The production of excess free radicals is common and leads to oxidative stress, closely associated with cancer, Amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, and with aging itself.[24-26] The complexity of redox chemistry surrounding free radicals and proteins is very important and will be discussed in detail below. The goal of this dissertation is to utilize mass spectrometry (specifically radical chemistry) in an unprecedented manner to provide innovative applications in protein characterization.

1.3.2 Gas Phase Radicals

As previously mentioned, radical chemistry has been leveraged in combination with mass spectrometry to become an indispensable tool for protein characterization. Protein sequencing using radical chemistry (ECD/ETD and RDD) in the gas phase is only one application. RDD (radical directed dissociation) is a unique method that was developed in the Julian lab.[20] In RDD, the photolabile property of the C-I bond is utilized. Typically, C-I bonds are strategically placed on chromophores that absorb in the UV region (Figure 1.3). Excitation of these chromophores using a 266nm Nd:YAG laser results in the homolytic cleavage of the C-I bond creating a radical. The modification of peptides/proteins with C-I containing chromophores (radical initiators) can be done both covalently and non-covalently (Figure 1.3). As the name implies, the radical can then direct the fragmentation pattern of the peptide/protein.

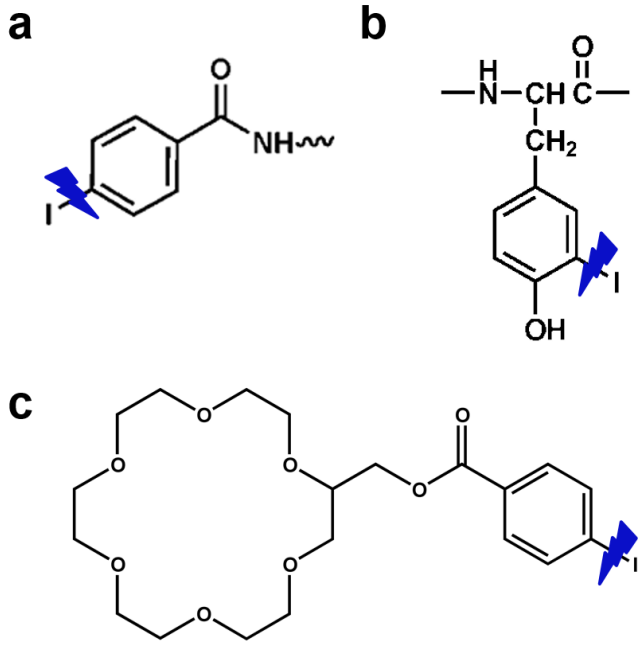

Figure 1.3 Radical Initiators can be covalent: a) directly iodinate tyrosine or b) modification of basic residues with iodobenzoic acid. Non-covalent radical initiators can be done using c) 2-(hydroxyl-methyl-iodobenzoylester)-18C6 which is also specific to basic residues (lysine and n-terminus).

RDD has been used to easily assign the presence of specific amino acids in a peptide/protein sequence. Each of the 20 natural amino acids has a unique side chain associated with a known mass. A common phenomenon seen with RDD is the loss of side chains from the peptide sequence (eg 59E, 56L, etc.) and thus making it a quick indicator of the presence of certain residues.[27] RDD has also been shown to specifically cleave at phosphorylated residues making the assignment of this post-translational modification (phosphorylation) facile in peptides/proteins.[21,28] Cysteines are one of the most reactive amino acids and can be found in proteins as part of a disulfide bond or in the free thiol form. Gas phase radical chemistry is once again applied to identify the presence of free

thiol cysteines and disulfide bound ones.[29] Most recently, RDD has been used to identify the presence of D-amino acids and differentiate between peptide epimers.[30]

1.4 Radicals and Antioxidants

Free radicals are a type of reactive species (RS). Examples include hydroxyl radical ($^{\bullet}OH$), superoxide radical ($O_2^{-\bullet}$), and nitric oxide ($^{\bullet}NO$). As discussed above, reactive species have devastating effects in biological systems, yet have roles that are necessary for cell survival. Excess reactive species are controlled by the presence of antioxidants; molecules that inhibit the oxidation of other species. Of extreme importance is the balance between antioxidant and reactive species. An excess of reactive species can result in the progression of different diseases. On the other hand, an excess of antioxidants could inhibit cellular processes that proceed through radical chemistry.

A balance of the amount of active reactive species is referred to as redox homeostasis. Understanding redox homeostasis is extremely important in biology and requires the identification of all contributing molecular species. Reactive species are relatively well defined. Redox contributions come from hydrogen peroxide, hydroxyl radicals, superoxide radicals, and nitrogen based radicals just to name a few. Antioxidants, however, are a little bit more elusive. Many of the most active participants (including metal ion chelators such as transferrin and lactoferrin, small molecule sacrificial scavengers such as vitamin C or uric acid, and catalytic scavengers such as superoxide dismutase) have been examined in detail.[31-33] However, it is more difficult to determine the extent to which highly abundant molecules (such as proteins or peptides that are neither catalytic scavengers nor chelators) contribute to overall antioxidant capacity. There is evidence that proteins may be

engineered to provide such protection as a secondary function to whatever primary role they may play in a cell. For example, methionine residues in proteins are particularly sensitive to oxidative stress and are easily oxidized. The existence of methionine sulfoxide reductase, which catalyzes the reduction of methionine in residue or amino acid form, suggests that proteins may have built-in defenses designed to protect themselves or the cellular milieu in general.[34] Furthermore, it is believed that proteins contribute 10-50% of the antioxidant capacity of blood plasma,[35] although the mechanistic details behind how this protection is afforded are incomplete. A more thorough understanding of the factors that mediate the antioxidant capacity of proteins is necessary to fully comprehend how biology maintains redox homeostasis.

A limitation to identifying antioxidant capacity of proteins has been the lack of a suitable method. Electron spin resonance (ESR), lipid peroxidation assays, and metal chelation are commonly used;[36,37] however all of these methods require considerable sample and cannot provide any specific information without being preceded by time consuming separations. As mentioned previously, mass spectrometry has become a huge part of protein characterization studies. Our goal was to develop a mass spectrometry-based method to identify antioxidants in proteins. Combining radical chemistry (commonly associated with oxidative species) in the gas phase with mass spectrometry was an attractive option. The development of a mass spectrometry method for the accurate identification of antioxidant proteins is described in detail in chapter 2. It is demonstrated that information derived from radical directed dissociation (RDD) MS can be leveraged to identify peptide regions within proteins that are antioxidant in aqueous solution. For

peptides with high antioxidant capacity, the radical that would typically facilitate peptide fragmentation is sequestered. This leads to dissociation that is distinctly not radical in nature and is initiated instead by proton driven pathways. Further details on this phenomenon are discussed in chapter 2.

The development of a mass spectrometry-based method that allows for the quick and facile identification of antioxidants in proteins provoked further investigation. Immediately, two different ideas come to mind. First, the analysis of many different proteins can provide a large enough database of antioxidant peptides where trends and residue content can be closely examined. This would make it easier to identify antioxidant peptide regions by genomic analysis if characteristic sequence or residue content could be determined. Approximately eighteen different antioxidant peptides from different proteins are identified (results in chapter 3). Sequence analysis for these peptides shows no trend with regards to residue content; displaying no specificity towards any specific amino acid. The second hypothesis, previously unexplored, is that all proteins have intrinsic protective regions. In addition to primary functions in biology, proteins might have a secondary role of providing antioxidant protection for itself and surrounding biomolecules. Multiple proteins with different primary functions are probed for antioxidant capacity in chapter 3. Using RDD coupled with liquid chromatography (LC), the antioxidant capacity of β-lactoglobulin, αA-crystallin, αB-crystallin, and myoglobin is mapped and attributed to specific peptide regions. The presence of antioxidant regions in all proteins investigated suggests that proteins have an intrinsic capacity to protect themselves against reactive species and oxidative damage. The successful application of RDD-MS for the

identification of antioxidant peptides can be applied in the future for a more comprehensive interrogation of proteins.

1.5 Higher Order Protein Structure in Mass Spectrometry

In many cases, elucidating protein structure is essential to understanding protein functionality. At the moment, MS is considered the top option for primary structure determination and can be done with ease. Higher order structure, however, is more elusive. The enhancement of current techniques for determining tertiary and quaternary structures of proteins is a continuously growing area of research. Nuclear magnetic resonance (NMR) and X-ray crystallography are at the frontier of three dimensional protein structure determination.[38,39] Both techniques are widely used and highly accurate, however, X-ray crystallography is considered the "gold standard" for determining 3D structure of proteins. The aforementioned techniques have proved to be very accurate and reproducible at high resolutions, but there are some limitations. NMR and X-ray crystallography require large amounts of protein sample, time consuming, and require complex data analysis. All issues that can be resolved when mass spectrometry comes to mind as a technique.

The analysis of higher order structures using MS has exponentially increased with the advent of ESI. Charge state distribution (CSD) has been used to identify different folding patterns of proteins.[40] As intact proteins are being ionized (with ESI), proteins will have different number of charges. The number of charges is closely related to the number of accessible basic residues (arginine, lysine, and histidine). To delve further, if a protein is in a natively folded state, many residues are buried and only residues on the surface of the protein are accessed. On the other hand, as a protein unfolds, previously unreachable

residues are accessible and the protein is more likely to carry more charges. Thus, higher charge states are commonly associated with unfolded states of the protein and lower charge states with a folded pattern. Hydrogen-deuterium exchange is another MS-based technique used to interrogate 3D protein structure.[41] Amide hydrogens (N-H) are the focus of protein structural determination using H/D exchange. Protein structural dynamics can be observed by monitoring specific amide hydrogen exchanges along the protein chain. Similar to CSD, only solvent accessible protons in the protein will be able to exchange. As a result, H/D exchange can differentiate between residues buried inside a globular protein vs solvent accessible residues exposed on the surface.

An additional MS-based technique that can be used for higher order structural interrogation is Selective Non-covalent Adduct Protein Probing Mass Spectrometry (SNAPP-MS). SNAPP-MS utilizes the ability of 18-crown-6 ether (18C6) to selectively target lysine residues (Figure 1.4).[42] Similar to the mechanism by which H/D exchange and CSD works, SNAPP-MS probes the solvent accessible lysine residues. SNAPP-MS shows both the number and intensities of 18C6 molecules that bind to protein ions at different charge states. Example SNAPP-MS distributions can be found in Figure 5.5. SNAPP is a unique tool and provides a simple and quick method to probe protein structural dynamics. Using SNAPP-MS, one is capable of observing very subtle changes in protein structure that might not produce any changes in CSD. Applications of SNAPP-MS include detecting protein conformational changes upon metal-ion binding,[43] differentiating between two native structures of lymphotactin,[44] and probe protein surface structure in highly conserved sequence variants.[45]

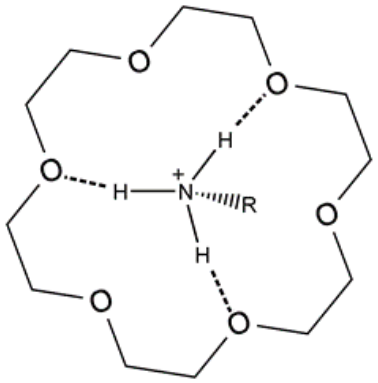

Figure 1.4 Hydrogen bonding interactions between 18C6 and amine side chain of lysine.

One of the challenges that comes with using MS for protein structure probing is the correlation between gas phase and solution phase structures; maintaining native structure of protein is key. Electrospray conditions and solvents are two factors that can commonly denature proteins prior to detection using MS. In a traditional SNAPP-MS experiment, the protein is electrosprayed using ESI as the ionization method. Solvents vary by experiment, however, water is used as the "native" solvent. Using 100% water typically reduces protein detection as signal drops due to hindrance of the electrospray process. Chen and coworkers reported that liquid DESI could be used to ionize proteins with apparent retention of solution-phase structure and enhanced sensitivity relative to ESI.[46] Of interest was trying to bring the newly founded liquid DESI sensitivity to SNAPP-MS. In chapter 5, liquid DESI is coupled to SNAPP-MS in order to maintain "native" structure of protein with better sensitivity. Indeed, liquid DESI proved to be a superior ionization method for SNAPP-MS experiments. To further enhance the ability to perform "native" SNAPP-MS experiments, the addition of buffers was investigated. Ammonium acetate is a MS

compatible buffer due to high volatility and is used in liquid DESI SNAPP-MS experiments. Comparing the different SNAPP distributions in the presence of buffer with ESI and liquid DESI provided some useful insight on the electrospray mechanism of both. Evidence suggests that liquid DESI proceeds through the charge reduction mechanism, where ions are more likely to retain native structures.

1.6 Biology and Mass Spectrometry

The complexity of biological systems drives many researchers today. All diseases and disorders originate from certain kinks in the system; problems that scientists strive to solve. There is a wide array of tools and techniques that can be used to understand biological systems. Unfathomable half a century ago, mass spectrometry has proved itself an indispensable tool for the study of almost anything biology-related. The focus of this dissertation is to develop more biologically-relevant MS-based methods for the study of proteins.

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Chapter 2

Identification of Inherently Antioxidant Regions in Proteins with Radical-Directed Dissociation Mass Spectrometry

2.1 Introduction

Redox homeostasis is incredibly important in biology and is achieved when oxidant and antioxidant species within a cell are properly balanced. Excess oxidants lead to oxidative stress, which is unfavorably linked with numerous diseases such as cancer, Amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, and with aging itself.[1-3] However, free radicals and other reactive species (RS) that can cause oxidative stress also have important nondestructive roles within cells.[4,5] For example, nitric oxide and other RS participate in numerous cell signaling cascades, regulating vascular tone and oxygen levels among many other things.[6] RS species are also a primary weapon employed by the immune system, and are thought to play a role in cell senescence.[6] Therefore, complete suppression of RS (for example by consumption of excess antioxidants) could potentially disrupt crucial cellular processes and may not necessarily be the key to preventing diseases related to oxidative stress.[7]

One of the difficulties associated with greater understanding of redox homeostasis is identification and quantification of the redox contributions from all relevant molecular species. Many of the most active participants (including metal ion chelators such as transferrin and lactoferrin, small molecule sacrificial scavengers such as vitamin C or uric acid, and catalytic scavengers such as superoxide dismutase) have been examined in

detail.[8-10] However, it is more difficult to determine the extent to which highly abundant molecules (such as proteins or peptides that are neither catalytic scavengers nor chelators) contribute to overall antioxidant capacity. There is evidence that proteins may be engineered to provide such protection as a secondary function to whatever primary role they may play in a cell. For example, methionine residues in proteins are particularly sensitive to oxidative stress and are easily oxidized. The existence of methionine sulfoxide reductase, which catalyzes the reduction of methionine in residue or amino acid form, suggests that proteins may have built-in defenses designed to protect themselves or the cellular milieu in general.[11] Furthermore, it is believed that proteins contribute 10-50% of the antioxidant capacity of blood plasma,[12] although the mechanistic details behind how this protection is afforded are incomplete. A more thorough understanding of the factors that mediate the antioxidant capacity of proteins is necessary to fully comprehend how biology maintains redox homeostasis. In addition to importance within biology, antioxidant peptides that can be derived from food sources are also the subject of significant interest in relation to nutrition and food preservation.[13]

The antioxidant capacity of proteins as a whole can be measured in solution by several methods. Electron spin resonance (ESR), lipid peroxidation assays, and metal chelation are commonly used;[14,15] however all of these methods require considerable sample and cannot provide any specific information without being preceded by time consuming separations. In contrast, recent advances in mass spectrometry (MS) have made it the method of choice for proteomics experiments due to speed, sensitivity, and sequencing capabilities.[16,17] A mass spectrometry based approach for measuring antioxidant capacity would therefore be

highly desirable. Interestingly, radical chemistry (which is frequently associated with oxidative stress) has recently been utilized beneficially for characterizing peptides in a variety of MS based experiments.[18-20] In these experiments, radicals are active agents that migrate within the peptide and ultimately initiate either backbone or side chain fragmentation that is unique and attributable to the radical. The potential relationship between radical mediated peptide chemistry in the gas phase and radical initiated oxidative stress in the typical biological context has not been previously explored.

Herein it is demonstrated that information derived from radical directed dissociation (RDD) MS can be leveraged to identify peptide regions within proteins that are antioxidant in aqueous solution. For peptides with high antioxidant capacity, the radical that would typically facilitate peptide fragmentation is sequestered. This leads to dissociation that is distinctly not radical in nature and is initiated instead by proton driven pathways. For peptides with low antioxidant capacity, RDD dominates and leads to the observation of a, x, c, z ions, and side chain losses. The intensity and type of ions produced for any given peptide can therefore be used to predict antioxidant capacity. The antioxidant capacity of a protein can be easily mapped to specific peptide regions by enzymatically digesting the protein. Using this approach, RDD coupled with liquid chromatography was used to rapidly identify regions of human serum albumin that have high antioxidant capacity. Subsequent solution phase experiments confirmed that several previously uncharacterized peptides exhibit high antioxidant capacity. Importantly, the presence of free cysteine or methionine residues is not requisite for high antioxidant capacity, suggesting that other factors such as structure or overall sequence are also important.

2.2 Experimental Section

2.2.1 Materials

Organic Solvents, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Linoleic Acid, Human Serum Albumin and Trypsin were purchased from Sigma-Aldrich (St Louis, MO). Iron (II) Chloride ($FeCl_2$) was purchased from Alfa Aesar (Ward Hill, MA). Water was purified using a Millipore 147 Direct-Q system (Billerica, MA). Fmoc protected amino acids and resins were purchased from AnaSpec (Fremont, CA). A biobasic C18 column from Thermo Scientific was used for all HPLC separations.

2.2.2 Peptide Synthesis

All peptides not purchased commercially or derived from protein digests were manually synthesized using standard Fmoc solid phase synthesis with Wang resins. Peptides are built from C-terminus to N-terminus in a fritted synthesis vessel with a three-way valve. The C-terminal residue is attached to a Wang resin. For each following residue, deprotection of the reactive N-terminus is done using a 20:80 mixture of piperidine:dimethylformamide (DMF). The resins are then washed with DMF multiple times to remove residual piperidine. The Fmoc-protected amino acid is then mixed with tetramethylaminium hexafluorophosphate (HCTU) and N-methylmorpholine (NMM) in DMF and added to the resins. HCTU is used as the coupling agent and NMM as the base to drive the reaction. This process is repeated for each residue in the peptide. Following the addition of the final residue, the peptide is cleaved off the Wang resin overnight in 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropylsilane (TIPS). The antioxidant peptide PHCKRM was purchased from American Peptide Co. (Sunnyvale, CA). Serum

Albumin peptides, DVFLGMFLYEYAR and VPQVSTPTLVEVSR were purchased from LifeTein LLC (South Plainfield, NJ).

2.2.3 Peptide Modifications

Peptides were modified to create a radical precursor in three ways. Iodination of tyrosine was carried out as previously described.[18] Sodium iodide and chloramine-T were added to peptides with a molar ratio of 1:2:1 of peptide/sodium iodide/chloramine-T. After 10 minutes reaction time at room temperature, the modified peptide was purified by removal of excess reagents using a peptide trap (Michrom Biosource Inc.). Peptides were modified with iodobenzoic acid prior to cleaving peptide off of the resin during peptide synthesis by addition of equimolar amounts of tetramethylaminium hexafluorophosphate (HCTU) and 4-iodobenzoic acid dissolved in dimethylformamide (DMF). 2-Hydroxymethyliodobenzoyl ester-18-crown-6 ether was prepared by the previously reported procedure.[21] Noncovalent attachment to the peptide was achieved by simply adding the crown to the electrospray solution and was used for HFGDPFH. Iodobenzoic acid modifications were used for the following peptides: PHCRKM, LQPGQGQQG, HGPLGPL, Aβ 1-40, Aβ 1-42, PSKYEPFV, NGPLQAGQPGER, LPHSGY, IQTGLDATHAER, SLHTLFDK, Glucagon-like Peptide, brain natriuretic peptide, and all HSA peptides. Tyrosine modifications were used for the following peptides: RPDFDLEPPY, HDSGYEVHHQK, YEVHHQKLVFF, DRVYIHP, and insulin B chain.

2.2.4 HSA Digest

Human Serum Albumin was digested with trypsin following reduction and alkylation of cysteine residues. HSA stock was dissolved in ultrapure water to a concentration of 0.5

mM. To 15 μL of 0.5 mM HSA, 1.5 μL of 100 mM dithiothreitol (DTT) and 15 μL of 50 mM ammonium bicarbonate were added and incubated at 95°C for 5 minutes. After allowing the sample to cool, 3 μL of 100 mM iodoacetamide was added to the sample tube and incubated in the dark at room temperature for 20 minutes. Acetonitrile (ACN) was added to adjust solution to 10% ACN. 4 μL of 87 μM trypsin was added to solution and left overnight at 37°C. Peptides in solution from the tryptic digest were modified with 4-iodobenzoic acid as described above.

2.2.5 Mass Spectrometry (MS) and Liquid Chromatography-Mass Spectrometry (LC-MS)

Solutions containing ~10 μM peptides in 49.5/49.5/1 water/methanol/acetic acid were directly infused into an LTQ linear ion trap with a standard electrospray ionization source (Thermo Scientific, San Jose, CA). The instrument has been modified with a quartz window to allow the beam from a 266 nm Nd:YAG laser into the ion trap. An isolation width of 4 Da was used for MS^2 and MS^3 isolations. LC-MS was carried out with a Thermo betabasic 18 column with 3 micron particle size. The instrument software was configured to fire the laser during MS2, followed by re-isolation and collision induced dissociation (CID) of the most abundant peak.

2.2.6 Lipid Peroxidation Assay

Linoleic acid was used as the substrate in the oxidation assay. A peptide solution (0.2 mL of 5 mM) was added to potassium phosphate buffer (0.5 mL of 0.1 M) and mixed with linoleic acid in 95% ethanol (0.5 mL of 50 mM). The reaction is initiated by adding 50 μL of 0.1 M AAPH and heated at 37°C for 200 minutes in the dark. After 200 minutes reaction time, 50 μL of reaction mixture was mixed with 2.35 mL of 75% ethanol, 50 μL of 20 mM

ferrous chloride solution in 3.5% HCl, and 50 μL of 30% ammonium thiocyanate. The amount of lipid peroxidation was determined by measuring absorbance at 500 nm due to the formation of $[FeSCN]^{2+}$.[22]

2.2.7 Assignment of RSS Scores

Application of a t-test to the results in Tables 2.1 and 2.2 suggests that 99% of peptides that are antioxidant will have RSS scores between 1.4 and 9.2 and peptides that are not antioxidant will yield RSS scores between 0.096 and 0.67. Peptides with RSS values above 9.2 are exceptionally good at radical sequestration, and are also clearly antioxidant. However, for peptides with RSS values between 0.67 and 1.4, the antioxidant status cannot be determined by RDD.

2.3 Results and Discussion

2.3.1 Identification of Antioxidant Peptides using RDD-MS

While investigating RDD of Amyloid β (Aβ), we noticed some very unusual behavior. Typically RDD produces a variety of fragments including both backbone dissociations and side chain losses. Furthermore, it has been demonstrated previously that backbone fragmentation yielding a/x or c/z ions occurs readily at aromatic residues and at serine and threonine.[23] Given the sequence of Aβ, there are numerous sites where backbone fragmentation would be expected to be observed (DAE**F**R**H**D**S**G**Y** EV**HH**QKLV**FF** AEDVG**S**NKGA IIGLMVGGVV, favorable backbone cleavage sites in bold). However, as seen Figure 2.1, collisional activation of radical Aβ 1-40 yields very few of the expected RDD fragments in either the +3 or +4 charge states. Even casual inspection reveals several significant differences between the spectra in Figures 2.1a and 2.1b. Fragments derived

from RDD are labeled in red and proton derived fragments are labeled in green. It is clear that RDD produces most of the fragment ions for the +3 charge state (Figure 2.1a); however, few dissociation channels are populated and very little backbone fragmentation is observed. This behavior can be rationalized by the fact that in the +3 charge state, all protons are sequestered at basic sites, which frustrates fragmentation pathways that require a mobile proton. Therefore, some RDD is observed; however, the paucity of RDD suggests that the radical is also not very active in facilitating dissociation of Aβ 1-40. The end result is that very few fragmentation channels are populated in Figure 1a.

Even more striking are the results from collisional activation of the +4 charge state, which results in a series of b ions with virtually no RDD fragments (Figure 2.1b). Of the hundreds of peptides investigated previously by RDD, none yielded a spectrum where typical RDD products were completely absent.[24] Furthermore, comparison with the CID spectrum for +4 nonradical Aβ 1-40 reveals a virtually identical fragmentation pattern as shown in Figure 2.1c. It is clear from comparison of the two spectra that the radical has very little influence on the dissociation observed in Figure 2.1b. Importantly, for the +4 charge state the number of protons exceeds the number of basic sites, meaning that a mobile proton should be available to facilitate proton derived fragmentation and lead to the formation of b and y ions (as is observed).

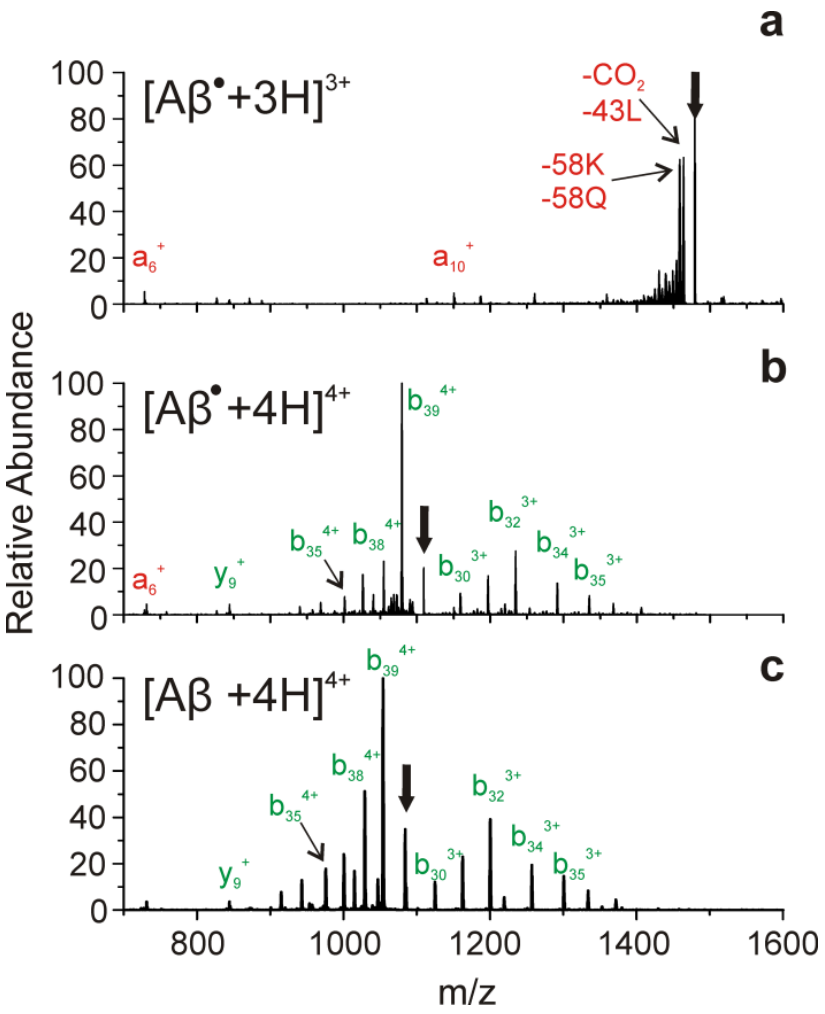

Figure 2.1. CID of iodobenzoyl-Aβ 1-40 radical. a) +3 Charge State and b) +4 Charge state. C) CID of non-modified protonated +4 charge state for the even electron ion. Note similarity between the final two spectra (mass shifts are due to iodobenzoyl modification).

It has been demonstrated previously Aβ acts as an antioxidant in solution via metal chelation and radical scavenging.[25,30] The results in Figure 2.1 suggest that the radical sequestration capacity of Aβ may also be reflected in the gas phase dissociation chemistry observed in an RDD experiment. The absence of significant RDD for Aβ, particularly when a mobile proton is present, suggests that the peptide is capable of sequestering the radical and inactivating it in the gas phase. Importantly, changing the initial starting position of

the radical from the N-terminus to the tyrosine side chain yielded very similar dissociation (Figure 2.2), indicating that efficient radical sequestration is a property of the peptide as a whole and not a function of a particular radical initiation site. These initial observations suggest that information from RDD spectra might be able to predict antioxidant capacity for peptides in solution.

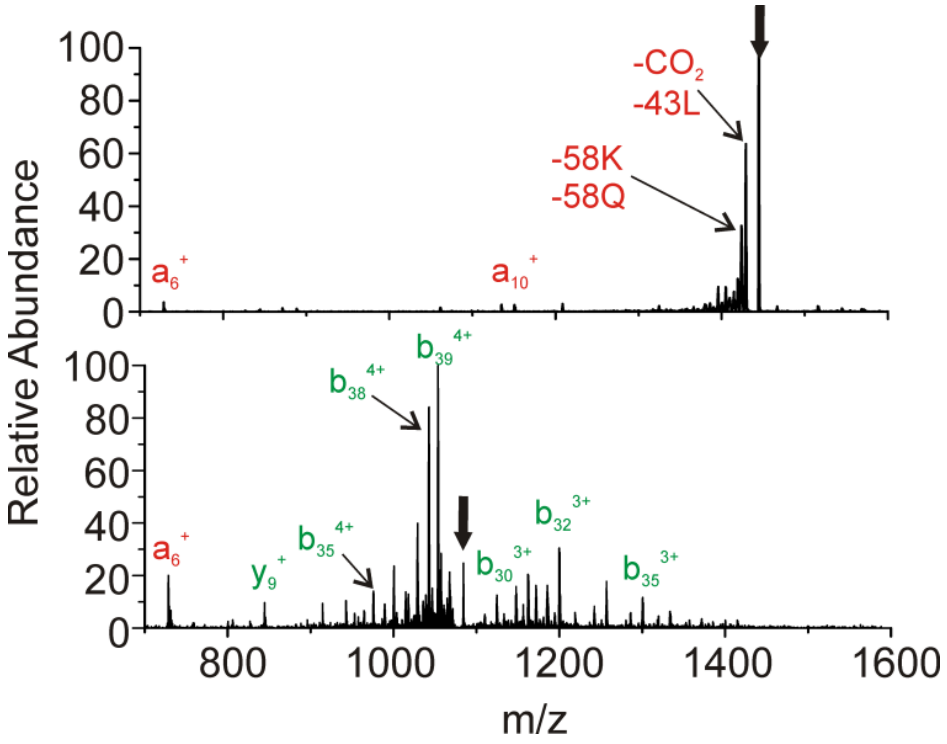

Figure 2.2 CID of Tyrosine radical in Aβ 1-40 radical. a) +3 Charge state and b) +4 Charge state.

In order to explore this avenue further, experiments were conducted on PHCKRM, a short synthetic peptide reported previously to be a strong antioxidant.[26] Very similar results to those noted for Aβ were obtained (Figure 2.3). In the +1 charge state few fragments are observed. With the addition of a mobile proton, the +2 charge state of PHCKRM fragments

to yield primarily b and y ions (the peptide is modified at lysine, which should make the second a proton mobile).

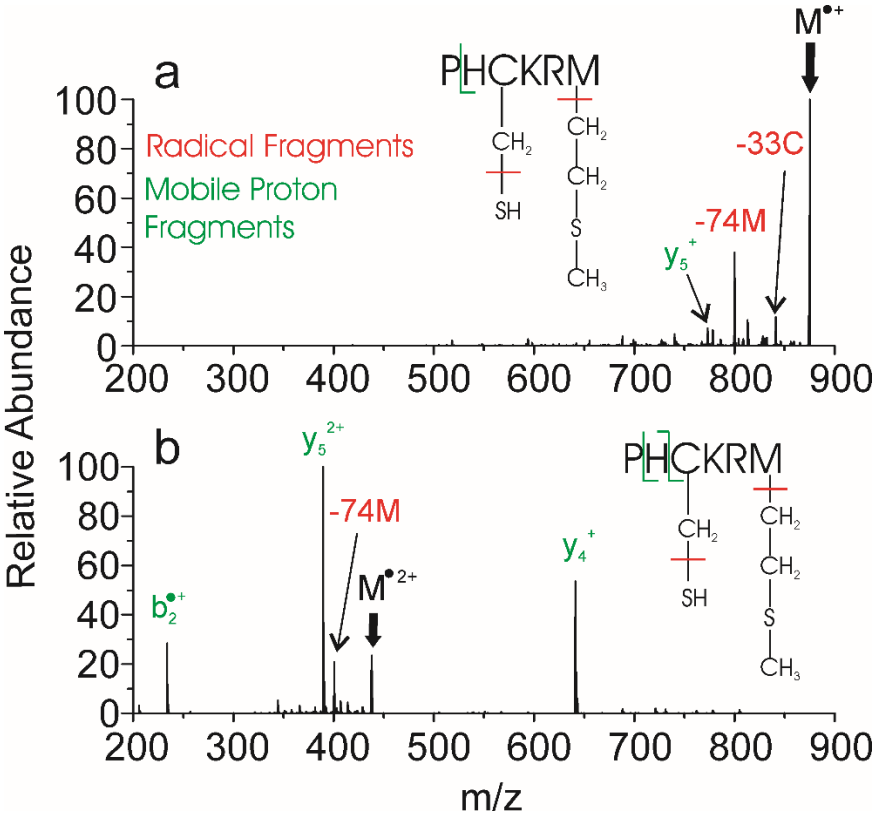

Figure 2.3 CID of PHCKRM Radical in a) +1 and b) +2 charge states.

Subsequently, a variety of known antioxidant peptides were investigated by RDD and the results are summarized in Table 2.1. For each target peptide, multiple charge states were examined (see below for further discussion). To simplify comparison between different peptides, a numerical value called the radical sequestering score (RSS) is reported for each peptide. The RSS is simply the ratio of mobile proton to radical directed fragmentation. An RSS value >1.4 (see experimental for statistical analysis) suggests that the radical is less active in directing peptide dissociation and that the peptide is likely an antioxidant. Importantly, RSS values >1.4 are obtained for almost all of the known

antioxidant peptides listed in Table 2.1. There are two exceptions. For RPDFDLEPPY, the RSS score is in the ambiguous range. However, it is not clear that the isolated peptide was ever independently examined for antioxidant capacity, which may indicate that this peptide is not actually an independent antioxidant.[35] For LPHSGY, the reported hydroxyl radical scavenging percentage (35%) is fairly low, consistent with a weak antioxidant.[34] It is possible that RDD fails to properly identify these peptides as antioxidants, or it is also possible that the antioxidant potential of these peptides has been overstated. In either case, as described in the introduction above, antioxidant capacity can manifest in more than one way, and it is unlikely that RDD will identify all antioxidant peptides. However, the results in Table 2.1 do reveal a strong correlation between gas phase radical fragmentation and solution phase antioxidant capacity. In order for a high RSS value to be obtained, the peptide radical must rapidly migrate from where it is initially created (which is always an unstable site) to a stable position without initiating any fragmentation along the way. This may serve to mimic the situation in solution where peptides must react quickly with radicals intermolecularly and then sequester the radical and prevent subsequent chain reactions.

Sequence	RSS Max*
PHCKRM[26]	5.94
LQPGQGQQG[27]	7.30
HGPLGPL[28]	9.89
Aβ 1-40[29,30]	5.24
Aβ 1-42	4.85
Aβ 1-40**	2.20
PSKYEPFV[31]	5.62
NGPLQAGQPGER[32]	4.15
HFGDPFH[33]	1.64
LPHSGY[34]	0.18
RPDFDLEPPY[35]	0.75
Glutathione	15.45

Table 2.1 RSS for peptides reported to be antioxidants. * Maximum observed RSS score. **with oxidized methionine

Several peptides that are not known to be antioxidants were also examined and the results are shown in Table 2.2. The peptides in Table 2.2 were chosen due to similarity in terms of size and sequence to the peptides in Table 2.1. The maximum RSS values for the peptides in Table 2.2 are all <0.7. Qualitatively, the results obtained for these peptides are similar to the behavior observed for most peptides in our experience. Taken together, the results in Tables 2.1 and 2.2 suggest that RDD is an effective tool for predicting antioxidant capacity.

Sequence	RSS Max*
IQTGLDATHAER	0.40
YEVHHQKLVFF (Aβ 10-20)	0.41
DRVYIHP	0.02
SLHTLFDK	0.66
FVNQHLC*[a]GSH LVEALYLVC*G ERGFFYTPKA (Insulin B Chain)	0.44
HAEGTFTSDV SSYLEGQAAK EFIAWLVKGRG (Glucagon Like Peptide)	0.15
NSKMAHSSSC FGQKIB[b]IDRIGA VSRLGCDGLR LF (Brain Natriuretic Peptide)	0.29

Table 2.2 RSS for peptides not known to be antioxidants. [a] Oxidized cysteine [b] Lysine modified with 4-iodobenzoic acid.

The charge state of the peptide subjected to RDD is important and worth further discussion. Previous work[36-39] has demonstrated that mobile protons are necessary for proton initiated dissociation to be competitive with RDD; however, prediction of the charge state where a proton will become sufficiently mobile is not very straightforward. Frequently it is assumed that protons will be sequestered by arginine and lysine, but it is certainly possible that other structural or sequence effects could lead to sequestered protons in the absence of these residues. For example, the antioxidant capacity of HGPLGPL only becomes apparent upon examination of the +2 charge state (even though the +1 charge state could have a mobile proton, the RSS is 0.07). The obvious explanation is that n-terminal histidine may sequester the proton; however, histidine in general does not appear to sequester protons in other peptides. Fortunately this complication is easily avoided by

simply examining the highest charge states that are available for any given peptide, as protons will be most mobile in this situation. Although this may lead to interrogation of charge states for which there is more than one mobile proton, this condition does not appear to be problematic. For example, SLHTLFGDK^{4IB} (lysine modified with iodobenzoic acid and not capable of sequestering charge) is not an antioxidant peptide which should have a mobile proton in the +1 charge state. RSS values for SLHTLFGDK^{4IB} do not change significantly even if a higher charge state is examined (+2 charge state still produces numerous radical fragments, see Figure 2.4). Indeed, examination of peptides that are not antioxidants does not yield high RSS values regardless of the charge state.

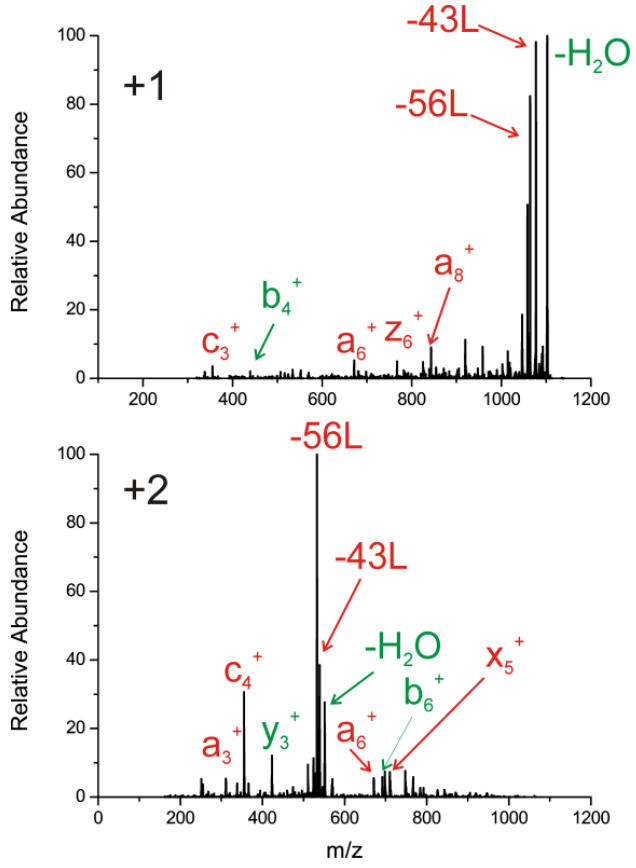

Figure 2.4 RDD of SLHTLFGDK^{4IB} in +1 and +2 charge states.

The results in Tables 2.1 and 2.2 also serve to highlight some of the advantages of a rapid MS based approach for antioxidant identification. For example, three variations of Aβ are examined in Table 2.1. Although all three are predicted to be antioxidant, the results also suggest that the methionine residue may be important for the antioxidant properties of Aβ. The addition of two residues to generate Aβ 1-42, which siginficantly influences the aggregation properties,[40] appears to have little influence on the antioxidant capacity. Furthermore, a partial sequence of Aβ (YEVHHQKLVFF) examined in Table 2.2 exhibits no antioxidant capacity. In fact, RDD yields abundant fragmentation of the peptide backbone at numerous sites for YEVHHQKLVFF (Figure 2.5). It is remarkable that when this sequence is included with the rest of Aβ, which is a significantly larger peptide, the radical is prevented from accessing any of those same sites.

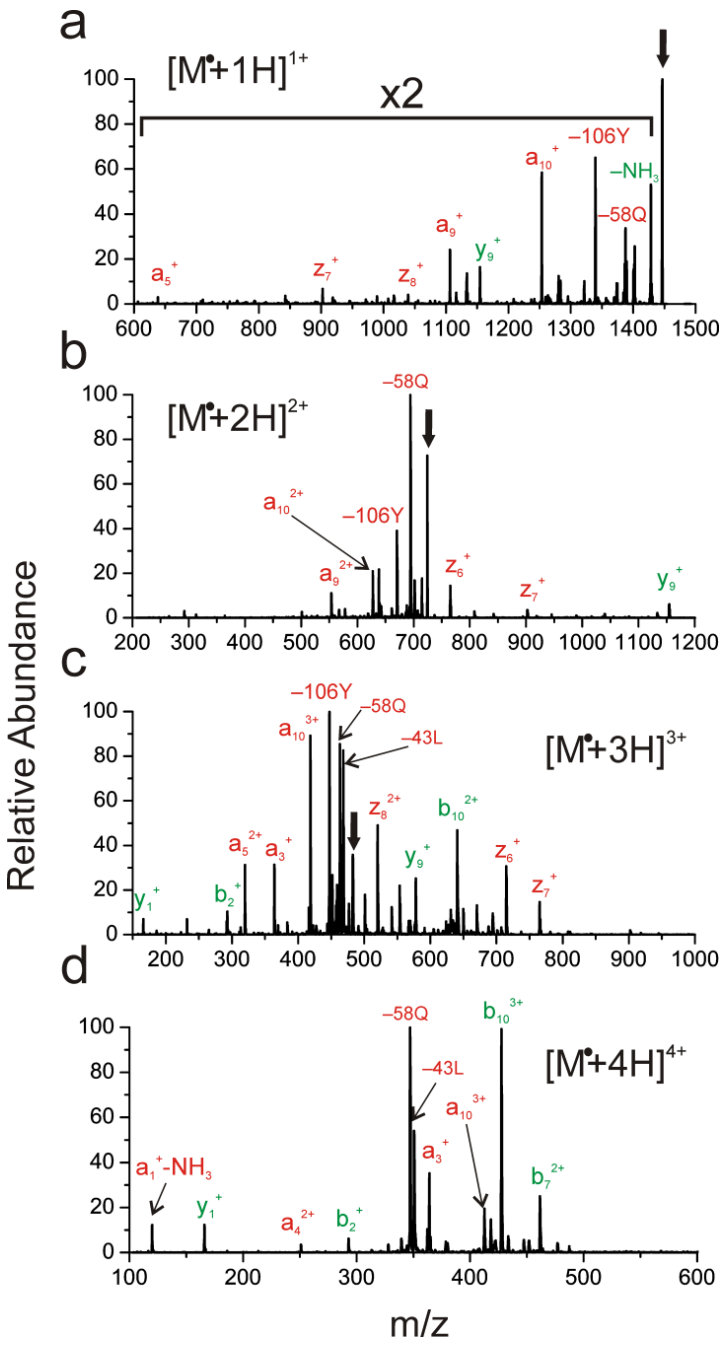

Figure 2.5 CID of YEVHHQKLVFF radical in a) +1, b) +2, c) +3, and d) +4 charge states.

2.3.2 Human Serum Albumin

Human serum albumin (HSA) is the main protein component in blood plasma and is known to have significant antioxidant capacity due to several synergistic properties.[11,12,41] For example, HSA is a metal ion chelator with one high affinity site that binds copper(II), potentially preventing generation of hydroxyl radicals via Fenton-like reactions. HSA may also derive antioxidant capacity from organic ligands such as bilirubin, which is a known antioxidant. HSA also contains several methionine residues and a single free cysteine (Cys34). Several experiments have confirmed the antioxidant role of Cys34,[11] which directly interacts with oxidants. Given the high reactivity of free thiols in general, it is likely that most will exhibit some degree of antioxidant capacity, and therefore they are not a subject of focus in the present work. We have previously identified a method for selectively identifying free cysteine residues in whole proteins that could be used to screen for such molecules.[42] The contribution of the methionine residues to the antioxidant capacity of HSA remains less clear. Some direct scavenging abilities are attributed to methionine, but metal ion binding has also been suggested as a primary function.[43,44] Interestingly, contributions to the inherent antioxidant properties of HSA by the remaining ~580 residues remain essentially unexplored.

In order to remedy this situation, a search for antioxidant peptides in HSA was carried out with RDD. Following reduction and alkylation of all cysteine residues, trypsin was used to digest HSA into peptides, which were then modified with iodobenzoic acid and subjected to LC-MS. 44 peptides were identified and characterized by RDD in a single LC-MS run. It should be mentioned that any traditional technique would require separation and

isolation of sufficient sample for individual analysis of each of these 44 peptides, which would likely require at least an order of magnitude more sample and several orders of magnitude more time. In addition to trypsin, Glu-C and Lys-C digests were performed to provide overlapping coverage and to reduce the possibility of cleaving in the middle of any antioxidant portions of HSA. A complete list of the peptides identified and examined for antioxidant activity are shown in Table 2.3.

Peptide Sequence	RSS Score	Peptide Sequence	RSS Score
TFLK	**14.67**	SISSKLK	0.15
VAHRFK	**5.21**	NYAEAKD	0.14
VPQVSTPTLVEVSR	**4.21**	ETCFAEEGK	0.14
DVFLGMFLYEYAR	**3.79**	YKAAFTECCQAADK	0.14
DDNPNLPR	**2.83**	AAFTECCQAADK	0.12
YLYEIAR	1.30	CCAAADPHECYAK	0.11
SEVAHR	0.89	KPLLEK	0.10
NYEAK	0.79	CFLQHKD	0.08
TPVSDRVTK	0.68	LVRPEVDVMCTAFHDNEETFLK	0.08
LVTDLTK	0.57	TCFAEEGKK	0.06
LVNEVTEFAK	0.55	AVMDDFAAFVEK	0.06
KQTALVE	0.53	TPVSDRVTKCCTE	0.05
EQLK	0.52	FQNALLVRYTK	0.05
KYLYE	0.51	NECFLQHK	0.05
FQNALLVR	0.51	ICTLSEKE	0.04
DAHKSE	0.49	VPQVSTPTLVE	0.04
AWAVAR	0.48	VMCTAFHDNEE	0.03
SLHTLFGDKLCTVATLR	0.46	VHTECCHGDLLECADDR	0.03
SLHTLFGDK	0.46	PLLEKSHCIAE	0.02
VSKLVTDLTK	0.43	CCQAADK	0.01
AACLLPK	0.41		
LDELRDEGK	0.39		
CASLQK	0.36		
KSLHTLFGD	0.35		
TYETTLEK	0.35		
SEVAHRFK	0.34		
QEPERNE	0.33		
AEFAEVSK	0.32		
ADDKETCFAEEGK	0.31		
HPDYSVVLLLR	0.29		
RPCFSALEVDETYVPK	0.27		
KVPQVSTPTLVEVSR	0.25		
FAKTCVADE	0.24		
DLGEENFK	0.24		
AEFAEVSKLVTDLTK	0.21		
TCVADESAENCDK	0.20		
ETYGEMADCCAK	0.20		
QEPERNECFLQHK	0.17		
VSRNLGK	0.16		

Table 2.3 HSA peptides identified and analyzed for antioxidant capacity. Peptides in bold have RSS values > 1.4.

The total sequence coverage from all digests was 72%. It is worth mentioning that no peptides containing Cys34 were identified; however, since all cysteine residues were alkylated we anticipate that the antioxidant capacity of any peptide containing Cys34 would have been significantly perturbed in any case. The final analysis of all results yielded 5 peptides with RSS values >1.4 (see Figure 2.6). None of these peptides have previously been identified as having antioxidant capacity. In order to establish the accuracy of the RDD results, we decided to measure the antioxidant capacity of the potential antioxidant peptides in Figure 2.6 by traditional means.

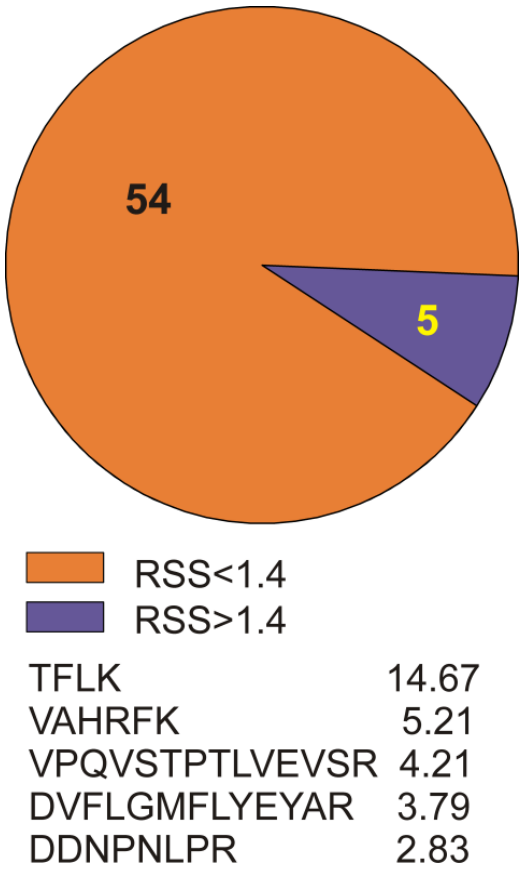

Figure 2.6 RSS values for peptides investigated using RDD.

2.3.3 Lipid Peroxidation Assay

The antioxidant capacity of peptides with RSS values >1.4 was measured using a standard lipid peroxidation assay in solution.[22] The degree of lipid peroxidation for each peptide and several controls is shown in Figure 2.7. The change in absorbance at 500 nm portrays the inhibition of lipid peroxidation. Glutathione, a known radical scavenger, was used as a positive control and shows the highest degree of protection. Three peptides (VAHRFK, DVFLGMFLYEYAR, TFLK) also inhibit lipid peroxidation to varying degrees and were confirmed to be antioxidants. Two peptides from HSA identified as potential antioxidants using RDD failed to show any inhibition of radical activity (DDNPNLPR and VPQVSTPTLVEVSR). DDNPNLPR presents a unique case with regards to gas phase fragmentation. Proline and aspartic acid are residues that are highly favorable sites for backbone fragmentation, producing b and y ions.[45] The presence of two proline and two aspartic acid residues that account for the majority of the observed fragmentation explains the anomalously high RSS value that was observed for DDNPNLPR. The second false positive peptide also contains an internal proline which corresponds to the site of dominant b/y ion fragmentation. These results suggest that in cases where potential antioxidants have high RSS values primarily due to extensive fragmentation at proline or aspartic acid, another method should be employed to verify antioxidant capacity. To clarify, this does not mean that the method cannot be applied to all peptides which contain these residues, rather, in the unique situation when fragmentation is dominant at these residues further experiments should be performed. For the majority of peptides containing Asp and Pro that were successfully examined this was

not an issue. The antioxidant capacity of a group of peptides with low RSS values was also evaluated using the lipid peroxidation assay (see Figure 2.8). These peptides with RSS <1.4 showed minimal ability to inhibit lipid peroxidation.

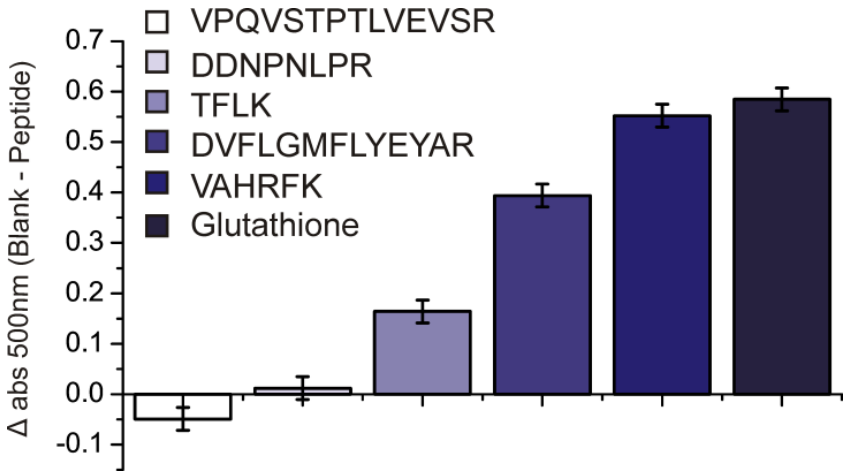

Figure 2.7 Lipid peroxidation inhibition in the presence of peptides (Larger difference in absorbance suggest higher degree of inhibition of lipid peroxidation)

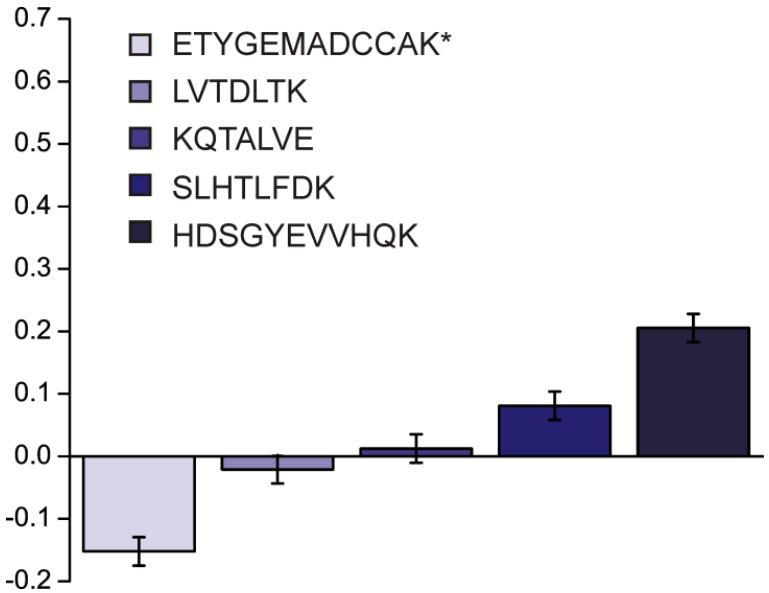

Figure 2.8 Lipid Peroxidation inhibition in the presence of peptides with RSS<1. *Cysteines blocked using iodoacetamide.

2.4 Conclusions

RDD is a viable method for rapidly assessing the antioxidant capacity of peptides. Comparison with solution phase methods reveals excellent agreement, with a few exceptions. A small percentage of antioxidant peptides will likely not be identified by RDD due to the chemically diverse ways in which antioxidants may manifest; however, this limitation also applies to more traditional assays that are also far slower and less sensitive. Using RDD, we were able to identify antioxidant regions of HSA that likely contribute to the overall antioxidant capacity of this important serum protein. Indeed, the peptide VAHRFK from HSA exhibits similar antioxidant capacity to glutathione without any cysteine or methionine residues. Furthermore, of the three antioxidant peptides that were identified from HSA, only one contained methionine. These results clearly indicate that significant antioxidant capacity is possible in the absence of these residues. The structural and sequence parameters that may influence antioxidant capacity are not completely understood; however, it is clear that RDD may provide a rapid avenue for further exploration of these interesting issues in the future. The application of this new technique is further expanded in chapter 3. RDD is used for the analysis of multiple proteins to identify new antioxidant peptides/regions.

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Chapter 3

The Innate Capacity of Proteins to Protect Against Reactive Species

3.1 Introduction

Redox chemistry is a necessary evil in biology. The production of reactive species (RS) such as free radicals is both inevitable and necessary for normal functioning of cells. For example, nitric oxide and other RS are employed for cellular signaling, regulating vascular tone, and destruction of invading species by the immune system.[1] Simultaneously, RS can modify and damage many desirable biomolecules, including proteins, lipids and carbohydrates. A common example affecting many lives today is coronary heart disease which is caused (in part) by circulation of oxidized lipids.[2] Excess RS leads to oxidative stress, which is associated with multiple diseases such as cancer, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease.[3-5]

It is also well established that proteins play important roles in biological systems. Enzymatic catalysis, cellular signaling, and ligand binding/transport are a few examples. The functionality of most proteins is dependent on structure, which can be disrupted following modifications due to oxidative stress. Protection of proteins and other biomolecules from oxidative stress is therefore important; however, the mechanisms which regulate redox homeostasis in cells are not entirely clear.

A thorough accounting of all contributors, both pro-oxidant and antioxidant, is a necessary starting point that remains currently unrealized. Much information has been acquired. For example, the production of free radicals and other reactive species that are

pro-oxidant is well established.[6] Many small molecule antioxidants have been identified and investigated; for example glutathione and ascorbic acid have been studied extensively.[7] However, highly abundant molecules such as proteins, which can serve dual purpose roles, have not been extensively examined as antioxidants. It has already been established that sacrificial radical scavenging properties are apparent in some proteins. For example, methionine is easily oxidized in the presence of RS. The existence of methionine sulfoxide reductase, which catalyzes the reduction of methionine back to native form, suggests that methionine can serve as a sacrificial RS scavenger.[8] In addition, a small number of peptide regions in proteins are known to behave as antioxidants.[9,10] For example, human serum albumin (HSA) is a blood plasma protein that is known to be antioxidant.[8,11] A question that has yet to be answered is whether all or most proteins have innate antioxidant capacity.

Recently, Mass Spectrometry (MS) has been leveraged for rapid identification of antioxidant peptides using radical directed dissociation (RDD).[12] RDD leverages radical chemistry to initiate fragmentation, which can be used to investigate chemical properties including epimerization or phosphorylation.[13-15] Peptides that are resistant to RDD, which always yields characteristic fragments such as side chain losses or a/x ions, have been demonstrated to behave as antioxidants in solution.[12] The fragmentation pattern of a radical peptide is therefore indicative of its ability to act as an antioxidant. When the radical is sequestered, typical proton initiated fragmentation will dominate as illustrated in Figure 3.1.

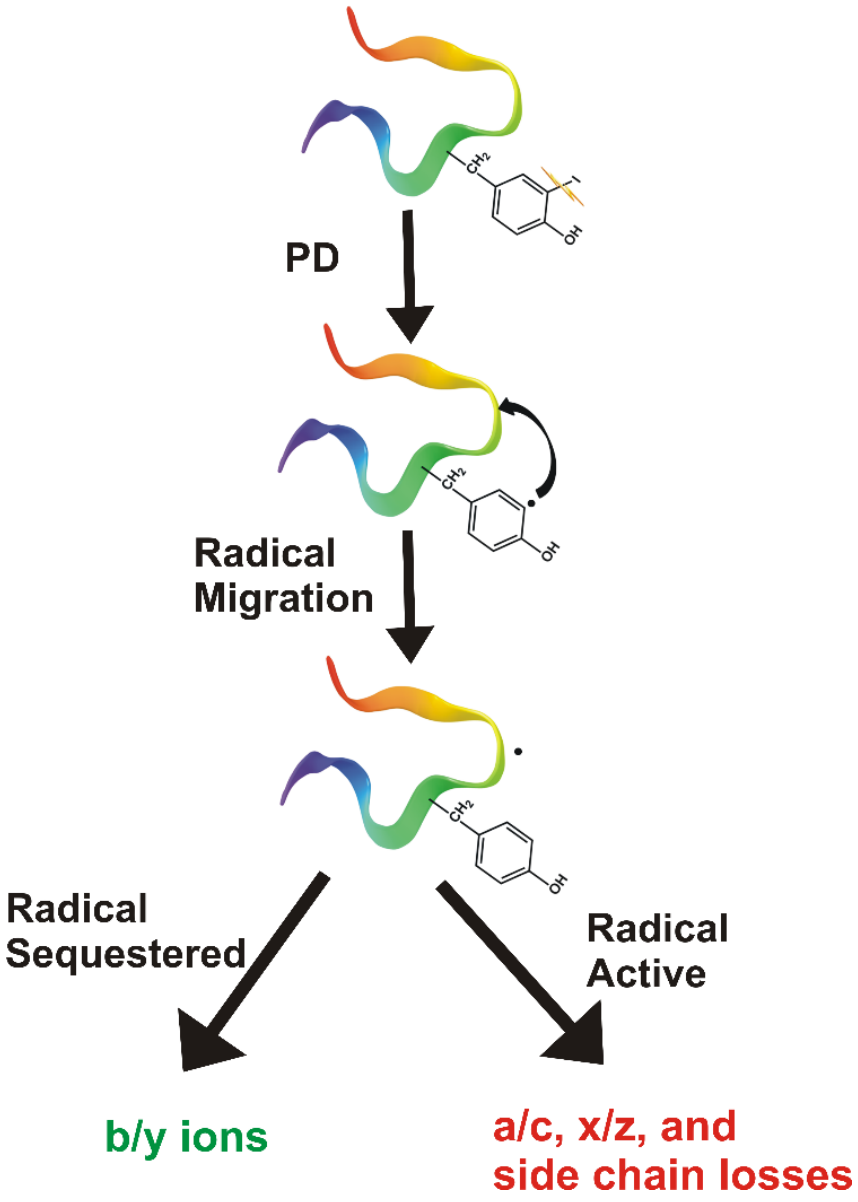

Figure 3.1 Using RDD to identify antioxidant peptides

The development of a quick and facile method for identifying antioxidant peptides/regions in proteins makes it possible to investigate a larger pool of antioxidant peptides from different proteins. Herein, multiple proteins with different functionalities are investigated. Using RDD coupled with liquid chromatography (LC), the antioxidant

capacity of β-lactoglobulin, αA crystallin, αB crystallin, and myoglobin is mapped and attributed to specific peptide regions. The presence of antioxidant regions in all proteins investigated suggests that proteins have an intrinsic capacity to protect themselves against reactive species and oxidative damage.

3.2 Experimental Methods

3.2.1 Materials

Organic Solvents, Human Serum Albumin, Myoglobin, α-crystallin and Trypsin were purchased from Sigma-Aldrich (St Louis, MO). β-Lactoglobulin was purchased from MP Biomedicals (Solon, OH). Water was purified using a Millipore 147 Direct-Q system (Billerica, MA). A betabasic C18 column from Thermo Scientific was used for all LC-MS experiments.

3.2.2 Protein Digests

Proteins were digested with trypsin following reduction and alkylation. 8-10 nmols of protein was dissolved in 15 uL of ultrapure water. 1.5 uL of 100 mM dithiothreitol (DTT) and 15 uL of 50 mM ammonium bicarbonate were added and incubated at 95°C for 5 minutes. After allowing the sample to cool, 3 uL of 100 mM iodoacetamide was added to the sample tube and incubated in the dark at room temperature for 20 minutes. Acetonitrile (ACN) was added to adjust solution to 10% ACN. 4 uL of 87 uM trypsin was added to solution and left overnight at 37°C. Peptides in solution from tryptic digest were modified with 4-iodobenzoic acid as previously described.[16]

3.2.3 Mass Spectrometry (MS) and Liquid Chromatography-Mass Spectrometry (LC-MS)

All LC-MS experiments were done on an LTQ linear ion trap with a standard electrospray ionization source (Thermo Scientific, San Jose, CA). The MS has been modified with a quartz window to allow firing a 266 nm Nd:YAG laser into the ion trap. LC-MS was done using a Thermo betabasic 18 column with 3 micron particle size. Buffers used were water with 0.1% formic acid and acetonitrile w 0.1% formic acid. The following gradient was used for the LC in terms of time (%B); 0 (5%) – 25 (20%) – 70 (50%) – 80 (95%) – 85 (5%). Software was configured to fire laser at MS^2 for photodissociation (PD) followed by reisolation and collision induced dissociation (CID) of the most abundant peak.

3.2.4 Identifying antioxidant peptides with radical sequestering score (RSS)

RSS value is simply the ratio of mobile proton to radical directed fragmentation. Peptides that are antioxidant will have RSS scores between 1.4 and 9.2 and peptides that are not antioxidant will yield RSS scores between 0.096 and 0.67. Peptides with RSS values above 9.2 are exceptionally good at radical sequestration, and are also clearly antioxidant. However, for peptides with RSS values between 0.67 and 1.4, the antioxidant status cannot be determined by RDD.

3.2.5 Comparing Sequence Homology

Sequence homology was done using the Clusta Omega program on uniprot.org. Human serum albumin was compared against bovine, rat, mouse, chicken, dog, pig, cat, and horse. Horse Myoglobin compared against human, bovine, pig, mouse, sheep, and chicken. Bovine β-lactoglobulin against water buffalo, goat, tammar wallaby, kangaroo, and pig.

Finally, bovine α-crystallin was compared with human, rat, rabbit, mouse, domestic pigeon, and golden hamster.

3.3 Results and Discussion

RDD is used to identify antioxidant peptides as described in the introduction. Illustrative data is shown for two peptides derived from β-lactoglobulin in Figure 3.2. IPAVFK behaves like most peptides. When a radical is created by photocleavage of a carbon-iodine bond and subjected to collisional activation, the resulting fragmentation is dominated by RDD. In contrast, activation of radical VYVEELKPTPEGDLEILLQK yields primarily b and y ions, indicating that the radical has been sequestered and the peptide is likely antioxidant. The degree of sequestration in each case is quantified by the RSS score (described in detail above), where values above 1.4 indicate the peptide is antioxidant. We use this approach to rapidly evaluate regions of proteins likely to be antioxidant.

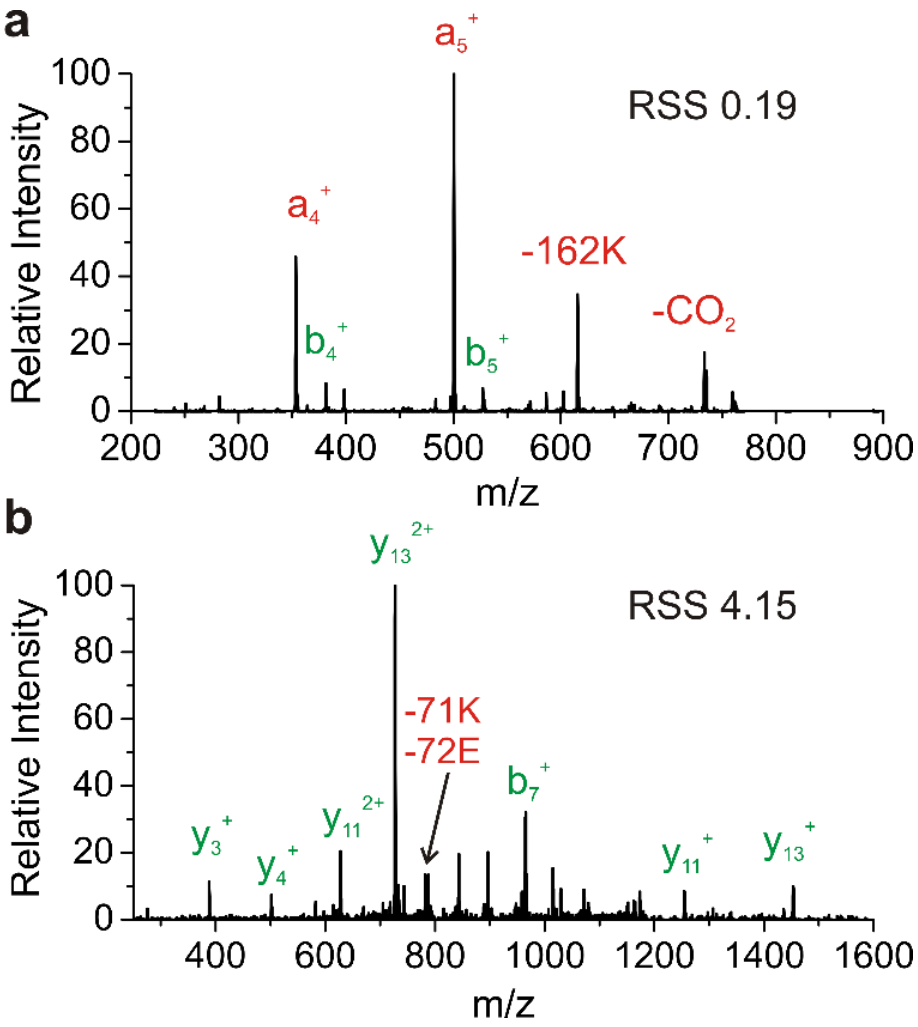

Figure 3.2 a) CID of +1 IPAVFK^{4IB} radical. b) CID of +3 VYVEELK^{4IB}PTPEGDLEILLQK radical

3.3.1 Antioxidants in Proteins.

Human serum albumin (HSA) has been confirmed to have antioxidant capacity in both solution and gas phase studies.[8,12] HSA is the major protein in blood plasma and primarily serves as a transporter of different ligands such as lipids. A previous search for antioxidant regions in HSA using RDD yielded multiple peptides with antioxidant capacity. These results suggest that HSA serves a secondary role in blood plasma as an antioxidant. Of

interest is whether this phenomenon is unique to HSA, or do most proteins have antioxidant regions?

To address this question, four proteins with varying biological functions and locations were examined for antioxidant peptides using RDD. The results are summarized and compared with those obtained previously for HSA in Figure 3.3, where the fraction of typtic peptides exhibiting antioxidant capacity is compared with the total sequence coverage for each protein. Interestingly, all five proteins contain peptides that exhibit antioxidant capacity. The highest fractional abundance (32%) of antioxidant peptides was found for β-lactoglobulin, with the lowest fraction (5%) found for αB-crystallin.

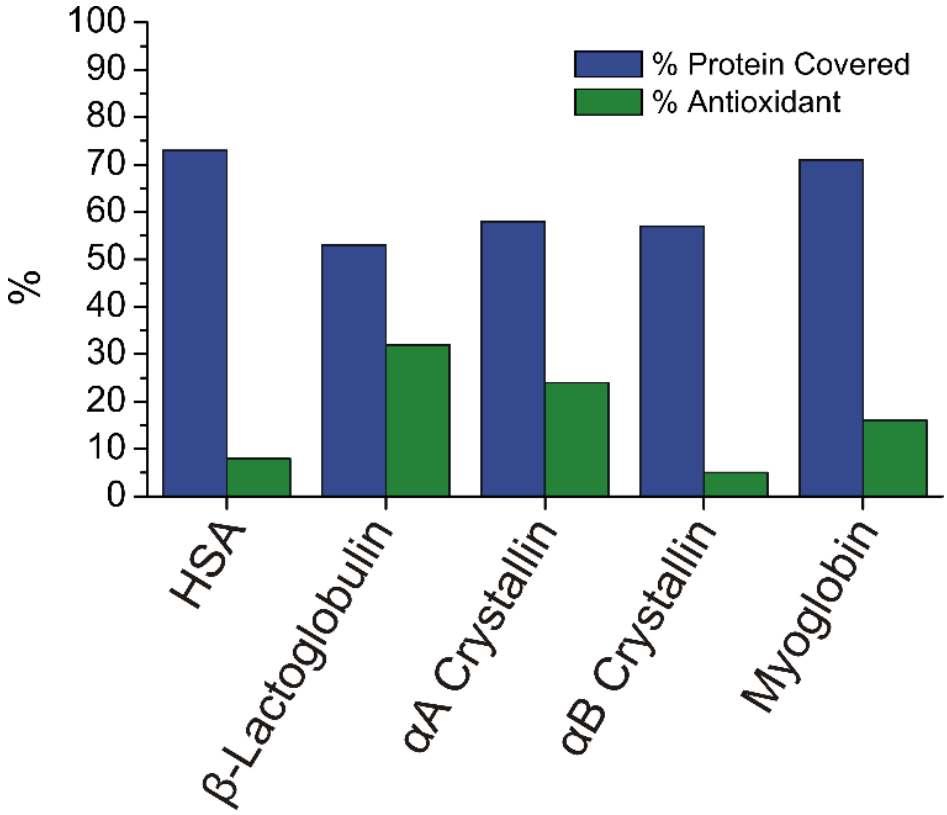

Figure 3.3 Antioxidant regions identified using RDD.

The presence of antioxidant regions in these proteins is intriguing, given the diversity of roles they play in biology. As previously mentioned, HSA is the major soluble protein circulating in blood plasma. Myoglobin is an iron and oxygen binding protein found primarily in mammalian muscle tissue.[17,18] It follows that HSA and myoglobin are frequently exposed to oxygen, which participates in many reaction pathways that lead to free radical generation and oxidative stress.[19] The presence of protective antioxidant regions in these proteins is therefore not unexpected.

β-lactoglobulin and the α-crystallins also contain peptide regions that are antioxidant. β-lactoglobulin is a whey protein abundant in most mammalian milk. A specific identifiable function for β-lactoglobulin is lacking, however, the ability of β-lactoglobulin to readily bind ligands (specifically hydrophobic molecules) suggests it may function as a transporter.[20] This information does not imply any specifically abundant interactions between β-lactoglobulin and ROS, but previous studies have shown it to have radical scavenging capabilities.[21] Additional detail regarding the mechanism of action or regions of β-lactoglobulin that confer antioxidant capacity have not been previously investigated. RDD results suggest antioxidant capacity for β-lactoglobulin originates from specific peptide regions (the specific sequences are discussed in greater detail below). αA-crystallin and αB-crystallin are structural and chaperone proteins. They constitute 90% of the protein content in the eye lens and are critical for maintenance of lens transparency.[22,23] Oxygen levels are relatively low in the eye lens; however, there is also no turnover of proteins, meaning that oxidative damage must be avoided for an entire lifetime. There is a correlation between increased production of ROS and the development of cataracts.[24] Both αA-

crystallin and αB-crystallin have antioxidant peptide regions, which is not unusual given the length of time that these proteins must survive without losing functionality. Interestingly, all five proteins that were examined have antioxidant regions regardless of function or location.

3.3.2 Are Antioxidant Peptides Similar?

Although RDD has greatly increased our ability to identify antioxidant peptides quickly, it would be even easier to identify antioxidant peptide regions by genomic analysis if characteristic sequence or residue content could be determined. Table 3.1 lists all peptides with high RSS values, which are likely to be antioxidant. Residue content among peptides is very diverse with no one amino acid being common in all. A high percentage of the antioxidant capacity of proteins and peptides is believed to come from residues such as methionine and cysteine.[8] However, the antioxidant peptides identified here suggest this is not always the case and many different combinations of residues can be radical scavengers. These results further justify previous findings; peptide sequence is an integral part of antioxidant capacity.

Peptide Sequence	Protein	RSS
VAHRFK	HSA	5.21
DVFLGMFLYEYAR	HSA	3.79
TFLK	HSA	14.67
NDIAAK	Horse Myoglobin	2.24
ASEDLK	Horse Myoglobin	5.58
ALELFR	Horse Myoglobin	8.54
ELGFQG	Horse Myoglobin	5.49
LIVTQTMK	Bovine β-lactoglobulin	2.73
TPEVDDEALEK	Bovine β-lactoglobulin	4.18
VYVEELKPTPEGDLEILLQK	Bovine β-lactoglobulin	4.15
EEKPSSAPSS	Bovine αA-crystallin	17.07
AIPVSREEKPSSAPSS	Bovine αA-crystallin	1.47
TVLDSGISEVRSDRDK	Bovine αA-crystallin	1.63
HFSPEDLTVK	Bovine αA-crystallin	3.05
TVLDSGISEVR	Bovine αA-crystallin	6.29
HFSPEELK	Bovine αB-crystallin	3.91

Table 3.1 Antioxidant Peptides with High RSS Values Identified from Different Proteins

While there is no clear-cut recipe for peptides to have antioxidant capacity, there are interesting trends observed. Methionine is present in only two of the antioxidant peptides identified. This suggests that the presence of methionine is not a necessity for a peptide to exhibit antioxidant capacity. In fact, some peptides that contain methionine residues are

not antioxidants. The absence of cysteine residues in any of the peptides observed is not an indication of the lack of antioxidant contribution from cysteines. However, it is important to note that all cysteine residues are blocked with iodoacetamide, which would naturally inhibit the ability of the side chain of cysteine to protect the peptide. As a general note, most cysteine residues in the proteins investigated are a part of disulfide bonds. As previously mentioned, radical directed dissociation is preferential at aromatic residues (His, Tyr, Phe, Trp), serine and threonine residues. Interestingly, aromatic residues are present in 70% of the antioxidant peptides identified. Additionally, threonine and serine are each present in seven (41%) of the peptides. The ability of the peptide to still act as an antioxidant in the presence of these radical favored fragmentation positions suggests the peptide has a stronger ability to capture the radical and sequester it. There appears to be no bias for peptides that lack residues that favor radical fragmentation. Comparing the presence of these residues in peptides that are antioxidant and ones that are not antioxidant is show in Table 3.2. There are minor differences between the two sets of peptides (antioxidants and not antioxidants). Once again, evidence suggests the sequence of the peptides is integral for the ability of the peptide to display antioxidant capabilities.

Residue	Presence in Antioxidants (%)	Presence in Not Antioxidants (%)
Leu	65	55
Met	12	7
Ser	41	30
Thr	41	49
Aromatic	71	78

Table 3.2 Antioxidant Peptides with High RSS Values Identified from Different Proteins

Amino Acid	Natural Abundance in Vertebrates (%)	Abundance in Antioxidant Peptides (%)	Deviation from Natural Abundance
Alanine	7.4	6.7	-0.7
Arginine	4.2	4.3	0.1
Asparagine	4.4	0.6	-3.8
Aspartic Acid	5.9	6.7	0.9
Cysteine	3.3	0.0	-3.3
Glutamic Acid	5.8	12.9	7.1
Glutamine	3.7	1.8	-1.9
Glycine	7.4	3.7	-3.7
Histidine	2.9	1.8	-1.1
Isoleucine	3.8	4.9	1.1
Leucine	7.6	9.8	2.2
Lysine	7.2	8.6	1.4
Methionine	1.8	1.2	-0.6
Phenylalanine	4.0	4.9	0.9
Proline	5.0	6.1	1.1
Serine	8.1	10.4	2.3
Threonine	6.2	4.9	-1.3
Tryptophan	1.3	0.0	-1.3
Tyrosine	3.3	1.8	-1.5
Valine	6.8	7.4	0.6

Table 3.3 Natural Abundances of Amino Acids vs Abundances in Antioxidant Peptides

Amino acid composition is subject to evolution and natural selection.[25-27] Evidence suggests certain residues (Cys, Met, His, Ser and Phe) are increasing in frequency at the expense of other amino acids (Pro, Ala, Glu and Gly).[28] A trend for amino acid substitution in antioxidant peptides has not been reported. It is possible that certain residues contribute more to the antioxidant capacity of proteins/peptides and as a result antioxidant peptides would show a significant difference over the natural abundance for such amino acids.

Table 3.3 shows the abundance of all 20 natural amino acids in the peptides found to be antioxidants and is compared to the natural abundance in vertebrates. The differences

in the abundances are not significant and there looks to be no preference for specific amino acids in antioxidant peptides. The largest deviation from natural abundance is seen in glutamic acid (+7.1) and glycine (-3.7). A study comparing the amino acid composition in a thousand different types of proteins (extracellular, anchored, membrane, intracellular, and nuclear) shows standard deviations ranging from 0.7 to 13.5 for each residue.[29] As a result, the deviations seen in antioxidant peptides can be attributed to bias in the small sample size of proteins. In addition, peptides that are not antioxidant have a very similar distribution of amino acid composition (see Table 3.4). Again, these results support the conclusion that peptide sequence and structure are major contributors to the antioxidant capacity of peptides and clear favoritism towards specific residues is lacking.

Amino Acid	Natural Abundance in Proteins (%)	Abundance in Not Antioxidant Peptides (%)	Deviation from Natural Abundance
Alanine	7.4	10.0	2.6
Arginine	4.2	2.6	-1.6
Asparagine	4.4	2.5	-1.9
Aspartic Acid	5.9	6.5	0.6
Cysteine	3.3	5.0	1.7
Glutamic Acid	5.8	10.6	3.8
Glutamine	3.7	2.9	-0.8
Glycine	7.4	3.7	-3.7
Histidine	2.9	3.5	0.6
Isoleucine	3.8	2.3	-1.5
Leucine	7.6	8.8	1.2
Lysine	7.2	10.7	3.5
Methionine	1.8	0.7	-1.1
Phenylalanine	4.0	3.7	-0.3
Proline	5.0	3.1	-1.9
Serine	8.1	3.2	-4.9
Threonine	6.2	7.0	0.8
Tryptophan	1.3	0.3	-1.0
Tyrosine	3.3	1.9	-1.4
Valine	6.8	6.9	0.1

Table 3.4 Natural Abundances of Amino Acids vs Abundances in Antioxidant Peptides

3.3.4 Are Antioxidant Regions Coincidental?

All proteins we have examined appear to have antioxidant regions. There are two likely explanations for the presence of antioxidant peptides. One is that due to inherent sequence diversity, some part of most proteins will be antioxidant by chance. The second explanation is that antioxidant sequences have been incorporated by evolutionary pressure. Regions of proteins that are important for functionality typically exhibit sequence similarity across different species. In order to explore this possibility, homology studies were conducted for multiple organisms for each protein.

Figure 3.4 shows overlap between highly conserved regions and antioxidant peptides. Myoglobin and the α-crystallins are highly conserved throughout their entire sequences. Thus, antioxidant peptides are unavoidably located within conserved regions. αA-crystallin and αB-crystallin have similar sequences. However, of the three antioxidant regions in αA-crystallin, only one (HFSPEDLTVK) is highly conserved when compared with αB-crystallin. A strikingly similar sequence is observed for the only antioxidant region (HFSPEELK) in αB-crystallin suggesting this region is engineered in both α-crystallins to provide innate protection. On the other hand, serum albumin and β-lactoglobulin have limited areas with high homology. A few of the antioxidant regions overlap with these highly conserved areas.

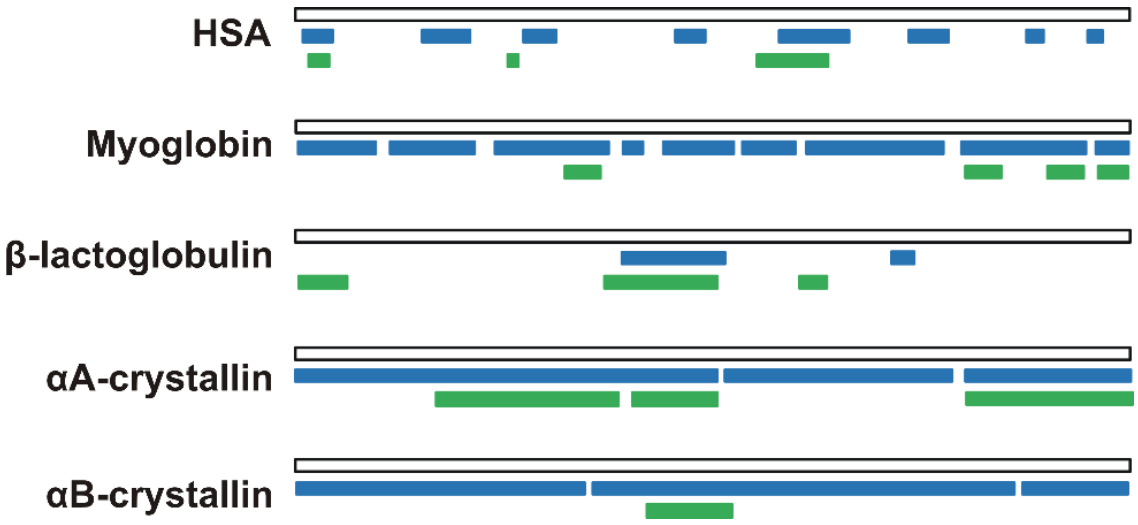

Figure 3.4 Highly conserved regions (blue) and antioxidant regions (green) in HSA, Myoglobin, β-lactoglobulin, αA-crystallin and αB-crystallin. The white bar represents the full protein sequence.

In β-lactoglobulin, there is one peptide region that is both conserved and antioxidant. This conserved region is the main ligand binding site in β-lactoglobulin.[20] A common ligand that binds to β-lactoglobulin is cholesterol; a molecule that is very sensitive to

oxidation. Oxidized cholesterol is closely related to arterial damage.[30] The presence of an antioxidant peptide in the ligand binding region of β-lactoglobulin suggests that in addition to functioning as a transport agent for cholesterol, β-lactoglobulin functions as a protective agent. While past studies have shown a radical scavenging ability of β-lactoglobulin, confining the antioxidant region to the only main conserved region in β-lactoglobulin is a novel discovery. Since the antioxidant regions are found in regions with other known functions, like ligand binding, no conclusions can be drawn regarding evolutionary pressure on antioxidant capacity. However, it is possible that these regions have evolved with dual functionalities.

The n-terminal antioxidant peptide in HSA (^{7}VAHRFK^{12}) is one that is highly conserved as seen in Figure 3.4. The n-terminal region of HSA has the ability to bind metal ions, specifically copper.[31,32] The n-terminal fragment (^{1}DAH^{3}) is the strongest binding site for copper. Studies show that copper binding to this region results in very little changes to the protein overall, but Ser5, Val7, and Arg10 seem to be affected.[31] The significance of this finding lies in the role of copper in the production of reactive species. Copper is a redox active metal; it is capable of producing hydroxyl radicals through the interaction with hydrogen peroxide 60 times faster than iron.[8] The presence of a conserved radical scavenging peptide in close proximity to the metal binding site would provide protection from the increased levels of ROS produced in this region.

3.4 Conclusions

RDD is an excellent method for quick identification of antioxidant peptides/regions in proteins. The five diverse proteins investigated show regions of antioxidant capacity,

regardless of location and function. From these results, one can conclude that in addition to protein primary function, they have an ability to provide protection from RS to both themselves and other biomolecules. The presence of conserved antioxidant regions in the proteins HSA and β-lactoglobulin can be explained by the functions of these two proteins and indicates that the antioxidant regions may be selectively engineered to serve that purpose. Additionally, the analysis of five different proteins has given a much larger pool of peptides with varying sequences. Comparing the natural abundance of residues versus that found in antioxidant peptides shows little differences. This provides support for the importance of the overall sequence of the peptide in producing antioxidant capacity, putting little emphasis on the presence of specific amino acids.

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Chapter 4

Insights into the Antioxidant Capacity of Peptides

4.1 Introduction

Antioxidants play essential roles in maintaining redox homeostasis. Understanding the contribution of all antioxidant species is necessary to better understand the redox environment in biological systems. A gap in understanding the contribution of large highly abundant biomolecules such as proteins is quite evident. The power of utilizing mass spectrometry in identifying antioxidant peptides quickly and accurately is described in detail in chapter 2.[1] Radical directed dissociation (RDD) is used to identify antioxidant peptides in multiple proteins (described in detail in chapter 3). The MS-based method allowed for the investigation of a larger set of peptides. As discussed in chapter 3, residue analysis showed no significant trends. The quick and facile nature of RDD as a method for identifying antioxidant peptides was motivating to investigate the effect of posttranslational modifications (PTMs) and take a deeper look at the effect of specific residues on the antioxidant capacity of peptides.

4.2 Experimental Details

4.2.1 Methionine Oxidation

Methionine oxidation was done simultaneously with the modification of tyrosine (iodination). For a typical peptide iodination reaction, 1 equivalent of chloramine-T is added to a 50-100 uL solution containing 1 equivalents of peptide (~1-50 nmol) and 1 equivalents of sodium iodide. To avoid side products, the reaction is quenched after 30

seconds with 2 equivalents of sodium metabisulfite. For my purposes, the reaction was allowed to react for 10 minutes to promote methionine oxidation.

4.2.2 Peptide Synthesis

All peptides not purchased commercially were manually synthesized using standard Fmoc solid phase synthesis with Wang resins. Peptides are built from C-terminus to N-terminus in a fritted synthesis vessel with a three-way valve. The C-terminal residue is attached to a wang resin. For each following residue, deprotection of the reactive N-terminus is done using a 20:80 mixture of piperidine:dimethylformamide (DMF). The resins are then washed with DMF multiple times to remove residual piperidine. The Fmoc-protected amino acid is then mixed with tetramethylaminium hexafluorophosphate (HCTU) and N-methylmorpholine (NMM) in DMF and added to the resins. HCTU is used as the coupling agent and NMM as the base to drive the reaction. This process is repeated for each residue in the peptide. Following the addition of the final residue, the peptide is cleaved off the wang resin overnight in 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropyl silane (TIPS). The antioxidant peptide PHCKRM was purchased from American Peptide Co. (Sunnyvale, CA).

4.2.3 Mass Spectrometry

Solutions containing ~10 μM peptides in 49.5/49.5/1 water/methanol/acetic acid were directly infused into an LTQ linear ion trap with a standard electrospray ionization source (Thermo Scientific, San Jose, CA). The instrument has been modified with a quartz window to allow the beam from a 266 nm Nd:YAG laser into the ion trap. An isolation width of 4 Da was used for MS2 and MS3 isolations.

4.2.4 Calculation Details

Calculations were performed using Gaussian 09 Rev. A.1 (Gaussian, Inc., Wallingford, CT). Structural optimizations and energy calculations were done with hybrid density functional theory B3LYP at the 6-31G(d) and 6-311G(d). For the sake of comparison to literature reported bond dissociation energy (BDE) values of native methionine, 6-31G(d) was used. BDEs were calculated in two different ways. First, the BDEs were calculated using the isodesmic route, similar to methods used by Moore et al.[2] In this method experimentally determined BDEs of reference molecules were used. Reference molecules were picked based on similarity to C-H of choice. For example, α carbon on methionine is most similar to that of glycine. Since glycine BDE was calculated experimentally, it was appropriate for our purposes. An example scheme used for the isodesmic route of α carbon is shown in figure 4.1. The BDE was then calculated by using the experimental BDE of the reference to offset the calculated values. Since the same bonds are present in both reactants and products, the errors in the calculations should cancel out. Propane was used as a reference molecule for the β carbon. For the γ and δ carbons, methanethiol (CH3SH) was used as a reference molecule to mimic the sulfur bond adjacent to those carbons in methionine. Since our interest revolves around hydrogen abstraction in a peptide/protein environment, methionine was modeled as being part of a typical backbone. A formyl group extended the N-terminus and the C-terminus was converted into an amide.

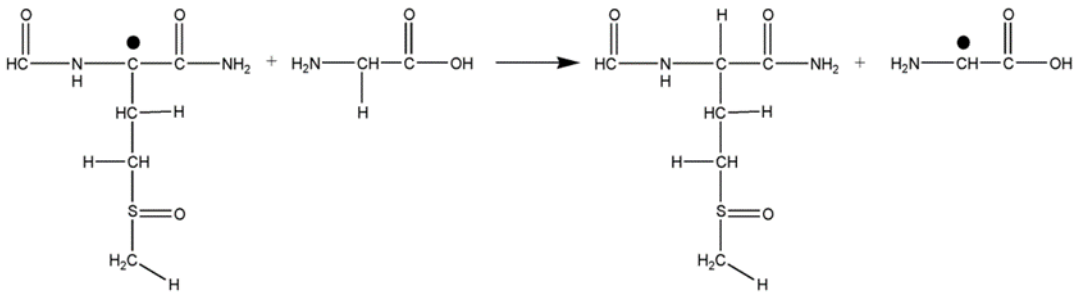

Figure 4.1 Example of BDE calculation of α carbon in oxidized methionine using isodesmic route

4.3 Methionine Oxidation in Amyloid Beta

The PTM investigated in this study is the oxidation of methionine in amyloid beta (Aβ) peptides. Dementia is the major and most devastating disease affecting the elderly in the world, estimated to be around 24 million and is expected to double over the next 3 decades.[3] The leading cause of dementia is believed to be Alzheimer's disease. Deposits of amyloid plaques in the brain composing of amyloid beta (Aβ) peptides are believed to play a major role in disease development. One of the two major hypotheses surrounding the development of the disease is that Aβ exhibits antioxidant activity and is upregulated in response to oxidative stress.[4,5] Additionally, it is believed that the variant of Aβ containing oxidized methionine plays a role in the toxicity.[6,7] As a result, it was of interest to investigate the radical sequestering ability of Aβ 1-40 with oxidized methionine in an attempt to support this hypothesis.

As previously discussed in chapter 3, collisional activation of Aβ 1-40 radical following loss of iodine (radical present) for the +3 and +4 charge states is shown in Figure 4.1. In the +3 charge state (Figure 4.2a), all protons are sequestered. Strikingly, there are very few fragments of any kind observed following collisional activation of the +3 radical.

A few side chain losses are the main fragments observed, along with a very limited amount of backbone fragmentation. Previous work has demonstrated that aromatic residues and serine/threonine are predisposed to favor backbone dissociation, meaning that there are numerous sites where fragmentation would be expected to be favorable (DAE**F**R**H**D**S**G**Y** EV**HH**QKLV**FF** AEDVG**S**NKGA IIGLMVGGVV). The absence of such fragments suggests that Aβ 1-40 is capable of quickly sequestering the radical, making it unavailable to facilitate RDD. Collisional activation of the +4 charge state of Aβ 1-40 results in a series of b ions with virtually no RDD fragments (Figure 4.2b). Mobile proton directed fragmentation becomes the main pathway in the +4 charge state, where again the presence of some RDD fragments would be expected.

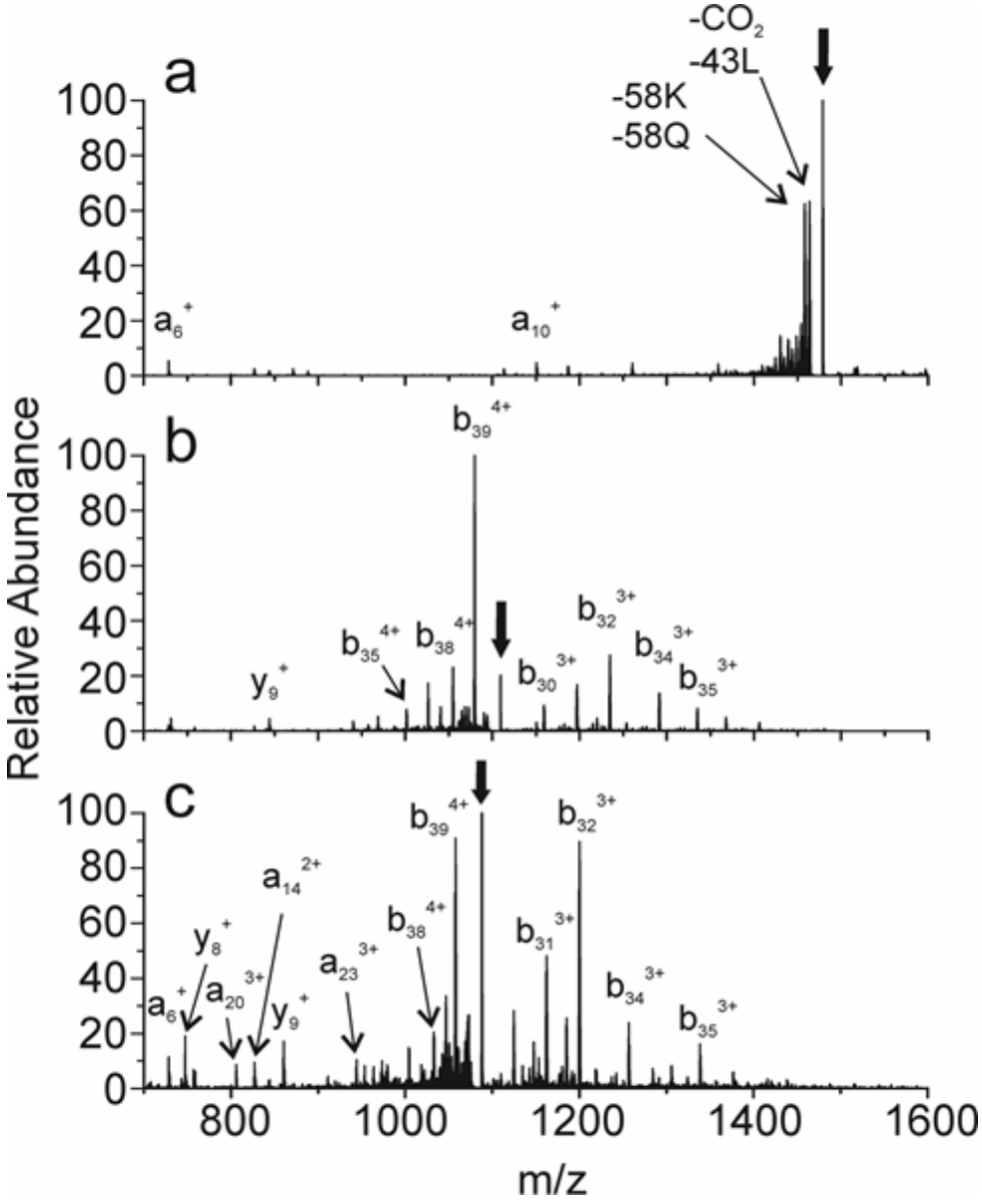

Figure 4.2 CID of iodobenzoyl-Aβ 1-40 radical. a) +3 Charge State and b) +4 Charge state. c) +4 with oxidized methionine.

In Figure 4.2c, collisional activation of the +4 radical of Aβ 1-40 where Met35 was oxidized to a sulfoxide is shown. Several a-type ions appear upon oxidation of methionine. These a-ions are generated at His6, His14, Phe20, and Asp23, which are consistent with favorable backbone RDD sites. The reappearance of RDD fragments following oxidation

of Met35 suggests that it plays an important role in radical sequestration. This idea is supported by previous reports that radicals are stabilized on methionine side chains[8] and that methionine can protect proteins from oxidative stress.[9] In addition, replacing Met35 with Leu showed weakened lipid peroxidation inhibition in previous work.[10]

To support experimental data suggesting weakened radical sequestering capabilities for oxidized methionine, theoretical work was also performed. One of the important factors that influence how a radical interacts with these biomolecules is bond dissociation energies (BDEs).[2] All X-H BDEs in all 20 amino acids has been calculated by Moore and Julian. The effect of methionine oxidation was not investigated. The BDEs of the four different C-H bonds in oxidized methionine were calculated using the isodesmic route as described in experimental section. Table 1 shows the BDE values corresponding to both the oxidized methionine and the native methionine using the isodesmic route using B3LYP/6-31G(d). Native methionine values were taken from Moore and Julian.[2] Comparing the two sets of values, it is apparent that the oxidation results in stabilization of all the C-H bonds in methionine. However, higher BDE values also suggest that radicals are less stable at that position. All BDEs increased in value ranging between 8 and 45 kJ/mol. This does agree with experimental data that has been collected. RDD on Aβ 1-40 and Aβ35 1-40 resulted in different patterns. Initially, it was apparent that Aβ 1-40 was able to sequester the radical and limit any radical activity. However, with the oxidation of methionine, $A\beta_{35}$ 1-40 resulted in additional radical fragments. This suggests that the oxidation of methionine promotes radical activity. Our BDE comparisons agree with such results. Increasing BDE values suggests that the C-H bonds become less likely to break, so radical will want to

migrate to other positions. The γ and δ values are most affected by the oxidation and this could be explained by the presence of oxygen. The γ and δ positions have been known to be stable positions for the radical due to their proximity to a sulfur atom, so addition of oxygen affects sulfurs ability to stabilize those two positions. The drastic increase in α C-H BDE is somewhat of a mystery at this point. It would be expected that both α and β would not change dramatically as they are far away from the oxidation. A possible explanation would be the flexibility of the methionine side chain causing oxygen to hydrogen bond to the backbone. However, the output structure shows no signs of this occurring so this hypothesis was refuted by results. Further evaluation is needed to explain this phenomenon.

	Native Methionine (kJ/mol)	**Oxidized Methionine (kJ/mol)**	**Change in BDE (kJ/mol)**
α C-H	338.9	360.81	21.91
β C-H	392.7	401.5	8.8
γ C-H	351.1	395.79	44.69
δ C-H	369.4	412.89	43.49

Table 4.1 BDEs of native methionine and oxidized methionine using isodesmic route

4.4 Residue Effects on Antioxidant Capacity of Peptides

Of interest is the contribution of individual residues to overall antioxidant capacity of peptides. In chapter 3, a thorough analysis of all antioxidant peptides identified from proteins suggested that no trend existed; no amino acid is significantly more or less abundant. A more tactical approach was preferred and is applied to PHCKRM (a known antioxidant peptide). Collisional activation of radical PHCKRM is shown in Figure 4.3. In Figure 4.3a, the proton is sequestered at the arginine residue, resulting in mainly side chain

losses from methionine and cysteine. With the addition of a mobile proton (the +2 charge state has a mobile proton because the lysine is modified with iodobenzoic acid), PHCKRM fragments to yield primarily b and y ions (Figure 4.3b). The absence of RDD suggests that the radical is sequestered. The trend in PHCKRM is very similar to that observed for Aβ 1-40 and 1-42. These results are consistent with RDD, or lack of RDD, being a useful indicator of antioxidant activity.

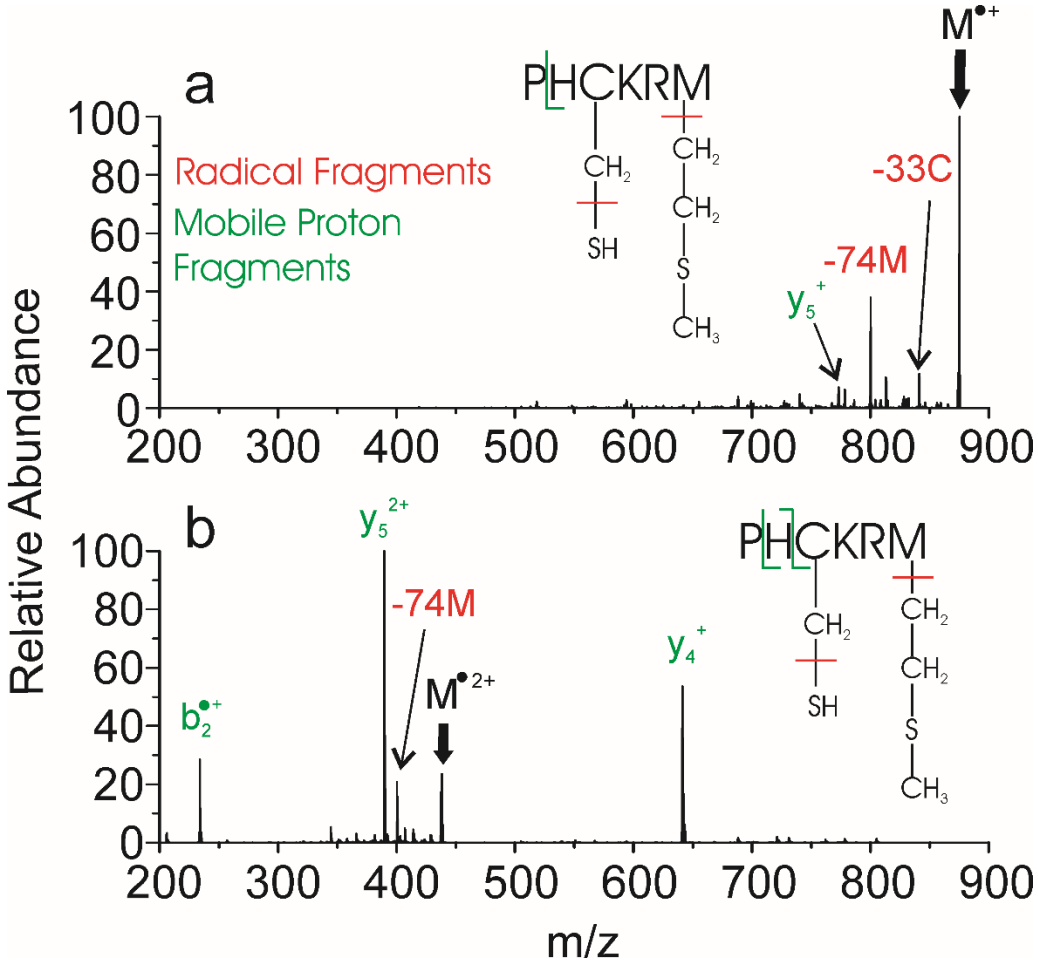

Figure 4.3 RDD of Iodobenzoic Acid modified PHCKRM. a) +1. b) +2.

In order to explore the origin of the antioxidant capacity of PHCKRM, two different experiments are conducted. First, single residue replacement is used. All residues except arginine and lysine are replaced with a glycine (simplest amino acid). Arginine and lysine remain unchanged due to their importance in maintaining consistent charge states with all the peptides and for the modification of lysine with iodobenzoic acid (necessary for RDD experiments). This first approach will give insight into the necessity of each one of the residues (proline, histidine, cysteine, and methionine) in maintaining antioxidant capacity. RSS values for peptides with single residue replacement are displayed in table 4.2. The only sequence that retains antioxidant capacity is PHCRKG, where methionine is replaced with a glycine. Removing proline, histidine, or cysteine all resulted in elimination of the antioxidant capacity of this peptide. This phenomenon can be justified in two different manners. One is that each residue replaced with glycine plays a crucial role in sequestering the radical. This explanation is in direct contradiction with results from the final peptide (PHCKRG), where methionine is replaced. Methionine is a sulfur containing peptide commonly believed to contribute significantly to the antioxidant capacity of peptides. As a result, it is difficult to conclude that a single residue can single handedly debunk the radical sequestering ability of a peptide. The more likely explanation to this observed trend is that these single residue mutations drastically change the structure of the peptide. Altering the structure of the peptide can change the local environment of these residues as well as limit access of the radical to its prime sequestering location.

Sequence	RSS (max)
PHCKRM	5.86
GHCKRM	0.15
PGCKRM	0.04
PHGKRM	0.27
PHCKRG	5.79

Table 4.2 RSS values for single residue replacement in PHCKRM. Residues labelled in red represent location where glycine is used as a replacement.

The second set of experiments serve to support the hypothesis that peptide structure is crucial in maintaining antioxidant capacity and that the presence of certain residues such as cysteine and methionine do not automatically classify peptides as antioxidants. The six residues present in PHCKRM are in all peptides tested, however, are scrambled at random (see table 4.3). The results provide secondary validation to my hypothesis on antioxidant peptides; structure and local environment hold greater value than residue content. PCMHRK is the only sequence where antioxidant capacity is maintained similar to the original peptide. Additional evaluation of these peptides is needed to make a strong conclusion. Theoretical Gas phase structure calculations could be an avenue worth pursuing, giving us information on the proximity of different side chains to each other as it is likely that more than one residue sequester the radical.

Sequence	RSS (max)
PHCKRM	5.86
PMRKCH	0.47
HCKMPR	0.28
PCMHRK	4.30

Table 4.3 RSS values for scrambled sequences of PHCKRM.

4.5 Conclusions

The ultimate goal in biology-related redox chemistry is to tightly control levels of oxidative stress, a situation often closely associated with disease development. Knowledge of the contributions of many antioxidant species is currently missing. My focus is on larger biomolecules such as proteins, which likely have significant contributions. In this chapter, the origin of the antioxidant capacity of peptides is taken a step further. The limiting effect of methionine oxidation on radical sequestering capability is portrayed using amyloid beta peptides and is justified with theoretical calculations of BDEs. Single residue mutation and sequence scrambling of the antioxidant peptide, PHCKRM, reveals the importance of peptide structure (rather than residue content). In addition, the notion that any sulfur-containing peptide can act as an antioxidant is disparaged. The findings discussed in this chapter open new avenues for further investigation of the origin of antioxidant peptides. Detailed dissection of more antioxidant peptides is necessary to make stronger conclusions and are a future direction in this field.

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Chapter 5

Protein Structure Evolution in Liquid DESI as Revealed by Selective Noncovalent Adduct Protein Probing

5.1 Introduction

Properly folded, biologically active proteins have secondary and tertiary structures essential to their role in the cell. A change in protein conformational structure is often associated with a change or loss in protein function. Determination of protein structure has been an area of interest especially given the therapeutic aspect of using small molecules or other conditions to activate, deactivate, or alter the function of proteins with the ultimate goal of treating disease. Traditionally, protein structure has been examined by solid and liquid phase techniques such as crystallography[1], NMR[2], FRET[3], fluorescence[4], and circular dichroism.[5] In recent years, mass spectrometry has been increasingly used to both directly and indirectly probe protein structure. Charge state distributions,[6,7] selective non-covalent adduct protein probing (SNAPP),[8] covalent labeling,[9] H/D exchange,[10] ion mobility,[11] electron capture dissociation (ECD),[12] and radical directed dissociation (RDD)[13] have all been successfully used to examine protein structure and monitor how conformational changes occur in response to external stimuli. These mass spectrometry based techniques all rely on electrospray ionization (ESI), which is particularly important for those methods that examine protein structure in the gas phase. For experiments where protein structure is probed *in vacuo*, the ideal outcome is successful transfer of the solution

phase structure into the gas phase with high fidelity. Unfortunately, conditions which favor this outcome typically reduce sensitivity significantly relative to standard ESI.

Recently, Chen and coworkers reported that liquid DESI could be used to ionize proteins with apparent retention of solution-phase structure and enhanced sensitivity relative to ESI.[14] Liquid DESI, a technique related to solid surface DESI developed by Cooks and coworkers,[15,16] consists of a simple experimental setup (see Figure 5.1) where the sample and electrospray solutions are decoupled. The sample of interest is dissolved in one solution and pushed through a flat, open ended tube at a steady flow rate. A separate spray solvent, typically consisting of 50:50:1 water:methanol:acetic acid, is then electrosprayed and directed by high speed gas flow towards the sample solution tube. Charged droplets emerge from the intersection of the source and sample solutions, eventually generating protonated ions for detection in the mass spectrometer. Importantly, Chen and coworkers demonstrated that although denaturants such as methanol and acetic acid are used in the spray solvent, the protein charge states observed from liquid DESI ionization were similar to those obtained from much more "native" solutions.[17] In comparison, charge state distributions for proteins electrosprayed directly from 50:50:1 water:methanol:acetic acid exhibit significant shifts indicating denaturation of the protein. The apparent lack of denaturation in liquid DESI is of interest because decoupling the spray and sample solvents potentially enables maximization of ion count simultaneously with preservation of protein structure. However, subtle structural changes are difficult to detect by shifts in charge state distribution, suggesting that a more sensitive method may be required to reveal whether smaller changes in structure take place in liquid DESI.

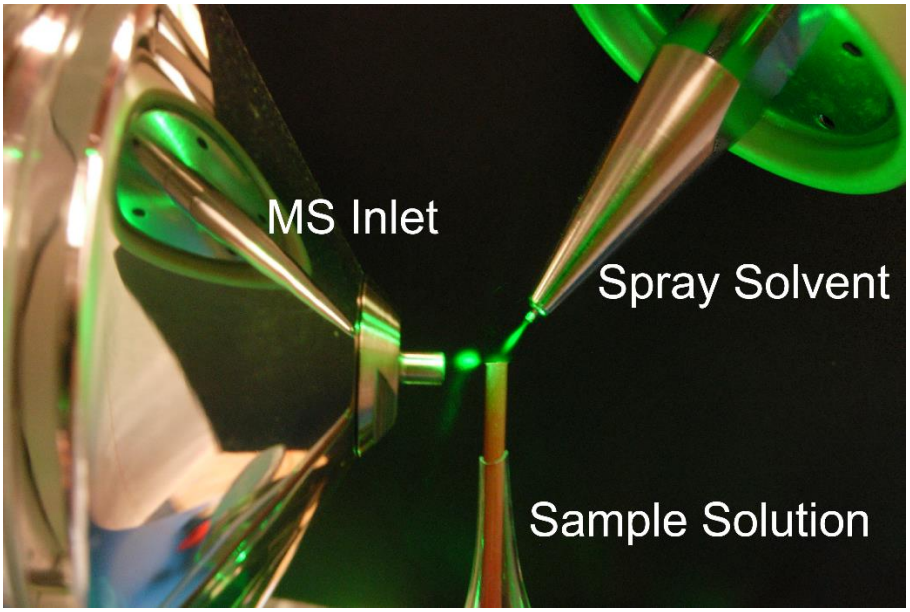

Figure 5.1 Composite image of the liquid DESI source setup. A green laser pointer is used to illuminate the droplet clouds between the ionization source, sample tube, and mass spectrometer inlet.

SNAPP is a simple mass spectrometry based method for investigating protein structure that excels at identifying conformational changes. SNAPP is a comparative method that utilizes non-covalent attachment of probe molecules during electrospray ionization (ESI) to monitor protein structure. SNAPP has been successfully used to reveal the effects of metal ions on the structure of alpha-synuclein,[18] to monitor structural changes induced by single amino acid mutations,[19] and to distinguish small structural differences in highly homologous proteins from different species.[20] 18-crown-6 (18C6) is typically the probe molecule used in SNAPP experiments due to its ability to non-covalently bind to the protonated side chains of lysine and arginine, or to the N-terminus. Side chains which are not buried or sequestered by intramolecular interactions are generally available to bind 18C6. Side chain availability is dictated by protein structure, and changes in protein

conformation lead to different numbers of 18C6 adducts. In a typical experiment, 18C6 is simply added to a protein solution and then electrosprayed directly.

Herein, SNAPP will be utilized to examine the effects of the ionization process on protein structure for both liquid DESI and standard ESI. A methanol induced conformational shift in the protein cytochrome C (Cytc) will be used as a model system to compare the two ionization processes. This system has been previously studied by SNAPP in ESI[8] and is examined here by SNAPP in liquid DESI. The protein structure of myoglobin ionized by liquid DESI from ammonium acetate buffer is examined by SNAPP to evaluate the performance of liquid DESI with additional buffer. The results with both Cytc and myoglobin suggest that liquid DESI is a viable method for examining protein structure. Finally, liquid DESI is used for a new system to study dynamic structural differences in the natively disordered synuclein proteins. Alpha synuclein is the principal component of amyloid fibrils associated with neurodegenerative Parkinson's disease and undergoes a high rate of fibril formation relative to the highly homologous proteins, beta and gamma synuclein. Examination of these three proteins by liquid DESI SNAPP is performed in order to gain insight into the link between fibril formation rate and dynamic protein structure.

5.2 Experimental Details

5.2.1 Materials

Horse heart cytochrome c and myoglobin were purchased from Sigma-Aldrich (St. Louis, MO). Distilled water was purified by a Millipore Direct-Q filtration system before

use. Methanol, acetic acid, and ammonium acetate were purchased from Thermo-Fisher Scientific (Waltham, MA). 18-crown-6 was purchased from Alfa Aesar (Pelham, NH).

5.2.2 ESI and DESI Setup.

All ESI spectra were acquired on a Thermo LTQ mass spectrometer with the standard IonMax™ ESI source supplied with the instrument. Liquid DESI spectra for myoglobin were acquired on a Thermo LTQ mass spectrometer and liquid DESI spectra for cytc were acquired on a Thermo LCQ mass spectrometer. The liquid DESI source was constructed in-house using a partially modified nozzle assembly from an existing Thermo ESI source. The assembly was modified by removing its enclosure and replacing the existing silica capillary (200 µm O.D., 100 µm I.D.) with a larger capillary (240 µm O.D., 100 µm I.D.) in order to increase sheath gas velocity at the spray tip. The spray assembly was properly grounded and attached to a firmly secured aluminum lab jack. The assembly was oriented in such a way that the emitted solvent plume impacted the flat cut end of a securely mounted portion of PEEK tubing (0.0625" O.D., 0.005" I.D.). A syringe pump was used to deliver sample solution through this tubing at 3 µL/min. The samples consisted of 5 µM protein and 100 µM 18C6 (1:20 ratio) in water unless otherwise noted. A second syringe pump was used to pump the spray solvent through the ESI needle at 3 – 6 µL/min. The spray solvent consisted of 50:50:1 water:methanol:acetic acid unless otherwise noted. The nitrogen gas pressure going into the spray assembly was set to 80 psi. A picture of this setup is shown in Figure 5.1. Sheath gas pressure, sample and spray flow rates, relative angles, and distances between the elements of the setup were optimized for ion count. For SNAPP experiments by both ion sources, ionization conditions and ion optic voltages were

initially optimized to improve signal and 18C6 attachment to the protein. These conditions were then kept constant throughout the experiments. The optimal ion optic voltages were not substantially different between the two sources.

5.3 Results and Discussion

Cytc is known to undergo a conformational shift when exposed to increasing amounts of methanol, and is therefore an excellent test subject to examine the effects of ionization in liquid DESI. Figure 5.2 shows fluorescence and circular dichroism spectra for solutions of Cytc and 18C6 as a function of increasing methanol content at neutral pH. Cytc contains a single tryptophan residue which fluoresces at 350 nm when excited at 285 nm. In the native protein structure this amino acid is buried within the hydrophobic core and does not fluoresce.[21] In Figure 5.2a the measured fluorescence of Cytc at 350 nm increases dramatically when the protein is exposed to >60% methanol. The increase in fluorescence indicates a structural rearrangement resulting in the exposure of the tryptophan residue to solvent. The native fluorescence of the amino acid is shown as a control (red line) and is noted to decrease in intensity with increasing methanol content. Figure 5.2b shows the circular dichroism at 222 nm of Cytc under the same solvent conditions. Shifts in circular dichroism at 222 nm are also indicative of change in protein secondary structure. At 50% - 60% methanol, a significant shift towards increased helicity is observed. It is well known that the secondary structures of Cytc and many other proteins undergo conformational changes resulting in increased helicity when exposed to methanol and the changes observed here are in agreement with previous results.[21,22]

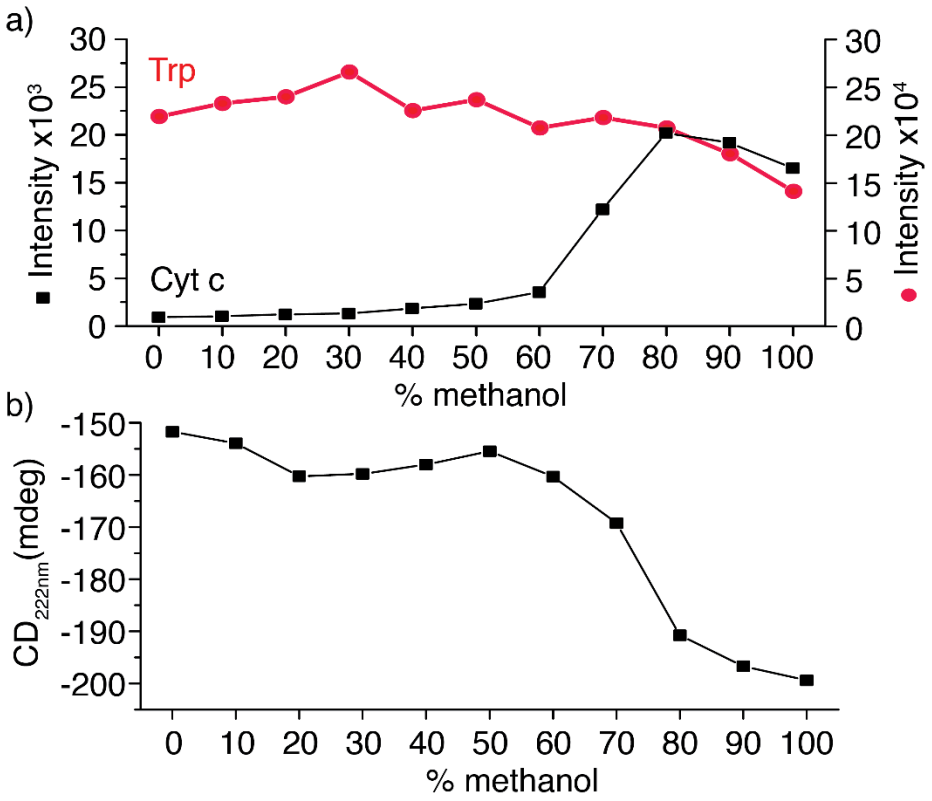

Figure 5.2. a) Fluorescence at 350nm of Cytc and tryptophan and b) circular dichroism at 222nm of Cytc in increasing concentrations of methanol.

SNAPP spectra of Cytc in varying amounts of methanol obtained by ESI and liquid DESI are shown in Figure 5.3. Since SNAPP is a comparison technique, it is only relevant to compare data originating from the same ion source under the same operating conditions, meaning that direct comparison of the ESI and DESI data is not meaningful. Rather, changes among the various DESI spectra can be compared to changes among the ESI spectra. For both liquid DESI and ESI, the number of 18C6 adducts increases substantially for the 50% methanol solution compared to the 0% methanol solution. This is consistent with the structural changes observed in solution in Figure 5.2, although the transition is observed at a somewhat lower percentage of methanol. This is likely due to the stability of

alpha helices in the gas phase, which may shift the balance of forces in favor of helix formation at a slightly lower percentage of methanol. There is not a dramatic shift in charge states for either the liquid DESI or ESI spectra at 50% methanol, although the charge states in both spectra are broadened somewhat. At 90% methanol, the ESI spectrum is very similar to that obtained at 50%; however, the liquid DESI spectrum has shifted notably towards higher charge states. This shift indicates that Cytc in liquid DESI is beginning to unfold at 90% methanol, a transition which is not expected to occur with just addition of methanol. The most likely explanation for this difference is the presence of acid in the liquid DESI spray solution. At lower concentrations of methanol, this acid is not sufficient to lead to denaturation of the protein within the timeframe of the experiment, which corresponds to the several milliseconds required to achieve complete desolvation. After desolvation, the 18C6 adducts are locked in place and further changes to the SNAPP spectra are unlikely. However at 90% methanol, protein denaturation becomes feasible and leads to a partial shift of the charge state distribution.[23] It should be noted that the charge state distribution would be shifted substantially more if the acidic solution were sprayed directly, indicating that the liquid DESI source has still preserved some native structure even under these conditions.

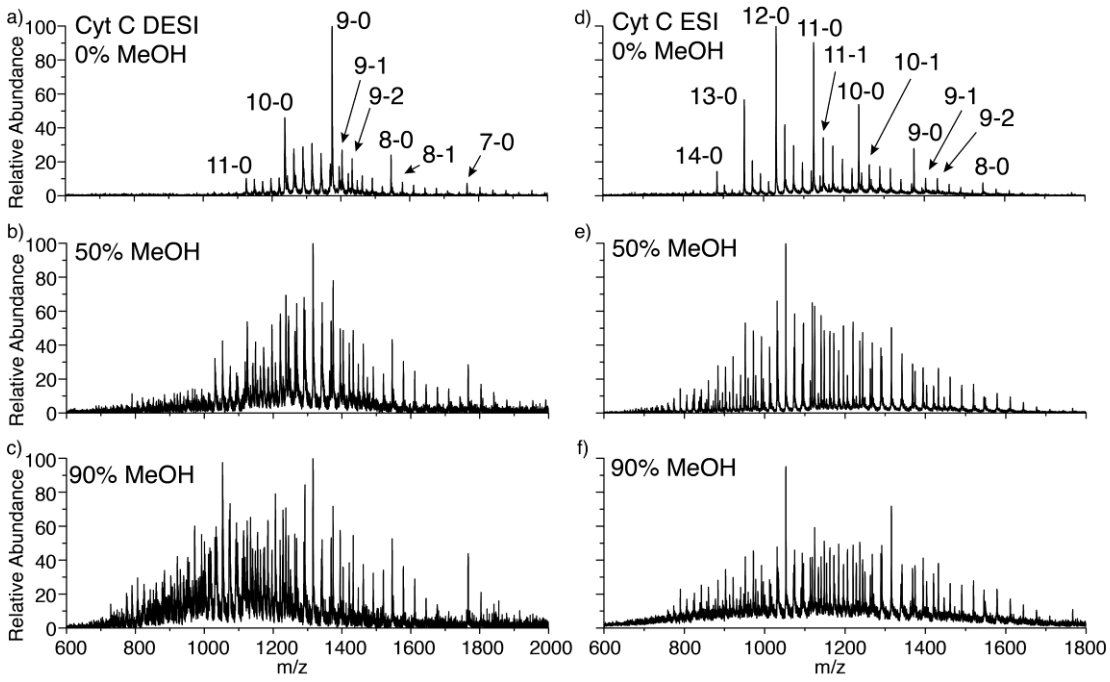

Figure 5.3 Cytc in solutions containing increasing amounts of methanol ionized via (a,b,c) DESI and (d,e,f) ESI. Peaks are identified by protein charge state and number of attached 18C6 molecules i.e. charge state - #18C6.

Buffers are frequently added to electrospray solutions in order to help preserve native structures,[24,25] and could potentially counter the effects of adding acid to the liquid DESI spray solution. Liquid DESI and ESI SNAPP spectra for myoglobin in various concentrations of ammonium acetate buffer are shown in Figure 5.4. When no buffer is present, both liquid DESI and ESI ionize the protein with 18C6 adducts attached, as expected. In ESI a significant amount of apo-myoglobin is observed, which is formed by loss of the noncovalently bound heme during ionization.[26] Heme loss is not prevalent in liquid DESI indicating a gentler ionization process.[17] ESI of myoglobin in the presence of buffer results in complete loss of 18C6 adducts even at the lowest concentration of buffer, as shown in Figure 5.4f. With increasing buffer concentrations, the protein signal in ESI

decreases significantly (Figure 5.4, right). In contrast, 18C6 binding to the myoglobin decreases only slightly in liquid DESI as buffer concentration increases. Even at the maximum concentration of buffer examined, 1000μM, 18C6 adducts are still observable by liquid DESI.

Several competing factors influence the results in these experiments. 18C6 can form noncovalent complexes with any small cation, including ammonium. Given the relative concentrations of buffer and protein in these experiments, competitive binding of 18C6 by ammonium is unavoidable. In ESI, this leads to loss of all myoglobin-18C6 adducts, although the $[18C6+NH_4]^+$ peak becomes quite intense. Another important factor to consider is that there are a finite number of charges associated with each droplet in both ESI and liquid DESI. Therefore, if the number of charges consumed by one species increases, it must be accompanied by a decrease in another. For ESI, it would appear that the addition of buffer leads primarily to generation of $[18C6+NH_4]^+$. However in liquid DESI, 18C6 adducts to protein are still observed at high relative concentrations of buffer. Clearly a different mechanism is operating to generate ions in the case of liquid DESI. Let us consider the differences between the two sources more carefully. In ESI, the initially produced charged droplets go on to produce ions without significant further interaction with other solvents or surfaces. In liquid DESI, the charged droplets impact a surface, combine with another solution, and then depart from the surface. All of these interactions will serve to decrease the charge density of the droplets which eventually go on to form ions in liquid DESI. In terms of the two extremes that are frequently invoked to describe the mechanism of ESI, charge residue and ion evaporation, dilution of charge will strongly

favor the charge residue mechanism. The results suggest that, in ESI, charge partitioning due to ion evaporation of $[18C6+NH_4]^+$ is significant and that insufficient 18C6 remains to form adducts with myoglobin. In liquid DESI, the data suggests that the charge residue mechanism dominates. Given that the protein is much more basic than ammonia, myoglobin is the eventual charge carrier in the shrinking residue droplet and is therefore able to compete for 18C6 adducts.

There are several important conclusions from the data in Figure 5.4 with respect to liquid DESI and preservation of protein structure. One, liquid DESI does not appear to form ions via ion evaporation. Two, the results imply that protein ions formed by the charge residue mechanism are more likely to retain native structures. This observation is important regardless of whether ESI or liquid DESI is to be employed. Three, addition of buffer will likely counteract any denaturation induced by acid from the spray solvent.

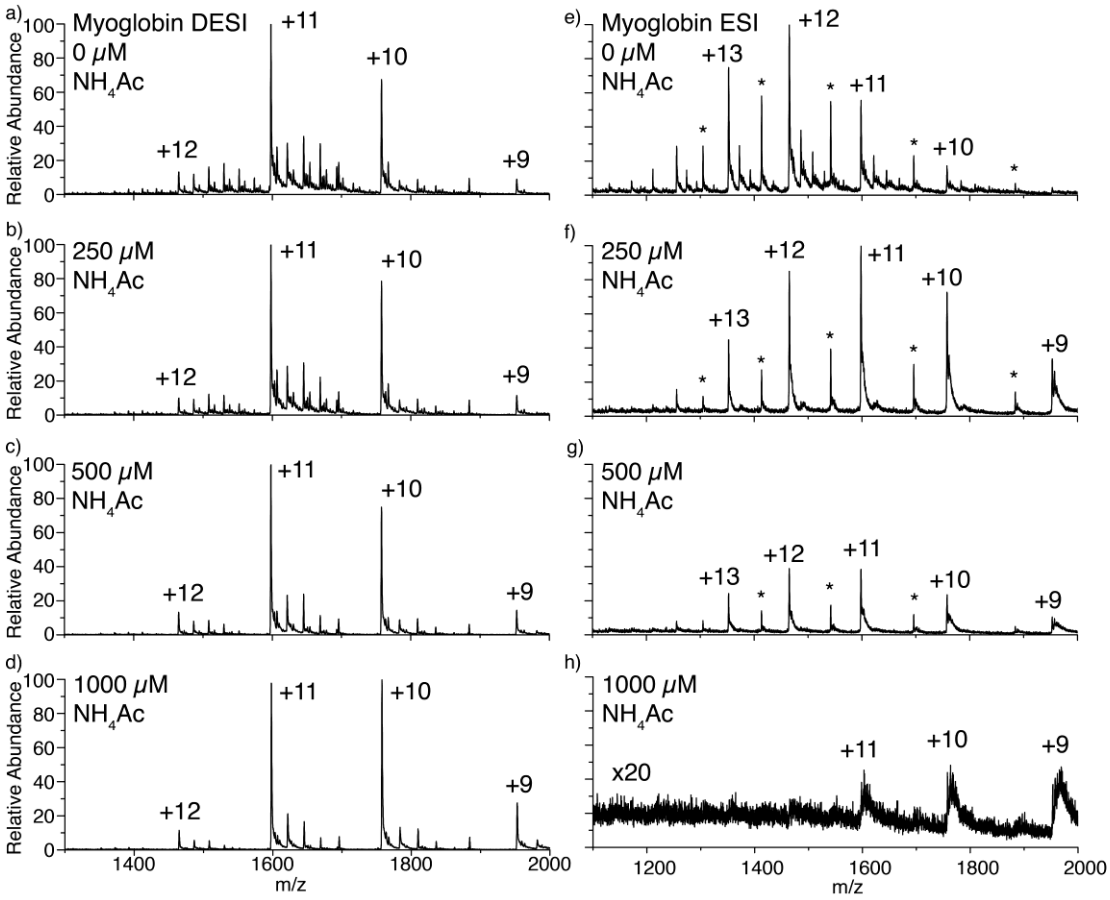

Figure 5.4 Solutions of the protein myoglobin with increasing concentrations of ammonium acetate buffer ionized via (a,b,c,d) DESI and (e,f,g,h) ESI.

Liquid DESI appears to be a viable method for gently ionizing proteins and appears to be well suited for SNAPP experiments. Therefore, we employed liquid DESI SNAPP to examine the natively disordered proteins alpha, beta, and gamma synuclein. All three of the synuclein proteins are natively disordered meaning that they lack tertiary structure and rapidly interconvert between a variety of unique conformations. Alpha synuclein is known to be the principle component of fibril plaques present in Parkinson's disease.[27,28,29] Beta and gamma synuclein, although highly homologous to alpha, are not related to the disease

and form fibrils at significantly slower rates and have been shown to actually inhibit alpha synuclein fibril formation.[30] The disparity in fibril formation rates of the synuclein proteins suggests that transient structural properties may be key to explaining their observed biological activity.

Liquid DESI SNAPP distributions extracted from the raw mass spectra are shown in Figure 5.5 for each of the synucleins in the 14+, 13+, 12+, and 11+ charge states. In all charge states, alpha synuclein binds a significantly larger number of 18C6 molecules compared to both beta and gamma synuclein. This difference cannot be explained by primary sequence alone as these highly homologous proteins contain a similar number of potential 18C6 binding sites; alpha synuclein: 17, beta synuclein: 15, gamma synuclein: 18. Instead, it is likely that alpha synuclein can access a substantially different, perhaps more extended, set of conformations compared to beta and gamma synuclein. This difference in dynamic conformation may be related to the relatively high fibril formation rate of alpha synuclein.

An important factor in the ability of natively disordered proteins to oligomerize and form fibrils is the accessibility of their basic and acidic side chains.[31,32] At physiological pH, a protein with a high rate of fibril formation will contain an equally large number of both positively and negatively charged residues that are solvent accessible and not participating in intramolecular electrostatic interactions. Intermolecular interactions can then cause the protein to form oligomers and eventually fibrils. Binding of 18C6 is a direct indicator of this kind of protonated basic side chains accessibility. Therefore, it can be

inferred that the increase in 18C6 binding to alpha synuclein relative to beta and gamma synuclein is reflective of the observed rates of fibril formation of the three proteins.

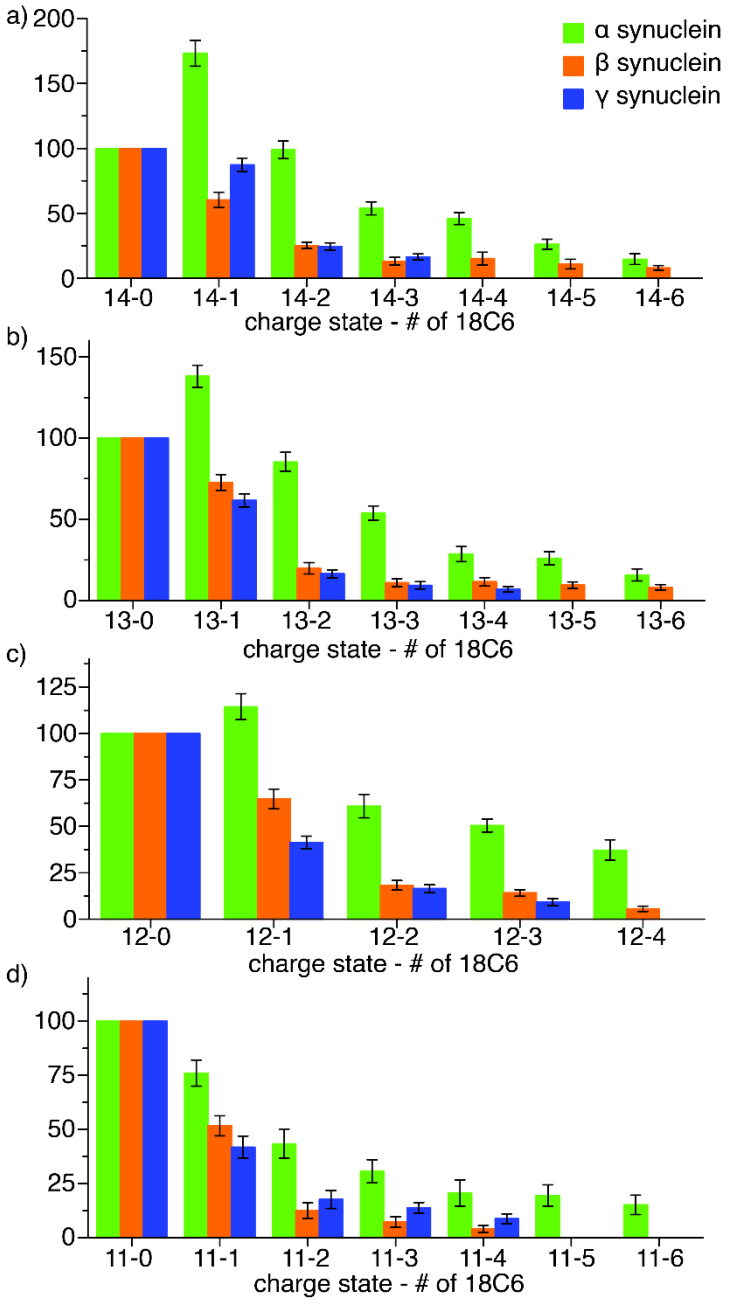

Figure 5.5 SNAPP distributions of alpha, beta, and gamma synucleins in H_2O via DESI.

5.4 Conclusions

The ability of liquid DESI to preserve solution phase protein structures has been examined. Protein conformation changes are observable with SNAPP in liquid DESI in a similar fashion to normal ESI SNAPP. Liquid DESI offers the advantage of higher ionization efficiency due to the use of an acidic spray solvent generally without denaturation of protein structure as would occur when using acidic spray solvent in ESI. In addition, liquid DESI was used to ionize protein in the presence of ammonium acetate buffer thus demonstrating the applicability of liquid DESI SNAPP under conditions that better reflect the cellular environment of proteins. Finally, liquid DESI was used to examine the natively disordered proteins alpha, beta, and gamma synuclein. Of the three proteins, alpha synuclein was shown to have a dynamic structure that bound a relatively large number of 18C6 molecules. The increased accessibility of protonated binding sites is likely related to the predisposition of alpha synuclein to undergo a high rate of fibril formation as compared to the other two synuclein proteins.

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Chapter 6

Reflections on charge state distributions, protein structure, and the mystical mechanism of electrospray ionization

6.1 Introduction

Electrospray ionization (ESI) is great. It can be used to gently transfer just about anything, except perhaps small animals, into the gas phase in an ionized state. [1-3] The full impact that ESI will have on chemistry, biochemistry, biology, medicine, and in other areas has not been fully determined, but will certainly be substantial. The utility and importance of ESI cannot therefore be reasonably disputed; however, ESI is not completely (or perhaps even well) understood. The basics can be inferred: a voltage difference is used to create charged droplets, these droplets fission, evaporate, and eventually yield ions. Exactly how these events occur, particularly at a molecular level, is not known.[4] Similarly, the influence that the electrospray process has on the molecules being ionized is not fully understood. Why not? There are experimental challenges, i.e. the droplets of interest are very small and exist only transiently in an awkward to access environment. Furthermore, methods for confident molecular level characterization of such droplets may not exist. There are also philosophical barriers- we know everything that we need to in order to make ESI useful, so why invest the time and money? Then there is the issue of source diversity, which is rarely addressed in relation to how ESI works. Electrospray itself can be found in numerous variations, all differing in some way with respect to liquid flow rates, gas flow rates, geometries, voltages, ion optics, materials, etc… In addition there are numerous closely

related methods such as nanospray, paperspray, electrosonic spray, and desorption electrospray (DESI), just to mention a few.[5-8] Do all of these variations operate under similar general principals, or are there important differences? The point is, at the moment, we do not know.

One of the interesting phenomena associated with ESI is that molecules are frequently observed in multiple charge states, especially larger molecules such as proteins.[9-12] The collection of charge states observed for a particular molecule under a given set of experimental conditions is referred to as the charge state distribution. Charge state distributions can be influenced by a variety of factors. For example, the addition of organic solvent and acid to an aqueous protein solution will frequently lead to a dramatic broadening and shift of the charge state distribution to lower m/z (higher charge states). In contrast, addition of buffer (such as ammonium acetate) to an aqueous solution frequently favors lower charge states and narrower distributions. These observations are presumably linked to protein structure, where unfolded proteins are able to accommodate more charge which leads to higher and broader charge state distributions, while folded proteins typically exhibit lower and narrower charge state distributions.

It is clear from ion mobility experiments that in the gas phase higher charge states do correspond to more unfolded proteins while lower charge states represent more compact structures.[13,14] It is therefore often inferred that such structures were present in the original solutions. It is entirely possible that this is the case; however, it is also possible that proteins may unfold during the process of ESI itself. This issue will be discussed further below, but it is sufficient for the moment to say that our ignorance about the mechanistic process of

ESI makes it dangerous to assume that structures present in solution are always (or perhaps even can be) directly transferred into the gas phase. This issue is particularly important given the increasing interest in the area of gas phase structural biology, where gas phase methods, including structural characterization *in vacuo*, are employed to examine protein structure. Proponents for this field argue that proteins can be transferred with structural fidelity into the gas phase and meaningfully examined within that environment, while others argue that the gas phase is a wildly unnatural medium that in no way mimics aqueous or cellular conditions and that examination of protein structure in the gas phase is a waste of time.

6.2 Nonaqueous Environments

Let us take a brief aside to examine this issue from another angle. Oddly enough, insight into the dilemma of protein structure in the gas phase can be acquired by examination of enzymatic catalysis in organic solvents, which is actually a fairly established field and has been reviewed.[15,16] Organic solvents (more specifically anhydrous organic solvents –at least to the extent which is possible) are not typically associated with biology or proteins, but are more comparable to the gas phase than water in many respects. Interestingly, even though most proteins are not soluble in organic solvents, they are frequently employed for catalytic transformations in organics despite this shortcoming. In fact, several applications in industry have been used to generate kilograms of product in this fashion.[15] Instinctively, one might assume that proteins would be denatured in organic solvents; however, experimental characterization, though limited, has suggested that this is not the case.[17,18] Furthermore, the ultimate test for structural fidelity with proteins is

generally considered to be functionality, which can obviously be retained and utilized in organic media. It is therefore reasonable to conclude that sufficient core structure is retained in organic solvents to allow for the observed catalysis.

Many of these experiments are carried out by lyophilization of the protein, which is then dispersed in an organic solvent by vigorous and constant agitation. Importantly, the conditions under which the protein is lyophilized can have significant impact on catalytic activity. If the protein is denatured prior to or during lyophilization, no catalysis is observed. This further supports retention of protein structure as being important for successful catalysis. In addition, experiments have shown that the addition of salts or crown ethers to solution prior to lyophilization greatly enhances catalytic activity.[19,20] There are multiple potential explanations for this observation. For example, it has been suggested that proteins become very rigid in organic solvents due to loss of the dynamic hydrogen bonding environment afforded by water. If this rigidity concept is correct, then salts or crown ethers may restore a degree of flexibility to the enzymes (which is necessary for catalysis). Another (not mutually incompatible) way of looking at this issue relates to charge solvation. It is likely that counterions or crown ethers enhance solvation of charged groups on the protein surface during and after lyophilization, which helps mitigate loss of structure due to Coulombic forces. Interestingly, solvation of the charge groups by retention of water itself is not a viable option. Addition of water, even in small quantities, is not beneficial for catalysis and leads to rapid denaturation.[15] When these observations are considered together, it would appear that proteins retain structure in organic media due to kinetic trapping. In other words, although native structures are likely not the lowest

energy states in organic solvents, the barriers to rearrangement to the lowest energy structures are sufficiently large to prevent this from occurring. The addition of water likely lowers the kinetic barriers, enabling transitions between different structural states.

To sum up, there are a few important take-home points. One, proteins can be kinetically trapped in their native state in nonaqueous environments and retain enzymatic activity under certain conditions. The theoretical possibility for gas phase structural biology is therefore possible. Two, water actually facilitates protein unfolding in denaturing environments. As a result, many of the water/organic solution combinations frequently employed in ESI are likely to be highly denaturing (ironically because of the combination, not just the organic). Three, lack of charge solvation can interfere with kinetic trapping of native states. The gas phase is even worse at charge solvation than organic solvents, making this a primary obstacle which will interfere with retention of native or native-like structures.

6.3 Timescale of Protein Denaturation in ESI

One issue which is often overlooked in relation to protein structure and ESI is time.[4,21] Proteins are large molecules, and require a certain amount of time to undergo structural transitions. The exact amount of time is dependent on the protein and the particular conditions which are present. Can proteins unfold within the timescale of ESI? For that matter, what is the relevant timescale? In most instruments, the time from droplet generation to naked ions will typically be in the millisecond timeframe, but at what point does the environment within a charged droplet appear to be substantially different to a protein? -Immediately? -After droplet fission?

Recent experiments by the Williams group offer compelling evidence that protein unfolding can occur within the timescale of ESI.[22] In experiments designed to elucidate the mechanism of supercharging (a phenomenon where the charge state distribution is pushed to very low m/z), proteins were demonstrated to be folded in bulk solution, yet unfolded in the gas phase. To demonstrate this, various amounts of DMSO was added to aqueous solutions that were examined by ESI and circular dichroism (CD). The onset of unfolding was observed at a much smaller percentage of DMSO in the ESI experiments. DMSO has a much higher boiling point than water and only acts as a denaturant at high concentrations.[23] It was postulated that the concentration of DMSO must increase during droplet evaporation in ESI. If this occurred, the bulk solution with a low concentration of DMSO would be non-denaturing (as confirmed by CD) while droplets generated by ESI would become more denaturing, leading to protein unfolding and a shift in charge state distribution.

We decided to test this hypothesis with a very simple experiment. A non-denaturing solution of water and DMSO (90/10) containing myoglobin was reduced in volume by sequential partial lyophilization. Indeed, as shown by CD in Figure 6.1, the protein unfolded after the volume of the solution was significantly reduced, presumably due to concentration of DMSO. In fact, if it is assumed that only water is lost, then the percent DMSO where denaturation occurs is in good agreement with previous experiments.[22,23] The point here is that the solution composition in ESI droplets is most likely evolving with time as evaporation and ionization occur. If one were to attempt to evaluate the amount of

organic solvent that causes denaturation of a protein using ESI, these considerations would need to be taken into account.

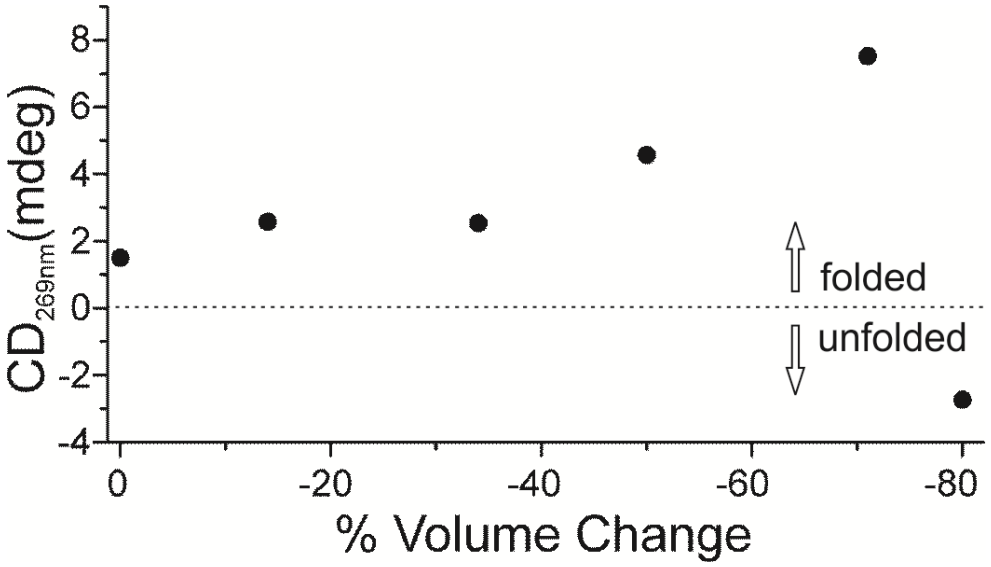

Figure 6.1 Circular dichroism at 269nm as a function of reduced volume following sequential lyophilization of 20μM myoglobin initially in 90/10 water/ DMSO. Unfolding occurs when the solution would hit ~50% DMSO (assuming only marginal DMSO loss).

Do proteins always unfold or otherwise undergo structural rearrangement within the timescale of ESI? This is a much more difficult question to answer, as proteins which retain compact structures may not represent native structures. For example, the addition of some acids to aqueous solutions can cause apparent charge state reduction and compaction, though the equilibrium has not been shifted towards the native state by such conditions.[24] However, there are experiments which demonstrate that mildly denaturing conditions may not be sufficient to enable protein unfolding within the timescale of ESI. Liquid desorption electrospray ionization, or liquid DESI, developed by the Chen group,[25] can be used to introduce the analyte of interest independently from the ionization solution as shown in Figure 6.2a. For example, an aqueous myoglobin solution can be intersected with an

ionized plume of water/methanol/acetic acid (50/50/1). When this is done with 2:1 (spray/sample) flow rates, the observed protein charge state distribution is very similar to that obtained by electrospraying the aqueous solution, as shown in Figure 6.2b. In fact, if anything the aqueous ESI spectrum (inset in Figure 6.2b) appears to be slightly more denatured than the liquid DESI spectrum. However, if a mixture containing proportional volumes of the spray and sample solutions is mixed and then directly electrosprayed, significant shifting of the charge state distribution is observed, as shown in Figure 6.2c. This suggests that the solvent mixture is denaturing in both cases, but that the timescale is too short for the protein to unfold in the liquid DESI configuration. If the proportion of water in the final mixture is reduced by decreasing the flow rate of the sample, protein unfolding can be observed in liquid DESI as well. These results suggest that protein unfolding can take place within the timeframe of ESI, but also that such unfolding is dependent on the severity of denaturing conditions. It should also be mentioned that due to the relative boiling points of water and methanol, in contrast to the DMSO experiments described above, the amount of organic solvent present will be decreasing with time when methanol is the organic component.

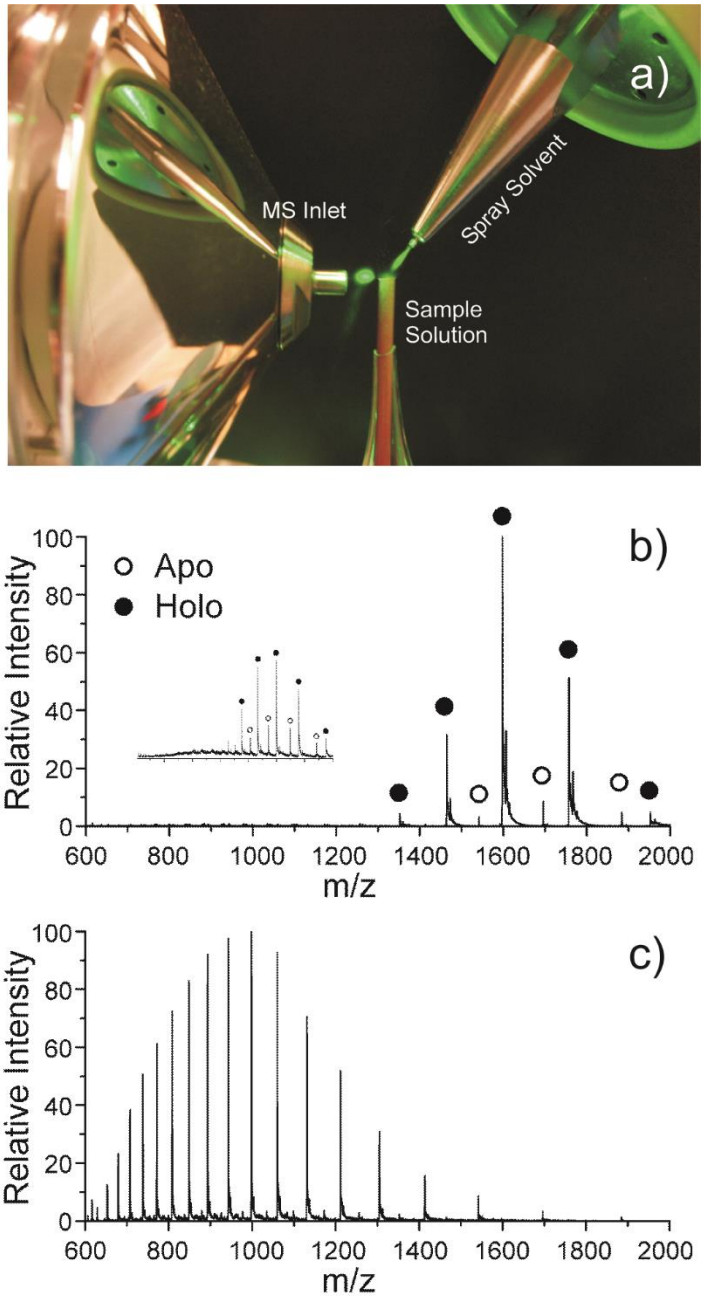

Figure 6.2 a) Photo of liquid DESI setup. A green laser was used to illuminate the initial and reflected droplet plumes. The sample is infused through the peek tubing in the center, while voltage is only applied to the spray solvent tip. The spray solvent is 50/50/1 water/MeOH/acetic acid and injected at twice the flow rate of the sample. b) Liquid DESI spectrum of myoglobin in water, which is very similar to that obtained by ESI of myoglobin from water (see inset of identical m/z range). c) Direct ESI of a solution corresponding to a mixture of the sample and spray solutions in b), demonstrating that indeed the mixture is denaturing.

6.4 Conclusions and Future Outlook

Although we may not know everything about ESI that we would like to, it is still possible to use information that we do have to steer future endeavors. If protein structure is denatured in solution, it is unlikely that the process of ESI will favor or allow refolding. Thus care should be taken to prepare samples in solvents which do not cause the protein to denature, if one desires to obtain information about the protein structure. It should also be taken into account that changes to the solvent composition may occur during ESI, and the effect that these changes might have on the folding state of the protein should be considered. Proteins can be kinetically trapped in their native state in very non-native conditions; however, insufficient charge solvation is a primary driving force which can interfere with or prevent kinetic trapping. It should be mentioned that the importance of charge solvation will scale inversely with the size of the protein. This can be rationalized by the fact that charged groups are almost always present at the surface of a protein. Proteins are almost all roughly globular in shape, meaning that uncharged stabilizing interactions will increase in proportion to volume while charged interactions will scale with surface area. As protein size increases, the volume scales according to $\sim r^3$ while the surface area increases by $\sim r^2$. Thus, for larger proteins, the fractional abundance of charged interactions becomes smaller and therefore represents a weaker driving force for structural rearrangement. This rationale assumes that internally stabilizing interactions, i.e. hydrogen bonds, Van der Waals interactions, etc.., maintain approximately similar relative contributions as protein size increases. Larger proteins should also fold and unfold more slowly, which should also favor kinetic trapping. Regardless of protein size, thermal

excitation post (or in the final stages of) desolvation is highly likely to overcome kinetic trapping and should be avoided. Similarly, protein structure should be examined on the shortest timescale possible.

A potential approach to reduce structural rearrangement due to insufficient charge solvation would be to provide partial solvation, similar to what is achieved by adding salts and crown ethers to the organic catalysis experiments. One possibility would be to examine proteins which are still partially solvated by water molecules; however, since water enables denaturation of proteins in organic solvents, this may have the opposite of the desired effect. Ironically, partially hydrated proteins may be less representative of solution phase structures than fully desolvated proteins because sustained partial hydration would likely facilitate structural rearrangement. Buffers may provide a better solution. The addition of buffers such as ammonium acetate likely reduces Coulombic effects up until the point that the buffer is lost in the gas phase (assuming that it is lost). Perhaps this is sufficient to provide for kinetic trapping for some proteins. Interestingly, crown ethers (particularly 18C6, which was found to be most effective in organic catalysis) have already found utility in variety of mass spectrometry experiments, but the potential of crowns as partial solvation agents has not been fully explored. These experiments are currently underway in several labs, so we may have more information on this exciting idea shortly. Finally, if the organic catalysis work is correct, then perhaps the best hope for retention of protein structure is to electrospray proteins directly out of organic solvents. Of course, that will require clever solutions to the problems of insolubility, among other things.

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Chapter 7

Concluding Remarks

Mass spectrometry has become a very powerful tool in research focused on protein structure and characterization. Method development for protein studies is continuously in demand. The overarching theme of this dissertation is to develop novel mass spectrometry-based methods with applications in biology, specifically protein biochemistry. A detailed analysis of radical directed dissociation (RDD) in peptides shows the capability to obtain information on radical sequestration. The ability of peptides to limit radical damage in the gas phase correlates well with solution phase antioxidant capacity. The facile and quick nature of RDD-MS experiments is unrivaled for the identification of antioxidant peptides. RDD-MS is used for the identification of previously known antioxidant peptides and extended to identify new ones. The properties/characteristics contributing to the ability of peptides to act as antioxidants is a gray area. In order to better understand this phenomenon, a much larger data set is required. Sixteen new antioxidant peptides from five different proteins (unique genomes) are identified and analyzed in detail. My analysis shows no significant trend among those peptides. Expanding the database of antioxidant peptides could still produce interesting results and would be a look at what the future holds. The RDD technique developed in chapter 2 is certainly of use in this scenario as large groups of peptides can be analyzed simultaneously in a single LC-MS run. One of the great assets of MS when studying proteins, is the ability to apply techniques in a high throughput manner. This was not investigated in this dissertation and is a possible direction for future

experiments. Pushing RDD-MS to its maximum capacity and investigating hundreds of peptides at once would be a remarkable and desirable feat. The identification of antioxidant peptides in food sources such as fish is a common area of investigation among food chemistry groups. Applying RDD-MS in a high-throughput manner on proteins extracted from food sources would speed up the process of identification by at least tenfold.

A new and exciting field in drug development is using the body's own protective mechanisms to fight against disease. Could naturally occurring antioxidant proteins/peptides be added as a possible therapeutic in disorders where oxidative stress is the culprit? This would be the end result and ultimate goal. A better understanding of redox homeostasis will be required. Reactive species will have to be accurately quantified. In addition, the quantity and contributions of all antioxidants in the system should be determined. While fundamental studies remain important and necessary, testing the feasibility of antioxidant proteins as a therapeutic is the next logical step. In-vitro and in-vivo studies can give a reasonable prediction of the effective nature of antioxidant proteins/peptides in inhibiting oxidative stress and a potential future direction for this project.

The continuous progress of MS in utility for protein characterization sparks an interest in improving "native" conditions. Electrospray ionization (ESI) is the most common method used to ionize proteins for MS detection. Chen and coworkers introduced liquid-DESI which is also capable of maintaining relatively "native" conditions with better signal. There is much debate concerning the mechanism of electrospray and maintaining "native" protein structure. The two hypotheses are the charge reduction mechanism and ion

evaporation. With the application of liquid DESI in combination with SNAPP, we were able to elucidate significant differences. Results in chapter 3 show that liquid DESI likely utilizes the charge reduction mechanism (CRM) where droplets shrink in size until the Rayleigh limit is reached. Another important discovery was that proteins ionized via the CRM were likely to retain more "native" structures. A better understanding of the electrospray mechanisms of ESI and liquid DESI help tailor protein characterization studies where native conditions are vital. In addition, increased knowledge on the fundamental aspects of electrospray can open up new areas of investigation in the development of ionization sources.