ABSTRACT

HU, YUZHE. Application of Hydrogen-Deuterium Exchange/Mass Spectrometry for Determination of Cytochrome c Orientation on SAMs. (Advised by Professor Edmond F. Bowden).

Progress towards the development of hydrogen-deuterium exchange/mass spectrometry (HDX/MS) methodology for determination of the orientation of adsorbed cytochrome c on COOH-terminated self-assembled monolayers (SAMs) is described. The premise of the approach is that the protected side of adsorbed cytochrome c will experience less deuteration compared to the solution state, a form of differential modification that can be characterized using peptide mapping. To obtain adequate amounts of adsorbed cytochrome for analysis, C_{10}COOH SAMs were prepared on gold particles having a minimum specific surface area of 69 cm^2/gram. After adsorption and exposure to D_2O, cytochrome c can be desorbed, enzymatically digested, and analyzed using ESI/LC/MS. Minimizing the deleterious extent of the deuterium-to-hydrogen back reaction requires low pH, low temperature, and sample handling times as short as possible. Towards this end, it was shown that the pH 2.4 formic acid solution used to quench the back exchange reaction was also effective in desorbing cytochrome c, thus efficiently combining two procedures into a single step. The extent of desorption was ~90% at 2-3 min exposure and ~99% at >5 min exposure. Peptide mapping of native horse heart cytochrome c was performed using 1:1(mol:mol) peptic digestion at pH= 2.4 for 5 minutes duration. These conditions enabled determination of 78% of the amino acid sequence. The unidentified part of the amino acid sequence, positions 1-21, contains the heme group, which is covalently attached to Cys14, Cys17, and His 18. Peptide mapping of deuterated cytochrome c was not successful due to experimental limitations that precluded adequate control of deuterium back exchange. However, using intact protein mass

analysis, it was shown that adsorbed cytochrome c exhibits a 23% reduction in overall deuteration extent compared to solution cytochrome c. This finding offers strong support for the premise that backbone amide groups located near the binding site are 'protected' with respect to HD exchange. In order to fully validate this premise and delineate the binding site and orientation of cytochrome c, detailed peptide mapping of deuterated samples will be needed. This in turn will require the use of automated HDX/LC/MS instrumentation for future experimentation.

Application of Hydrogen-Deuterium Exchange/Mass Spectrometry for Determination of Cytochrome c Orientation on SAMs

by
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DEDICATION

This work is dedicated to my loving family,

Dad, Mom and Xiaojing

Thank you for all your supporting and encouragement!

BIOGRAPHY

Yuzhe Hu was born in Zhejiang, China on June 17, 1989 to Zuyao and Xiaohong and she is the only child in her family. She grew up in Hangzhou, one of the most beautiful cities in China and spent most of her childhood near the famous city lake--West Lake. In 2007, she attended Zhejiang University majoring in pharmaceutical science. Her enthusiasm and interest in pharmaceutical industry was strengthened during the college study and she decided to pursue a higher degree in the analytical chemistry field. In her senior year, she was enrolled in the 3+3 honor graduate exchange program cooperated by Zhejiang University and North Carolina State University. In 2010 fall, she started her journey in graduate school at NC State and joined Dr. Bowden's research group. She was first experiencing the different American culture after she came to the United States and she was very excited to make a lot of friends in Raleigh. Thanks to all the friends and professors, she was able to adapt to the new environment and improve her English speaking quickly.

During her first year in graduate school, she also became interested in the biotech manufacturing and she decided to pursue a BTEC minor along with the Chemistry major. Near her completion of the MS program, she got a chance to have hands-on experience in the biotech industry working as a research Co-Op at Genentech Headquarter in San Francisco, where she was evolved in the research activities of protein analytical chemistry department and successfully applied the analytical techniques in new anti-cancer medicine process development.

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First of all, I would like to thank my advisor Dr. Edmond Bowden for guiding me and for helping me throughout this process. Thank you so much for inspiring me in the experimental design and for teaching me to think as a scientist. I have learned so much from you. Thank you!

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CHAPTER 1 BACKGROUND AND INTRODUCTION

1.1. Overview and Significance of Research

Protein orientation at solid/aqueous interfaces is critical to a number of scientific research areas including enzyme immobilization [1], biomaterials [2], biosensors [3] bioseparations [4] and protein electrochemistry [5,6]. The present study aims to evaluate the feasibility of a general methodology for characterizing protein interactions and orientation at solid/aqueous interfaces using hydrogen-deuterium exchange (HDX) coupled with mass spectrometry (MS). MS is a fast, sensitive, high-throughput methodology that has been successfully used for analysis of weak non-covalent protein-protein complexes [7], which can serve as a model for adsorbed proteins, i.e., protein-electrode complexes.

Our interest in pursuing development of this type of methodology stems from successful prior applications of HDX for characterizing protein-protein and protein-ligand interactions. Understanding these types of interactions is important for various aspects of bioanalytical chemistry. For example, studies of antibody-antigen binding are essential in the initial stages of assay development for drug discovery, for biomarker discovery, and rational drug or vaccine design [8-9]. To understand the mechanism of action of therapeutic antibodies, it is critical to identify the residues involved in this interaction, and in turn, to characterize the antigen-antibody structure/function relationship [10]. Differential HDX has recently been shown to be an effective means for the characterization of ligand binding and epitope mapping [11]. Application of HDX-MS to membrane proteins such as G-protein coupled receptor has also been successfully demonstrated by Zhang *et al*. [12]. Although HDX-FTIR has been applied to study the adsorption behavior of BSA monolayers on gold surfaces by

Smith *et al* [13], the application of HDX-MS in the characterization of protein-surface interactions has been very limited.

The inspiration for employing HDX-MS to characterize protein interactions at solid/aqueous surfaces originates from a previous set of experiments in Bowden's group, in which the binding domain of adsorbed horse heart cytochrome c (HHC) on a self-assembled monolayer (SAM) was delineated using a method based on differential modification of surface amino acids [6]. In that work, the protein was modified in the adsorbed state via *in situ* methylation of surface lysines using a reductive Schiff-base reaction, desorbed, digested using trypsin, and peptide mapped using LC/MS. Results were compared to solution cytochrome c (the reference state) that had been similarly modified, digested, and analyzed by LC/MS. Lysines involved in adsorptive binding were predicted to be less reactive than when full exposed in the solution phase, thus giving rise to different extents of modification. From differential modification results, a binding domain on the surface of cytochrome c was identified and an orientation was proposed that featured a heme tilt angle of approximately 35-40° with respect to the SAM substrate surface [6]. Differential lysine methylation does, however, have some limitations. Perhaps the lack of general applicability to other proteins is its most significant drawback. Horse heart cytochrome c is a lysine-rich protein, with 21 lysines dispersed fairly evenly over the entire surface. Whereas lysines thus comprise 20% of the amino acid content of cytochrome c, the natural occurrence of lysines across all proteins is only 5.8%. Thus, compared to cytochrome c, one should expect inferior resolution when using the same method to delineate protein binding domains and determine the orientation. Not only would fewer lysines be available for modification on most proteins,

but tryptic digestion would give rise to longer peptides. Using chemical modification of surface lysines (or other surface amino acids), one must also consider the possibility that protein binding properties might be altered to some degree depending on the modifier structure, location, and extent of modification.

The motivation for this thesis work was, therefore, to evaluate the feasibility of using HDX/LC/MS to determine the orientation of cytochrome c adsorbed on SAM/Au substrates and to evaluate the universality of HDX for characterization of protein-surface interactions. In this approach, differential protein modification would again be the basis of the determination, but with replacement of specific amino acid labeling by non-specific backbone amide labeling.

1.2. Hydrogen/Deuterium Exchange-Mass Spectrometry

1.2.1. HDX Principle

Hydrogen deuterium exchange coupled to mass spectrometry (MS) is widely used to study the conformational changes and structural aspects of individual proteins or protein complexes [14]. Exposure of a protein to D_2O can induce rapid amide H→D exchange in regions lacking stable hydrogen-bonding whereas protein binding regions or sites are much more 'protected' from HDX [1]. The exchange of amide protons for deuterons leads to a mass increase that is detectable in MS and the differential isotopic modification provides structural information for the given protein. The universal nature of HDX makes this technique, in theory, applicable for characterization of almost any type of protein interaction. Intact protein mass analysis is a straightforward approach for characterizing global changes in protein structure as a result of HDX [15]. Peptide mapping using proteolytic digestion, on

the other hand, can measure mass shifts for individual protein segments which provides detailed conformational information.

Hydrogen atoms in proteins are classified into three types based on HD exchange kinetics, as shown in Figure 1.1. Type I hydrogen atoms are located on certain side chain functional groups like –COOH or –CONH; they exhibit fast reversible exchange under all buffer conditions. Type III hydrogen atoms are carbon-bound atoms such as aromatic and aliphatic H atoms that show no exchange over the time frame of a typical HDX-MS experiment. Type II hydrogen atoms are the basis of HDX methodology. These are the backbone amide hydrogens whose intermediate exchange rates can be controlled by solution conditions (pH, temp), making them useful for studying protein stability and

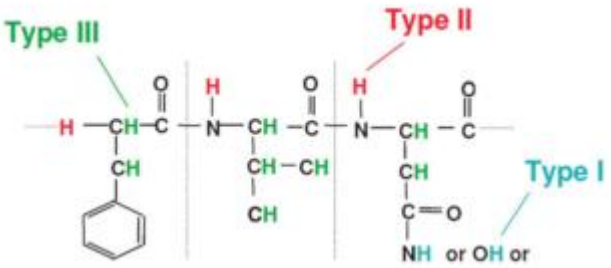

Figure 1.1 Three types of hydrogen atoms found in polypeptides. Type I: located on certain side chain functional groups such as amides and alcohols; Type II: amide backbone hydrogen; Type III: carbon-bound hydrogen. [14]

interactions [15,16]. Lowering pH and temperature from neutral pH and physiological or ambient temperatures will cause the exchange rate to decrease, with a minimum rate

observed in the pH range between 2 and 3 [16]. In HDX experiments, type II hydrogen-deuterium exchange is commonly quenched by lowering the temperature to 0°C and the pH to 2.5 [17].

Back-exchange of deuterium labels (D→H) is a persistent problem which degrades HDX information. Many factors during the digestion/separation/analysis processes can influence back exchange, such as solution pH, temperature, flow rates, and system volume [18]. Decreasing sample handling time to the absolute minimum and controlling pH and temperature are critical for limiting the impact of the back exchange reaction during the analytical process.

1.2.2. Electrospray Ionization

Electrospray ionization (ESI) is a nondestructive 'soft' ionization technique that can preserve non-covalent interactions in the gas phase. It has been widely applied to the study of large biomolecules since the mid-1980s [19]. One of its key features is the ability to produce multiply-charged ions, which allows large molecules to be detected in mass spectrometers having more limited m/z ranges [20]. ESI can be coupled with liquid chromatography, which assists the transfer of ions from solution into the gas phase before the ions are subjected to mass spectrometric analysis [21].

The principle of ESI is illustrated in Figure 1.2 [22]. A high voltage, usually 2-5 kV, is applied to the ESI needle. Analyte solution mixed with ESI solution is pumped through the needle at a slow flow rate. Due to the electric field gradient between the needle and the counter electrode, a Taylor cone forms at the needle tip with an excess of positive charge

accumulating on its surface. When the solution reaches the point at which the Coulombic force due to the surface charge is equal to the surface tension of the solution, a fine spray of charged droplets are dispersed from the tip of the Taylor cone [23]. As the droplets move towards the mass analyzer, solvent evaporation takes place and charged analyte molecules M^{n+} are produced that can be analyzed on the basis of their mass-to-charge ratios.

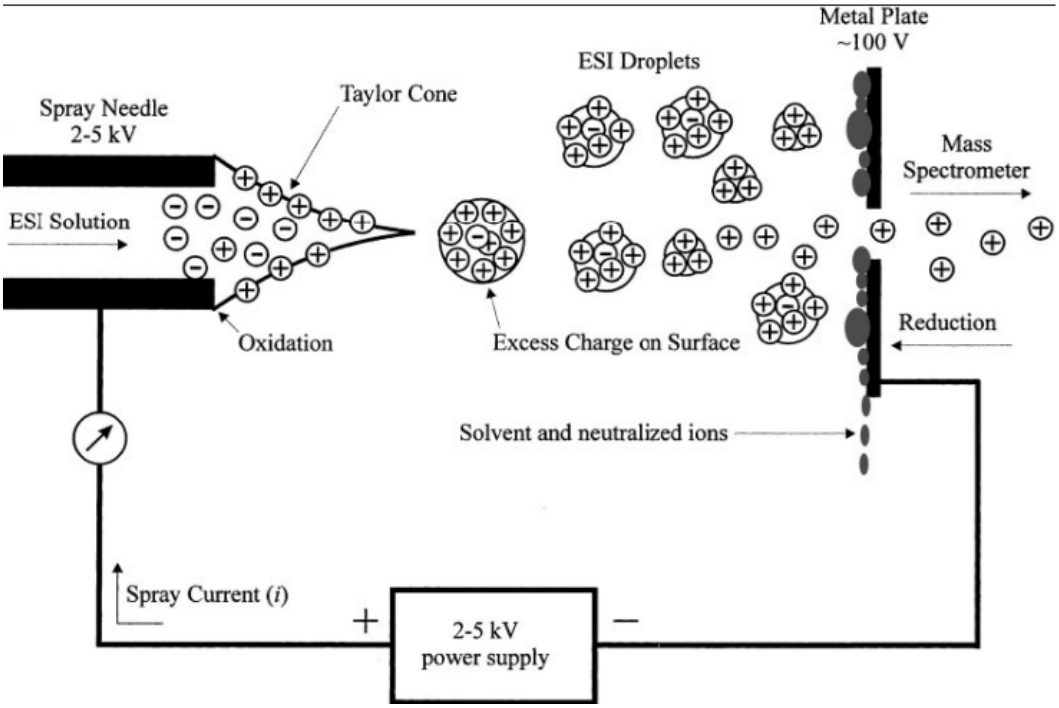

Figure 1.2 Schematic of the electrospray ionization process. [22]

1.2.3. Tandem Mass Spectrometry

Tandem Mass Spectrometry (MS/MS) involves multiple steps of mass analysis with fragmentation occurring between stages and plays an important role in peptide quantification

and identification. Tandem-in-time MS/MS utilizes a single mass spectrometer to trap the ions but with multiple MS steps taking place over time. For tandem-in-space MS/MS, individual analyzers such as ion-trap, quadrupole, time-of flight and orbitrap are physically separated. For the HDX-MS experiments in this thesis, a tandem-in-space MS/MS, the LTQ-Orbitrap, was used for peptide mapping, which provides the fingerprint of a given peptide. The Linear Trap Quadrupole (LTQ) is the first mass spectrometer that scans and selects the precursor ions while the Orbitrap functions as the second MS and is responsible for analyzing product ions after precursor ions dissociate in the gas phase [24].

1.3. Horse Heart Cytochrome c (HHC)

Cytochrome c, one of the simplest and best understood of all proteins, is the most extensively investigated electron transfer protein and has been the subject of extensive characterization [25]. Horse (equine) heart cytochrome c contains a 104 amino acid polypeptide chain and has a molecular weight of ~12,500 Daltons. Its redox center is a heme group, a macrocyclic complex with four nitrogen atoms of the porphyrin ring bonded to an iron atom; see Figure 1.3(d) [26]. The heme group in cytochrome c is held within the protein by four covalent bonds to the polypeptide chain, namely, two thioether linkages from porphyrin vinyl groups to Cys-14 and Cys-17, and two axial coordinate covalent bonds to the heme iron from His-18 and Met-80. One edge of the heme faces outward with approximately 5% of the porphyrin surface area exposed to solvent [27]. The exposed heme edge region is conventionally referred to as the front face, or front side, and is the site of electron transfer.

Cytochrome c possesses an asymmetric charge distribution on its surface. Positively charged lysines and arginines are distributed fairly evenly over most of the protein surface.

Negatively charged aspartates and glutamates, on the other hand, are located primarily on the back side of cyt c, which effectively render it neutral. The front face of cytochrome c has no carboxylate residues and, is accordingly dominated by lysines and positively charged as depicted in the electrostatic surface map shown in Figure 1.3(b). Cytochrome c interacts with its natural redox partners (cyt c reductase and cyt c oxidase) primarily through electrostatic interactions, with the lysine rich front face binding to carboxylate surface patches on its protein partners [28]. The excess positive charge concentrated around its heme edge makes cyt c an ideal candidate for immobilization on a negatively charged surface such as carboxylic acid terminated SAMs, as discussed below.

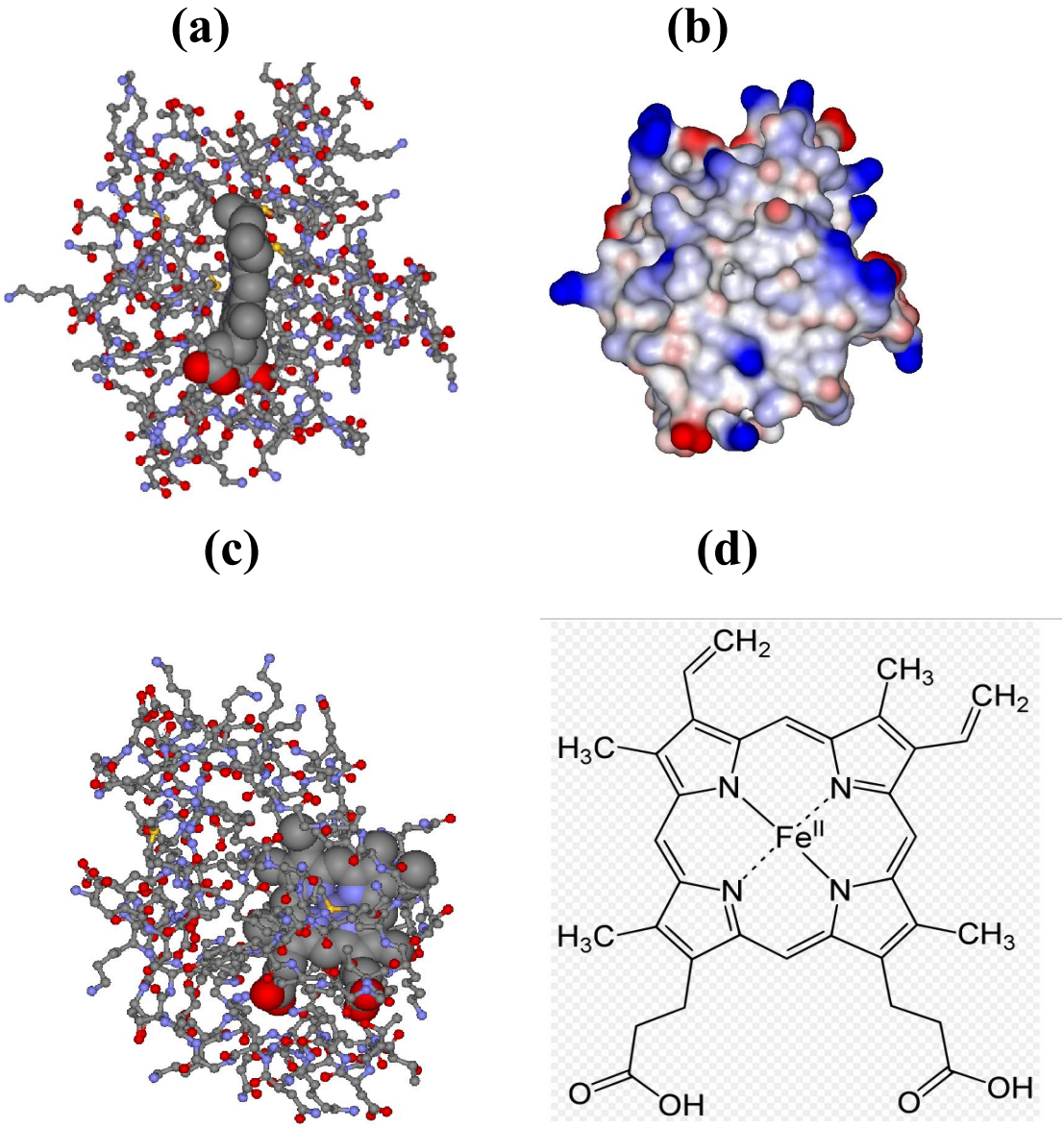

Figure 1.3 Structure of horse heart cytochrome c. (a) front face view with heme group displayed as CPK (b) electrostatic surface map, with positive charge shown in blue and negative charge in red; (c) side view with heme group displayed in CPK; (d) heme group.

1.4. The Cytochrome c / SAMs / Gold System

1.4.1. Self-Assembled Monolayers

Self-assembled monolayers (SAM) are formed by spontaneous adsorption of bifunctional organic molecules that typically feature a surface-active head group, an alkane chain and a terminal group. Among SAM-metal systems, gold has been the most frequently used metal because it does not undergo surface oxidation under ambient conditions [29]. Surface-active head groups are typically nitrogen, sulfur or phosphorous moieties, among which those containing sulfur give rise to the strongest, most stable attachment to gold through covalent bonding [30]. The terminal group of SAM-forming molecules can be designed to specifically mimic biological interfaces, for example, by imparting positive or negative charge.

1.4.2. Structure of Au/SAM/HHC

Carboxylic acid terminated alkanethiolate SAMs, $HS(CH_2)_nCOOH$, have been characterized extensively, with prior work in the Bowden group demonstrating their use as substrates for the adsorption of electroactive horse heart cytochrome c (HHC) [31]. Figure 1.4 illustrates in a simple manner the Au/SAM/HHC structure. The chain tilt angle has been reported to be approximately 30° for unsubstituted alkanethiols self-assembled on Au(111) [33]. In neutral pH solution, the COOH-terminated surface of the SAM is partially ionized and negatively charged, serving as an effective platform for adsorptive immobilization of HHC via its positively charged heme edge region. The adsorption of HHC to a SAM modified electrode is thought to be similar to the physiological docking between HHC and its physiological partners [34].

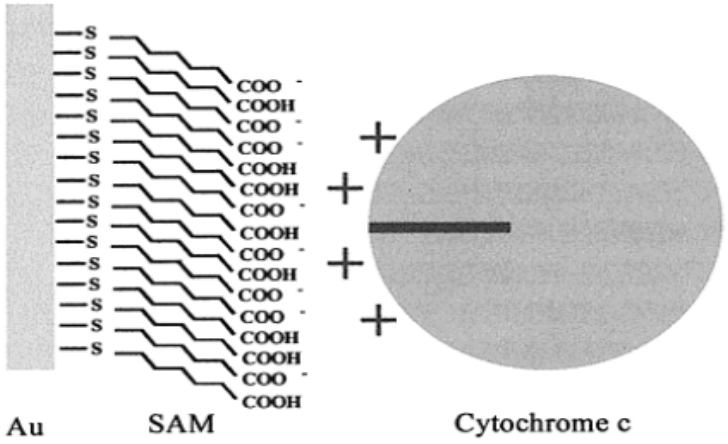

Figure 1.4 Schematic representation of HHC/SAM/Au system. [32]

1.5. Overview of Experimental Design

This project set out with the goal of developing a systematic HDX-MS approach for determining the orientation of HHC on COOH-terminated SAMs. Figure 1.5 is an overview of the envisioned experimental design, which is based on the notion of differential modification (i.e., labeling) of backbone amide groups, entailing the comparison of modification patterns for adsorbed proteins with those of solution-resident proteins. There are six main steps in this methodology for the adsorbed protein: 1) HDX deuterium labeling of the adsorbed protein on the SAM surface; 2) desorption from the SAM surface; 3) peptic digestion; 4) LC/MS analysis to determine the extent of deuteration for each peptide; 5) differential modification pattern analysis, i.e., determination of the binding domain via comparison with solution HHC reference samples; 6) orientation determination.

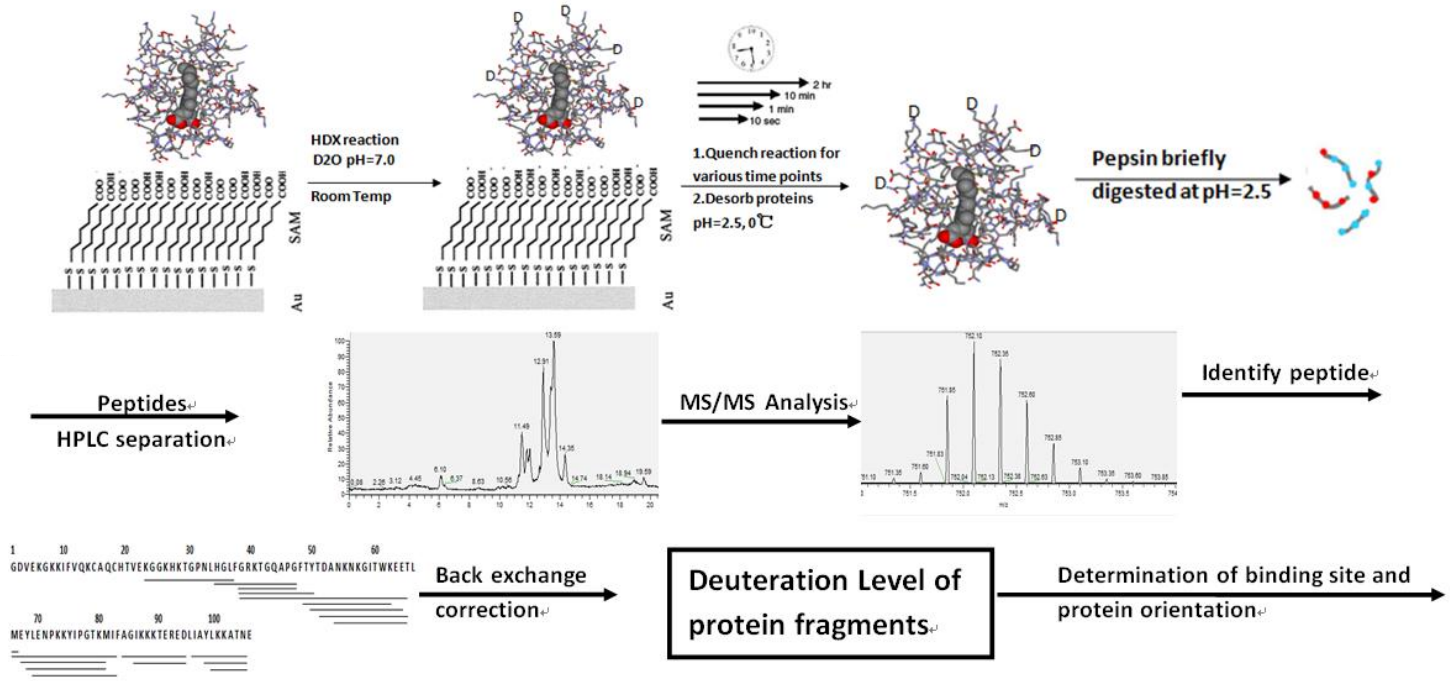

Figure 1.5 Overview of experimental design

1.6. Overview of Thesis Results

The ultimate goal of determining protein orientation by HDX/MS was not attained due to a number of experimental challenges. To perform state-of-the-art protein analysis using HDX/MS requires specialized instrumentation with automated sample handling to minimize analysis time while maintaining low temperature. With the capabilities that were available, however, progress was made in several areas, as described in the following chapters.

Chapter 2 describes experimental results that demonstrate the feasibility of a single desorption/quenching step. A more conventional and time-consuming approach would involve desorption of adsorbed cytochrome c using a high ionic strength solution, followed by desalting and addition of formic acid to quench the back reaction.

Chapter 3 describes the peptic digestion of native cytochrome c under acidic conditions and the identification of 36 peptides by LC/MS covering 78% of the amino acid sequence. The impact of the covalently attached heme group on the peptide sequencing is discussed.

Chapter 4 describes HDX/MS results obtained for two experiments, direct protein infusion and peptide mapping analysis. Direct infusion mass spectra revealed that a significant decrease in deuteration extent occurred as a result of cytochrome being adsorbed on COOH-SAMs relative to the solution state. This result offers strong support for the validity of the overall approach being pursued for the determination of protein orientation on

surfaces. Unfortunately, peptide mapping analysis was not successful due to inability to control the HD back exchange reaction.

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CHAPTER 2 ELECTROCHEMICAL CHARACTERIZATION OF THE ADSORPTION/DESORPTION PROPERTIES OF HORSE HEART CYTOCHROME C ON CARBOXYALKANETHIOL SELF-ASSEMBLED MONOLAYERS

2.1. Introduction

In the proposed HDX analysis scheme for adsorbed cyt c orientation determination, a critical challenge is to collect structurally intact protein molecules from the SAM surface that have been exposed to D_2O while minimizing the subsequent analysis time during which the back reaction can take place. In previous work from Bowden's group, it has been shown that the adsorption of cyt c on COOH SAMs at low ionic strength occurs primarily through electrostatic attraction between the negatively charged SAMs and the positively charged heme edge region of the protein. Accordingly, exposure of adsorbed cyt c to high ionic strength solutions of near neutral pH results in essentially complete desorption due to the interfacial charge screening that takes place [1]. For example, it has been shown that desorption of horse heart cyt c will result upon incubation in neutral 145 mM potassium phosphate buffer, which has an ionic strength (μ) = 300 mM [1]. A typical desorption solution such as this one, however, is not well suited for ESI mass spectrometry because the presence of salts will significantly decrease the ionization efficacy. Furthermore, although the desorbed sample could be desalted, this additional step would extend the analysis time and decrease the mapping resolution by allowing more back exchange of hydrogen with the deuterium labeled protein.

This chapter describes experimental results from a study undertaken to determine if adsorbed cyt c could be desorbed from COOH SAMs in a manner that did not require the

usual step of exposure to a high ionic strength buffer solution. The idea was to test whether a pH 2.4 formic acid solution, which is used to quench the HD exchange reaction as described in Chapter 1, would also result in desorption of cyt c. At this low pH, COOH SAMS are expected to be fully protonated, or nearly so, and thus uncharged. Because cyt c adsorption is largely electrostatic in nature, a change in solution conditions to a low pH would be expected to eliminate electrostatic attraction between cyt c and the SAM. Formic acid, which has a pK_a = 3.77, is commonly used in some chromatographic mobile phases as an ion pair reagent and can be tolerated in electrospray ionization without a significantly reduced detection sensitivity [2]. As illustrated in the Introduction chapter, the hydrogen/deuterium exchange (HDX) reaction is sensitive to temperature and pH. A low pH environment (e.g., pH~2.4) combined with low temperature (~ 0°C) is the optimal quenching condition for slowing down the deuterium→hydrogen back reaction [3]. If successful, this strategy would result in quenching of the HDX reaction and desorption of cyt c in a single step. This chapter aims to evaluate the feasibility of this single step quench/desorption strategy.

2.2. Experimental Procedures

2.2.1. Materials and Reagents

Horse heart cytochrome c was purchased from Sigma Chemical Company. Water for all experiments was purified on a Milli-Q/Organex-Q system. 11-Mercaptoundecanoic acid ($C_{10}COOH$) was purchased from Aldrich Chemical Company. Three pH = 7 potassium phosphate buffer (KPB) solutions were prepared at concentrations of 40 mM (ionic strength, μ = 90 mM), 70 mM (μ = 160 mM) and 4.4 mM (μ = 10 mM).

2.2.2. Purification of Horse Heart Cytochrome c

Horse heart cytochrome c was purified via column chromatography at ~4°C. Approximately 50 g of 'preswollen' carboxymethyl cellulose (CM52, Whatman, Clifton, NJ) was allowed to equilibrate over a 48 hour period in ~200 mL of 40 mM, pH 7 potassium phosphate buffer (KPB), with occasional gentle stirring. The gel slurry was added to a column having a height of 30 cm and an internal diameter of 1.5 cm, and three bed volumes (one bed volume ~19 mL) of 40 mM KPB were passed through once the gel had settled. A 1 mL aliquot of 0.2 mM cyt c in 40 mM KPB (pH 7) was loaded onto the column and eluted with 70 mM KPB (pH 7) over 15-24 h. Samples typically separate into three bands: 1) deaminated cyt c; 2) purified cyt c; and 3) cyt c dimer (see Figure 2.1) [4]. The deaminated protein elutes first, appearing as a very light red band, and is followed by a dark red band which comprises the purified cyt c. The third band will not elute from the column using the 70 mM KPB buffer. This band can be eluted during a later step with high ionic strength buffer (also containing sodium azide as an antibacterial agent for column storage). The purity of native cytochrome c can be complimentarily measured by cyclic voltammetry as previously reported [5].

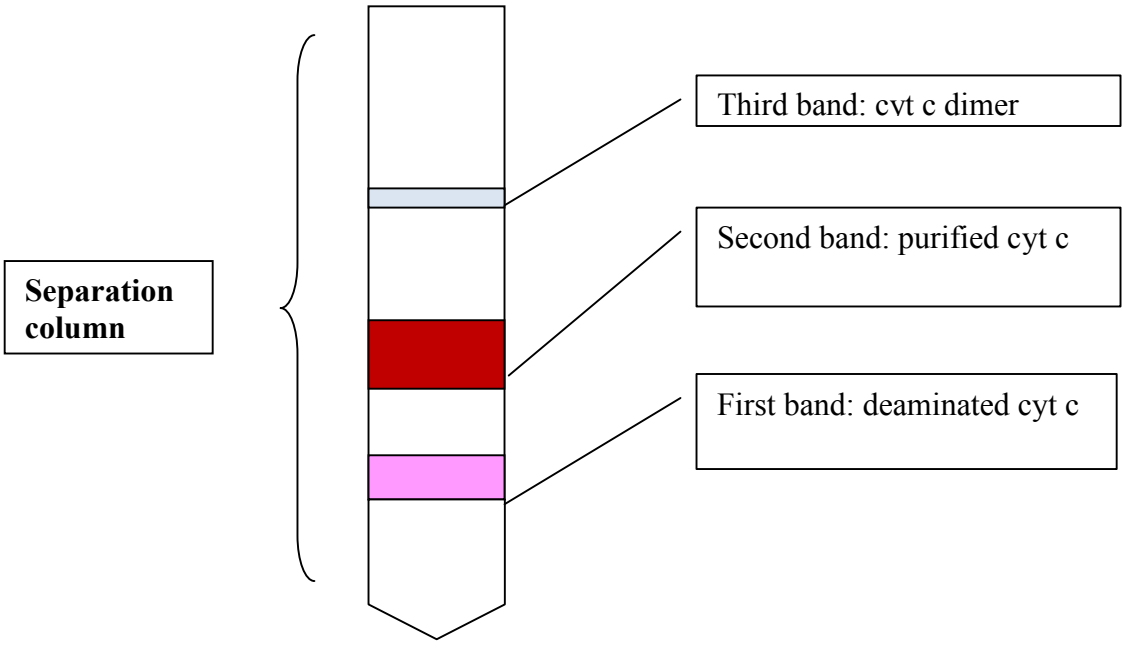

Figure 2.1 Schematic showing the three elution bands that typically result from chromatographing a commercial sample of cyt c.

2.2.3. Electrode Preparation

The gold working electrodes consisted of 1000 to 3000 Å films of evaporated gold deposited on a 50 Å titanium adhesion layer atop glass slides. They were purchased from EMF Corporation (Ithaca, NY). A thorough cleaning process prior to SAM assembly on the electrodes is required. The gold slides were immersed in 1% Liqui-Nox detergent and subjected to a 10 min ultrasonication followed by two 10-min sonications in Milli-Q water. After drying the slides and assembling the electrochemical cell, the solution-exposed portion of the electrode was subjected to a rigorous electrochemical pretreatment. After filling the cell with approximately 0.5 mL of 0.1 M H_2SO_4/0.01 M KCl solution, the potential was scanned linearly in a cyclic fashion from 0.0 V to +1.5 V vs Ag/AgCl. A total of 10

successive cycles was performed. This pretreatment procedure results in some dissolution of the gold on the positive going scans, followed by partial re-deposition of fresh gold on the return scans. The pretreated gold electrode was rinsed 3 times with Milli-Q water, followed by rinsing 3 times with 95% ethanol.

Surface modification by a COOH-terminated self-assembled monolayer was achieved by exposing the pretreated gold electrode to a solution of 11-mercaptoundecanoic acid in 95% ethanol for at least 12 h at room temperature. After SAM formation, the cell and electrode were rinsed 3 times with 95% ethanol, followed by 3 times with Milli-Q water. Cytochrome c was adsorbed onto the SAM/Au substrate at 4℃ by exposure to a 30 μM solution containing 4.4 mM potassium phosphate (μ = 10 mM, pH = 7) for 30 min. After the adsorption step, the cell and electrode were first rinsed 3 times with Milli-Q water, and then rinsed 2 times with 4.4 mM potassium phosphate (μ = 10 mM, pH = 7). The gold electrode was immersed in the 4.4 mM potassium phosphate for further analysis.

Cyclic voltammetry (CV) was performed in a previously described 3-electrode cell [6] equipped with a platinum auxiliary electrode and a Ag/AgCl reference electrode (glass barrel filled with 3M KCl); see Figure 2.2. The active geometric electrode area was 0.32 cm^2.

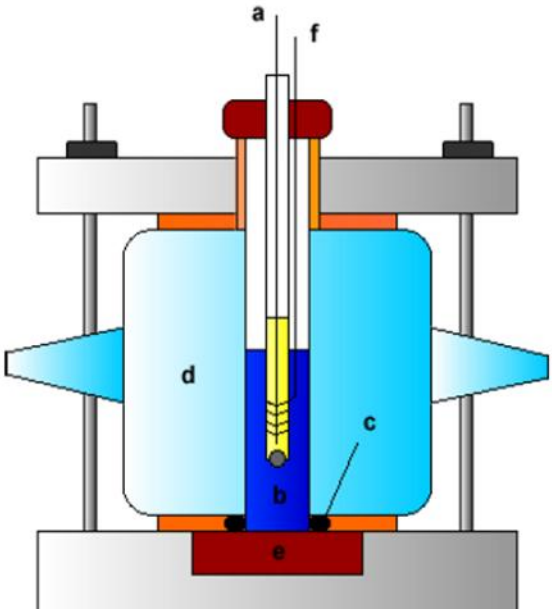

Figure 2.2 Diagram of the electrochemical cell. (a) Ag/AgCl reference electrode (glass barrel filled with 3M KCl); (b) buffered electrolyte solution; (c) Viton O-ring; (d) glass cell body; (e) SAM modified gold electrode; (f) platinum wire auxiliary electrode. [6]

2.3. Results and Discussion

2.3.1 Voltammetry of Adsorbed Horse Heart Cyt *c*

Cyt c undergoes an outer sphere 1-electron transfer reaction as its low spin heme iron cycles between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states as shown here:

$$Fe^{2+}\text{Cyt c} \rightleftharpoons Fe^{3+}\text{Cyt c} + e^-$$

A typical CV obtained for cyt c adsorbed on a C_{10}COOH SAM is shown in Figure 2.3. The background CV for the SAM-coated gold electrode was acquired following self-assembly of the C_{10}COOH SAM and is shown as a blue trace. After adsorption of cyt c, the red trace was obtained. The observed voltammetric peaks are consistent with the presence of native adsorbed cyt c, as documented elsewhere [7].

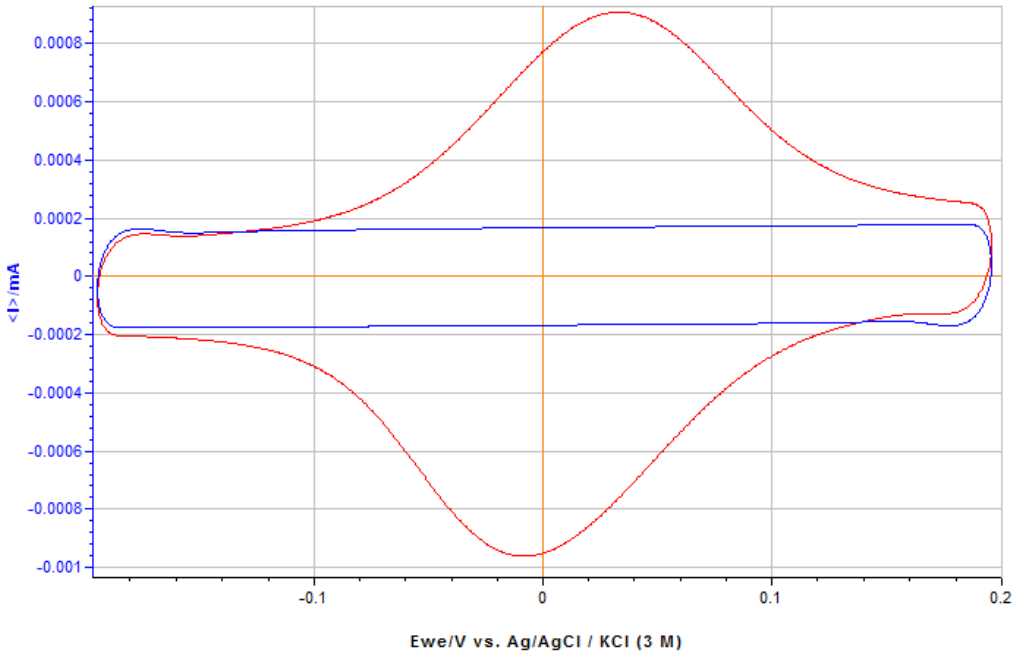

Figure 2.3 Cyclic voltammogram of horse cyt *c* adsorbed onto an evaporated gold film modified with a 11-mercaptoundecanoic acid SAM ($HS(CH_2)_{10}COOH$). Solution conditions are 4.4 mM potassium phosphate buffer (μ = 10 mM, pH = 7), and the sweep rate is 200 mV/s.

The surface concentration of cyt c was calculated using the following equation. The maximum theoretical surface concentration (Γ) is 15 mol/cm^2 on 11-mercaptoundecanoic acid SAM ($HS(CH_2)_{10}COOH$). [8]

$$i_p = \frac{n^2 F^2 A \nu \Gamma^*}{4RT} \qquad \text{equation (1)}$$

i_p : voltammetric peak height corrected for background current (A)

Γ^*: surface concentration (mol/cm^2)

ν: sweep rate (V/s)

A: electrode area (cm^2)

F: Faraday's constant = 96500 C/mol e^-

R: molar gas constant = 8.314 J/K·mol

n = 1

2.3.2 Effect of low pH on adsorbed horse cyt c

(a) Desorption experiment

After acquiring CVs for adsorbed cyt c (Figure 2.3), the cell was rinsed 3 times with Milli-Q water. A 0.5% formic acid (0.133 M) solution, pH = 2.4, was then introduced to the cell for times ranging from 2 to 8 minutes. Following each formic acid exposure, the cell was rinsed with Milli Q water 3 times. The 4.4 mM phosphate buffer (μ=10 mM, pH 7.0) was then re-introduced into the cell, and cyclic voltammograms were again obtained. Figure 2.4 shows cyclic voltammograms obtained following formic acid exposures of 0 to 8 min. Table 2.1 presents a summary of the cyt c surface concentration results.

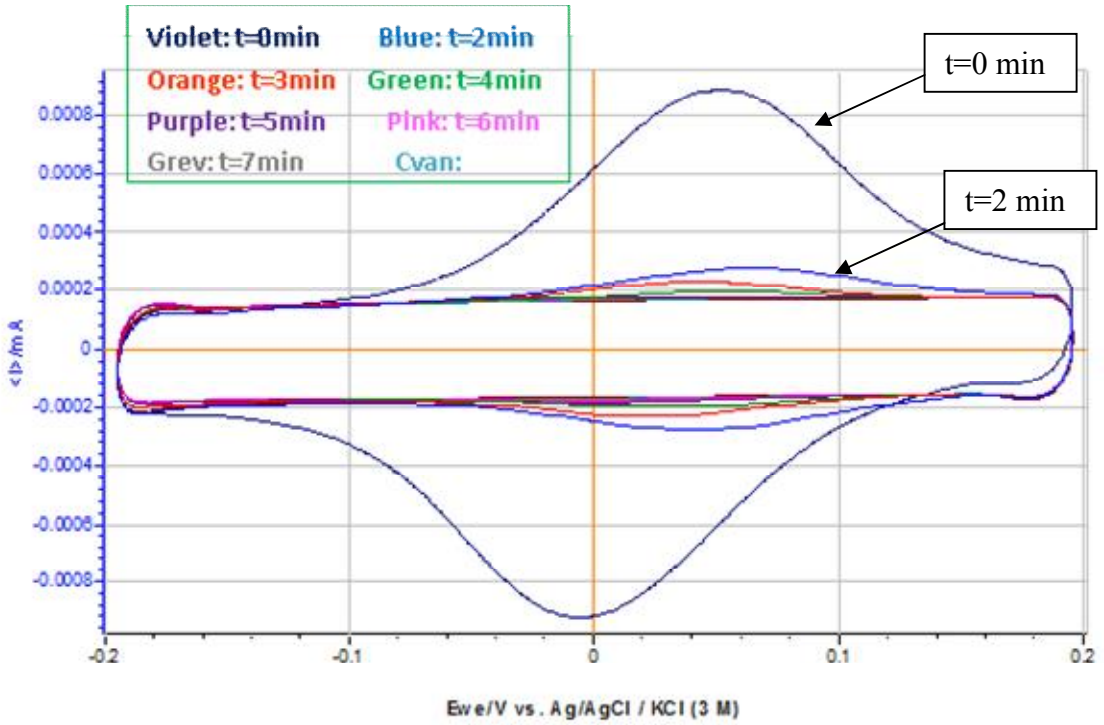

Figure 2.4 Desorption cyclic voltammetry. The CV at t = 0 min was acquired after adsorption of cyt c at pH 7 and before exposure to 0.5% formic acid (c = 0.133 M, pH = 2.4). The other CVs were acquired after exposure to the formic acid solution for the indicated times. All CVs were acquired in 4.4 mM potassium phosphate buffer (μ = 10 mM, pH = 7) at a sweep rate of 200 mV/s.

Table 2.1 Electroactive cyt c surface concentration (Γ) following exposure to formic acid

t / min[a]	$i_{p,c}$ / μA[b]	Γ / pmol·cm^{-2} [c]	% coverage[d]
0	0.737	13.09	100
2	0.110	1.95	14.9
3	0.0066	1.17	9.0
4	0.0028	0.49	3.8
5	0.0012	0.21	1.6
6	0.0007	0.12	0.9
7	0.0004	0.07	0.5
8	0.0003	0.06	0.4

[a]Exposure time to 0.5% formic acid (c = 0.133 M, pH = 2.4).

[b]Cathodic peak current corrected for background current.

[c]Electroactive surface concentration of cytochrome c calculated using equation (1).

[d]Surface concentration reported as a percent of Γ (t = 0).

(b) Re-adsorption experiment

To evaluate whether the apparent loss of adsorbed cytochrome c documented in Figure 2.4 and Table 2.1 was actually the result of desorption as opposed to denaturation of the adsorptively immobilized protein molecules, a re-adsorption experiment was performed. Exposure of adsorbed cytochrome c to formic acid for more than 8 min results in essentially complete loss of electroactivity (See Table 2.1). The cell was subsequently rinsed 3 times with Milli-Q water, and a standard cytochrome c adsorption solution (30 μM cyt c, 4.4 mM phosphate buffer, pH 7.0, 4°C) was re-introduced to the cell for 30 minutes. The cell was

then rinsed and CVs were acquired in the usual buffer employed for adsorbed cyt c electrochemistry. The CV results are shown in Figure 5, and the calculated surface concentrations are given in Table 2.2. Re-exposure of the formic acid treated electrode to cyt c adsorption solution resulted in restoration of the original CV response due to cytochrome c. Thus, we can conclude that exposure to pH 2.4 formic acid results in essentially complete desorption of adsorbed cytochrome c molecules, leaving behind a clean $C_{10}COOH$ SAM surface available for re-adsorption.

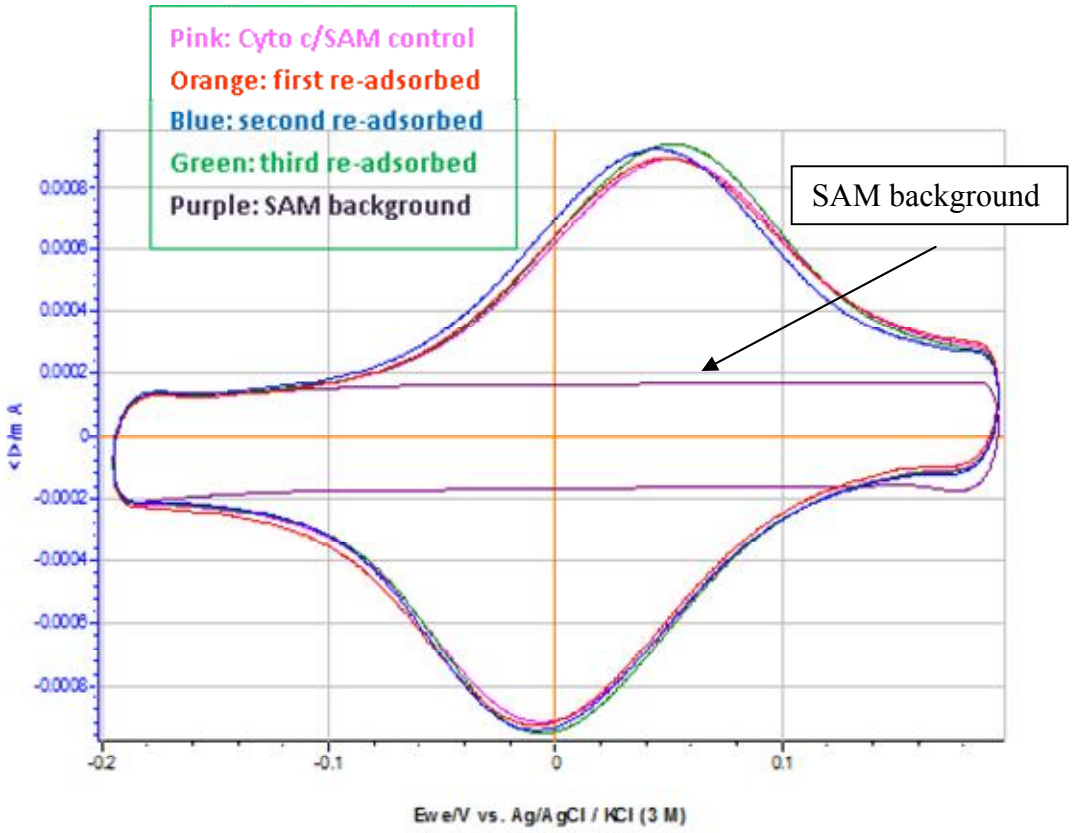

Figure 2.5 Cyclic voltammetry of re-adsorbed cytochrome c. Initially adsorbed cyt c gave rise to the control CV (pink). Following an 8 min exposure to 0.5% formic acid (c = 0.133 M, pH = 2.4), a complete loss of signal due to electroactive cytochrome c resulted (see Figure 2.4). Three subsequent exposures of the electrode to cyt c adsorption solution resulted in the three other CVs shown in the figure. All CVs were acquired in 4.4 mM potassium phosphate buffer (μ =10 mM, pH = 7) at a sweep rate of 200 mV/s.

Table 2.2 Electroactive cyt c surface concentration (Γ) following re-adsorption

Experiment	$i_{p,c}$ / μA[a]	Γ / pmol·cm^{-2} [b]	% coverage[c]
Control	7.37E-04	13.09	–
re-adsorption #1	7.56E-04	13.42	103%
re-adsorption #2	7.38E-05	13.10	100%
re-adsorption #3	7.77E-05	13.80	105%

[a]Cathodic peak current corrected for background current.

[b]Electroactive surface concentration of cytochrome c calculated using equation (1).

[c]Surface concentration reported as a percent of Γ (control).

2.4. Conclusions

The results show that adsorbed cytochrome c can be cleanly desorbed from the C_{10}COOH SAM surface upon exposure to the pH 2.4 formic acid solution used to quench the HDX back reaction. The extent of desorption is ~90% at 2-3 min exposure and ~99% at >5 min exposure. Following desorption, cytochrome c can be quantitatively re-adsorbed on the same surface, demonstrating that the loss of electroactivity observed due to formic acid exposure is indeed a result of desorption and not a result of denaturation of surface-retained cytochrome c molecules. Desorption appears to result from the elimination of electrostatic attraction of cytochrome c due to protonation of the C_{10}COOH SAM surface, which renders it neutral.

The application of these findings to the eventual scientific goal would be aimed at seeking optimal conditions for formic acid treatment of adsorbed cyt c. There are two

opposing needs in this regard, namely, to desorb the maximum amount of cytochrome c for the digestion/LC/MS analysis, and to minimize the time during which the D→H back reaction can occur. More extensive desorption occurs at longer exposure times, as evident from Table 1. It appears that a 2-3 min treatment with 0.5 formic acid solution would be a reasonable choice. This would result in desorption of ~90% of the adsorbed cyt c. For future experiments, the short end of the exposure time range could be further evaluated in the event that a more-than-adequate analytical signal was obtained at a 2-3 minute exposure. Any lessening of the sample handling time would be desirable for minimizing the negative impact of the back reaction.

2.5. References

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CHAPTER 3 PEPTIC DIGESTION AND MASS SPECTROMETRY OF HORSE HEART CYTOCHROME C

3.1. Introduction

Mass spectrometry-based proteomics, one of the major approaches for studying structural modification in proteins, involves enzymatic cleavage of the protein to yield a collection of peptides that are then fractionated by high-performance liquid chromatography and further analyzed by tandem mass spectrometry [1]. In this project, we sought to utilize differentially modified peptides of cytochrome c to reveal and map surface binding sites that are involved in its adsorptive interaction with COOH-terminated self-assembled monolayers. Thus, it is essential to characterize horse heart cytochrome c enzymatic digestion prior to using it with the hydrogen-deuterium exchange (HDX) isotopic labeling technique for investigating protein surface interactions. As mentioned in the previous chapter, HDX is very sensitive to pH and temperature, and the reaction can be quenched in an acidic environment where pH is adjusted to ~2.4, which also inhibits the back reaction. Although tryptic digestion of HHC has already been studied in Bowden's group [2], it is not suitable for HDX application since trypsin is most active at pH = 7.5-8.5 and at an optimal temperature of ~37°C [3]. In other words, trypsin has little activity at the low pH needed to inhibit the HDX back reaction. Pepsin A, on the other hands, is an enzyme first discovered in the stomach which has an optimal pH for protease activity of approximately 2.0 [4]. The aim of this chapter, therefore, is to evaluate the peptic digestion of horse heart cytochrome c using pepsin A and identify the resulting peptides.

LTQ-Orbitrap, the type of tandem mass spectrometer (MS/MS) used in this experiment, is widely used in all major applications of life science mass spectrometry [5]. It provides high mass accuracy and sensitivity for analysis of low level components in complex mixtures [6], and is commonly interfaced to liquid chromatographs. Figure 3.1 shows a schematic of an LTQ-Orbitrap. The molecules are first ionized via ESI (Electrospray Ionization), after which the ions in a selected M/Z range are captured or 'trapped' by the LTQ (Linear Trap Quadrupole, also known as Linear Ion Trap) for a certain time interval, and then transmitted into the C-trap for high-energy dissociation before entering the Orbitrap for fragmentation analysis [7].

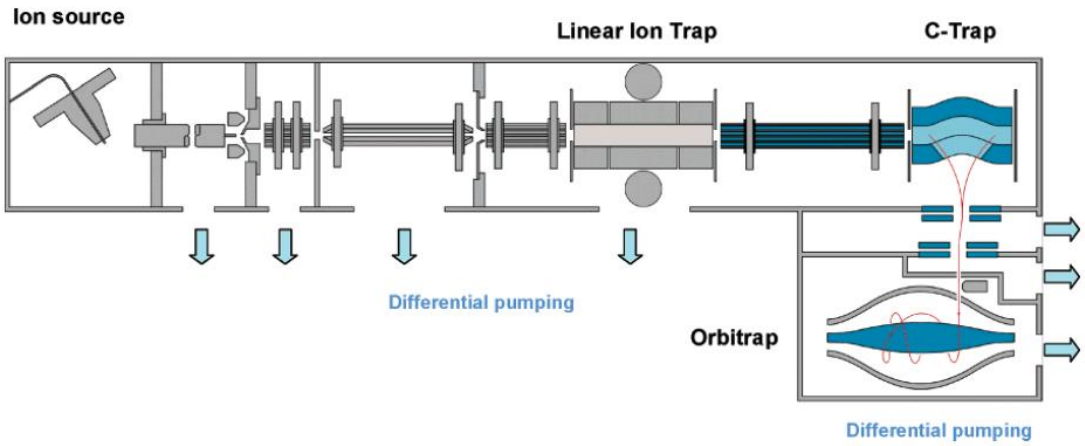

Figure 3.1 Schematic for the Thermo LTQ-Orbitrap XL used in this project. [7]

The protein sequences of porcine pepsin A and horse heart cytochrome c were obtained from Swiss-Prot, a freely accessible protein sequence database widely used in

proteomic studies. Swiss-Prot was first developed by the Swiss Institute of Bioinformatics in 1986 to provide reliable protein sequences associated with a high level of annotation [8]. In our study, the MS/MS results were analyzed using Mascot software with a predefined Swiss-Prot database to identify peptides based on mass fingerprints. Differences between tryptic digestion and peptic digestion in Mascot database search settings are also discussed in this chapter.

3.2. Experimental Design

3.2.1. Materials

Horse heart cytochrome c and formic acid were purchased from Sigma Chemical Company. Porcine pepsin was purchased from VWR International. Water for all experiments was purified on a Milli-Q/Organex-Q system.

3.2.2. Sample preparation

Formic acid was used to adjust the pH of aqueous solutions of native horse heart cytochrome c (HHC) to a value of 2.40 in order to provide the acidic environment for peptic digestion. A 60 μL volume of 1% formic acid was first added to 60 μL of 20 μM HHC in H_2O. The native HHC was then briefly digested by pepsin A at room temperature (~21°C) by introducing 20 μL of 60 uM porcine pepsin A in H_2O to the acidic HHC solution (pepsin : HHC = 1:1 mol/mol) for 5 min. The digested sample was diluted 1:50 using mobile phase A (see Section 3.2.3. for details) to make a final HHC concentration of 0.17 μM. The injection volume of peptide mixtures was 10.00 μL for the LC/MS/MS analysis. Figure 3.2 diagrams the sample preparation procedure.

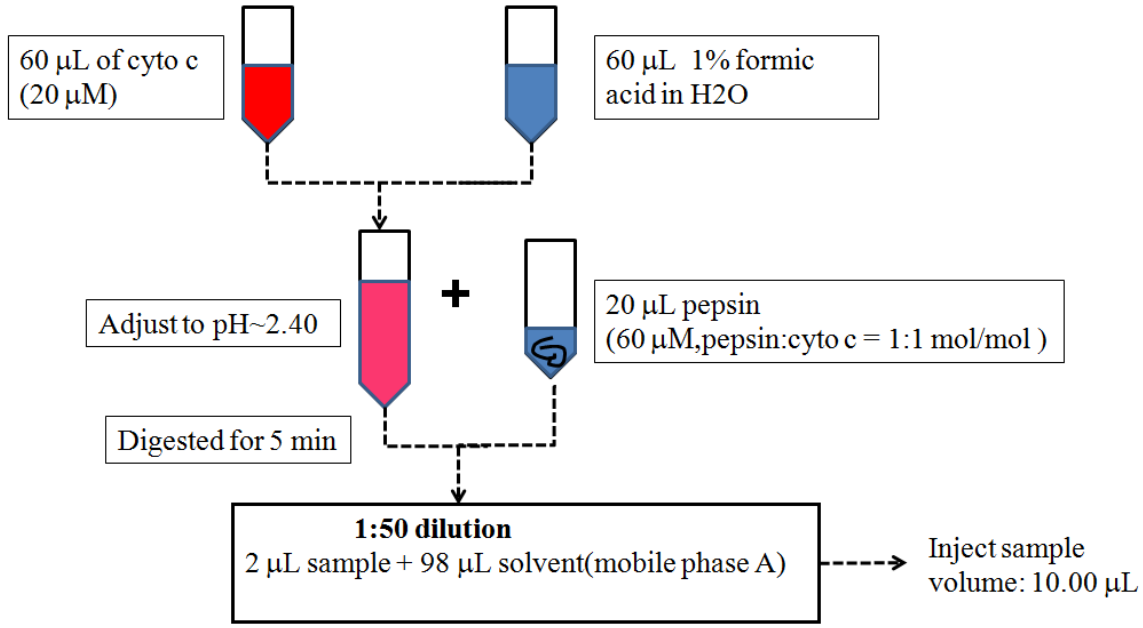

Figure 3.2 Flow chart for peptic digestion of native horse heart cytochrome c.

3.2.3. Liquid Chromatography

The digested peptides were separated by liquid chromatography (Eksigent cHiPLC-Nanoflex System). The injected peptides were first captured by a C18 trap column (ChromXP 200 mm*0.5 mm C18-CL 3 mm 120 Å, volume = 15.7 nL) and washed using 6000 nL of 100% mobile phase A with a flow rate = 4000 nL/min. The peptides were then moved through the C18 trap column and separated on a C18 analytical column (ChromXP 75 mm*15 cm C18-CL 3 mm 120 Å, volume = 662.5 nL) using a gradient mobile phase with a flow rate = 350 nL/min. The injection loop is shown in Figure 3.3. Aqueous mobile phase A contained 0.2% formic acid (FA) + 10% acetonitrile (ACN) + 90% H_2O; organic mobile phase B contained 0.2% FA + 90% ACN + 10% H_2O. The gradient profile is shown in Figure 3.4.

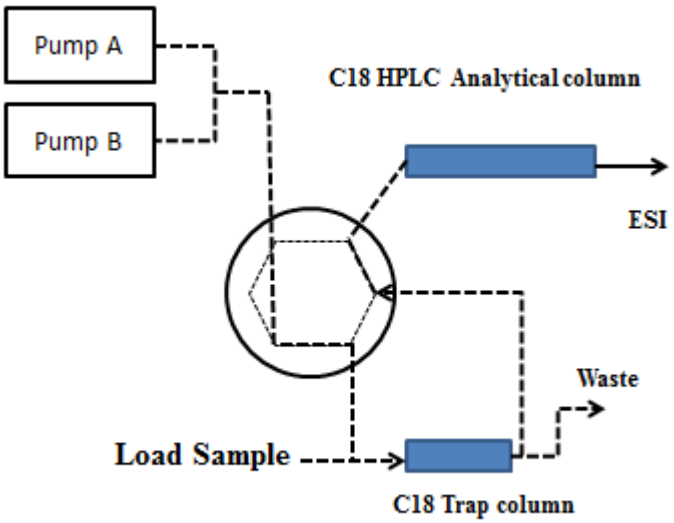

Figure 3.3 Eksigent cHiPLC-Nanoflex System Injection Loop

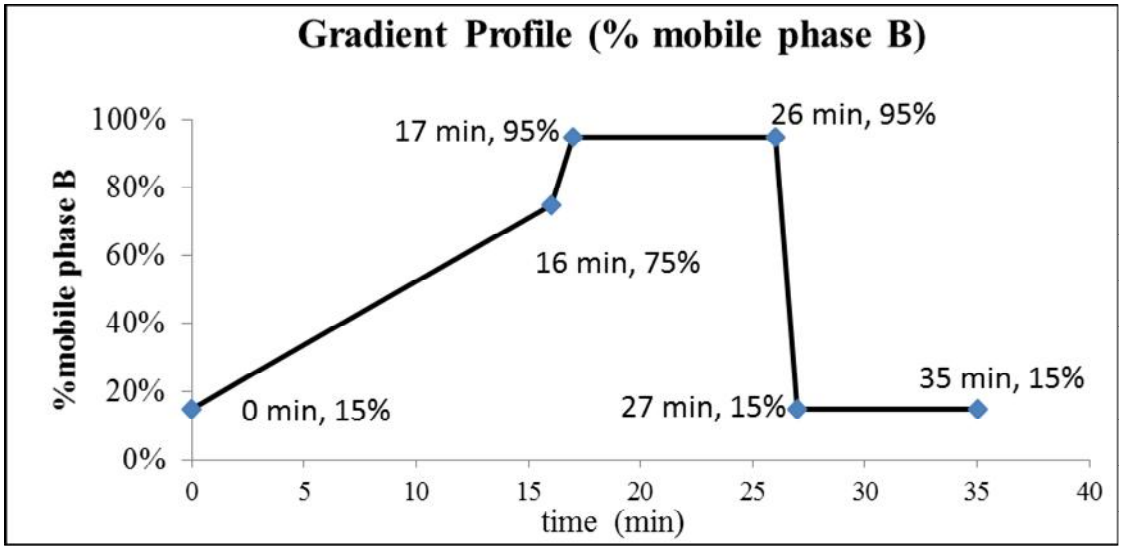

Figure 3.4 Gradient profile for liquid chromatographic separation

3.2.4. Tandem MS/MS

The Eksigent cHiPLC-Nanoflex System was coupled to a tandem MS/MS Thermo Fisher Scientific LTQ-Orbitrap XL. The peptides eluting from the C18 analytical column were injected and ionized by electrospray (ESI) followed by mass spectrometric analysis. The mass fingerprint data generated from tandem MS/MS was analyzed using a Mascot database search to identify the digested peptides in the compound mixtures.

3.3. Results and Discussion

3.3.1. LC Separation

The samples contained peptides from horse heart cytochrome c after it was briefly digested by pepsin for 5 min at 25 °C. Figure 3.5 shows a typical TIC (Total Ion Current) liquid chromatogram for the mass/charge range from 200 to 2000 Da. The time t = 0 denotes the completion of the 6000 nL 100% mobile phase A washing step and the start of the gradient profile shown in Figure 3.4. The total gradient elution lasted 35 min. On the TIC chromatogram, the peaks of interest are located between 6 min and 26 min, with the major peaks falling in the range of 11 to 14.5 min. A given peptide and its sequence can be identified from its intact mass value based on the m/z data and further confirmed by its MS/MS fingerprint determined through the database search. A typical MS/MS result is shown in Figure 3.6 for the peptide that eluted with RT = 14.35 min.

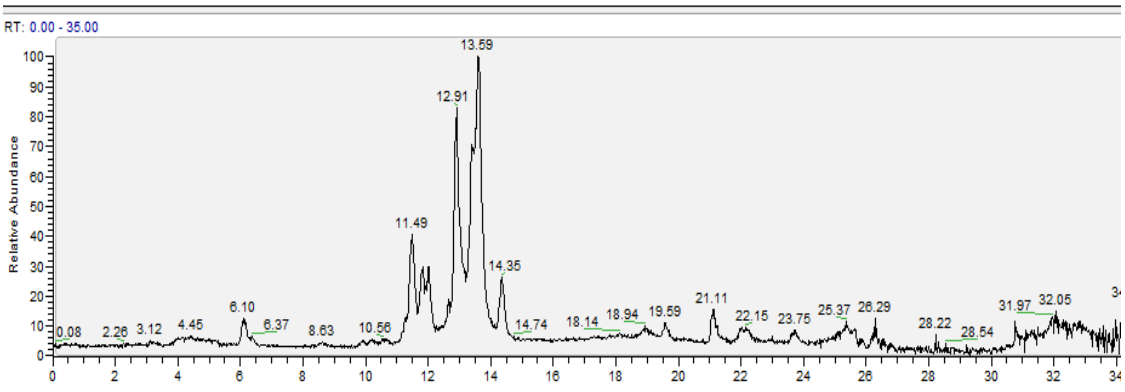

Figure 3.5 Total ion current liquid chromatogram of pepsin-digested cytochrome c from 0-35 min. Digestion time = 5 min.

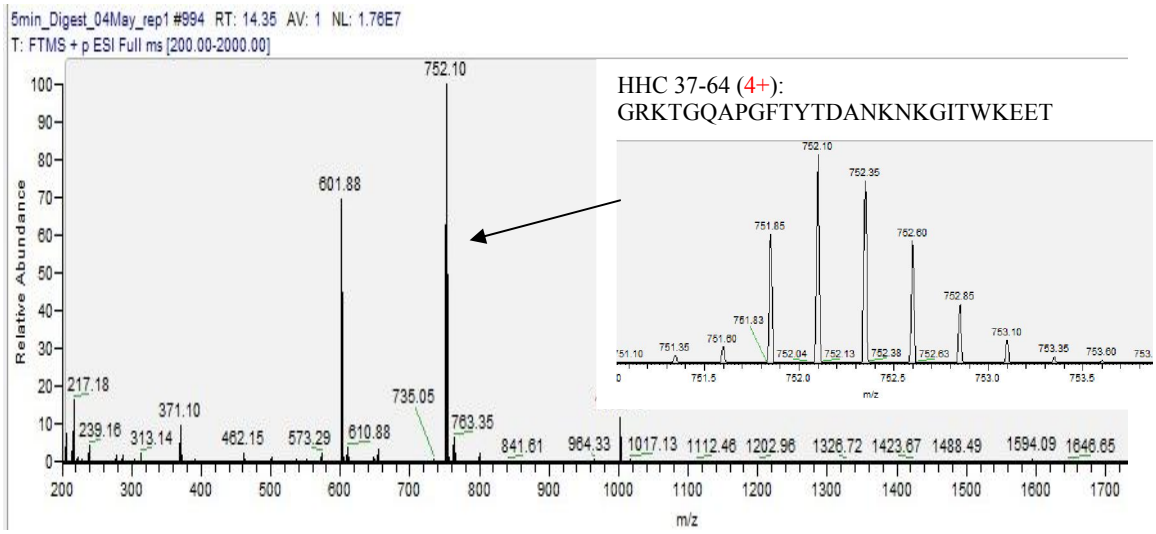

Figure 3.6 Typical MS and MS/MS data shown for the peptide with RT = 14.35 min

3.3.2 Peptide Identification

MS/MS data analysis revealed that the sample mixture contained peptides from both pepsin A and horse heart cytochrome c, thus demonstrating that pepsin is able to digest itself

to some extent under these experimental conditions. Table 3.1 lists the peptides identified from pepsin A. The results confirm that pepsin A prefers to cleave at the carboxyl side of phenylalanine (F) and leucine (L) since 5 of the 6 identified peptides were cleaved at these sites.

A total of 37 different peptides from horse heart cytochrome c were identified, covering 78% of the protein sequence from amino acid 22 to 104, as shown in figure 3.7 (bold red). Table 3.2, which lists Mascot-identified peptides categorized according to cleavage site, reveals that pepsin A does not strictly obey specific cleavage rules when digesting HHC. Although pepsin A prefers to cleave at the carboxyl side of phenylalanine (F), leucine (L), and glutamic acid (E), some cleavages sites were also found on the carboxyl side of alanine (A), isoleucine (I), methionine (M) and others. Each of three replicate experiments on different samples resulted in the same set of identified peptides, which indicates that, although peptic digestion of HHC is not specific, it is reproducible. A segment of the polypeptide chain representing 22% of the sequence was not identified from the MS/MS analysis. The missing sequence, comprising positions 1-21 along with the heme group covalently attached at Cys14, Cys17 and His18, will be discussed in Section 3.3.5 (Microperoxidase).

Table 3.1 Identified peptides originating from porcine pepsin A (Swiss-Prot Entry number = P00791)

Pepsin A	Peptide Sequence	M(calc)	M/Z(exp)	Z	M(exp)
41-49	(L)KTHKHNPA	931.4988	466.7558	2	931.497
41-50	(L)KTHKHNPAS	1018.5308	340.5174	3	1018.5305
75-87	(F)GTIGIGTPAQDFT	1276.63	320.1651	4	1276.6314
372-381	(F)DRANNKVGL	985.5305	493.7723	2	985.53
372-385	(F)DRANNKVGLAPVA	1323.7259	662.8706	2	1323.7267
378-385	(N)KVGLAPVA	753.4749	377.7452	2	753.4758

Figure 3.7 Protein sequence coverage of horse heart cytochrome c based on Mascot search

Table 3.2 Peptides identified from peptic digestion of horse heart cytochrome c as analyzed by Mascot peptide mass fingerprint search

Number	Cleavage sites	Peptides(z)			
1	22(E-K)	22-36(4)			
2	33(L-H)	33-46(3)			
3	37(F-G)	37-46(2)	37-47(2)	37-49(2)	
		37-56(4)	37-60(4)	37-64(4)	
		37-65(4)	37-66(4)	38-68(4)	
4	47(F-T)	47-61(3)	47-63(3)	47-65(3)	47-66(3)
5	48(T-Y)	48-63(3)	48-64(3)	48-65(3)	48-66(3)
6	50(T-D)	50-65(3)			
7	52(A-N)	52-65(3)	52-66(3)		
8	65(L-M)	65-82(3)	65-80(2)		
9	66(M-E)	66-80(3)	66-82(3)		
10	67(E-Y)	67-80(4)	67-82(3)		
11	68(L-E)	68-82(3)			
12	81(I-F)	81-94(3)	81-96(3)		
13	83(A-G)	83-94(3)	83-96(3)		
14	85(G-I)	85-94(3)			
15	95(L-I)	95-104(2)			
16	97(Y-L)	97-104(2)			
17	98(L-K)	98-104(2)			

3.3.3 MASCOT Analysis Optimization

Mascot Peptide Mass Fingerprint provides a powerful tool to analyze MS/MS data for peptide identification. In order to utilize this analysis, the specificity of the digestion enzyme needs to be evaluated before determining the parameters and search method. Peptic digestion, used in this work, employs an optimized method with no enzyme search and selection of multiply-charged precursor ions of +2, +3 and +4 charge. These critical parameters/factors are discussed further in the following section.

3.3.3.1 Cleavage Sites: Enzyme vs. No Enzyme Search

Figure 3.8 is a typical search window displayed in Mascot Peptide Mass Fingerprint. Trypsin cleaves polypeptide chains primarily at the carboxyl side of the amino acids lysine and arginine, except when either is followed by proline [9], which makes tryptic digestion predictable and specific. In traditional tryptic digestion analysis, the enzyme search method is used and the cleavage sites are fixed. The major difference between peptic and tryptic digestion is that pepsin A does not adhere to strict cleavage of specific amino acids as does trypsin. The experimental results in Table 3.2 show that pepsin A prefers to cleave on the C-terminal side of F, L and E but there are also peptides derived from cleavage at other amino acids. For the default Enzyme setting used in Database Swiss-Prot, however, the cleavage sites of pepsin A are predefined as the C terminus of only F, L, E, and A. This setting will result in missed peptides due to cleavage at other amino acids. Accordingly, a No Enzyme search setting that has no cleavage limitations is strongly recommended for non-specific enzymatic digestion.

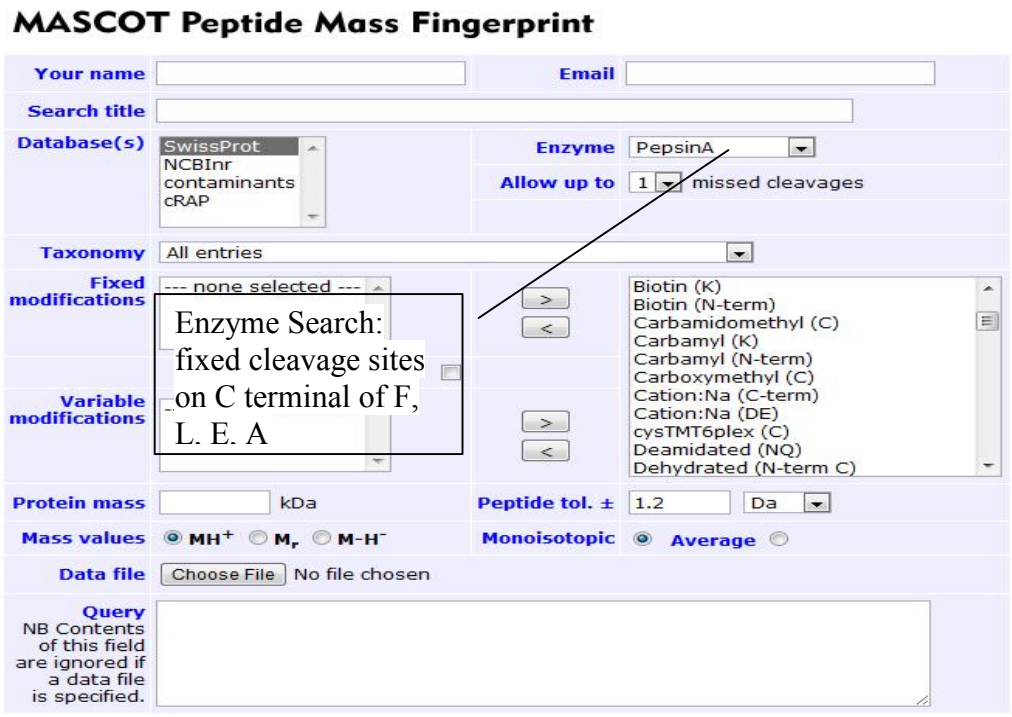

Figure 3.8 Mascot Software Peptide Mass Fingerprint Search Window

3.3.4.2 Charge Effect

Among all natural amino acids, the average occurrences of lysine (K) and arginine (R) are 5.9% and 5.1%, respectively [10], which indicates that, on average, trypsin can cleave at about 11% of amino acid sites in a given protein sequence. Therefore, tryptic digestion generates peptides with average sequence length on the order of 8 amino acids. Peptides of such length usually carry +2 and +3 charges when ionized by ESI. Since singly charged and highly charged ions may increase the noise level and provide limited contribution to the analysis, only ions with +2 and +3 charges will be selectively transmitted from LTQ for further high energy dissociation when using default MS/MS settings for tryptic digestion.

Peptide lengths resulting from peptic digestion, however, are less predictable and tend to produce longer peptides with charges of +4 or higher. Furthermore, in the present study, the peptic digestion of HHC was incomplete, lasting only 5 min, which tends to result in longer peptides. In fact, peptides with +4 charges are found among the results in Table 3.2. For cytochrome c peptides produced by peptic digestion, multiply charged precursor ions having +2, +3 or +4 charges were all injected into the tandem MS for analysis.

3.3.4 Microperoxidase

The method that was optimized for 5 min peptic digestion of HHC was unable to identify any peptides from the first 21 amino acids. The heme group, a covalently attached ligand, is the major cause for this result based upon prior literature. Microperoxidase (MP) is the name given to any of several heme-containing peptide fragments of cytochrome c that display peroxidase activity. The heme porphyrin ring in cytochrome c is covalently bonded to Cys14 and Cys17 via thioether bonds, and the heme iron is 6-coordinate with His18 and Met80 as the axial ligands. In microperoxidase itself, only the His18 ligand remains bound, and the iron atom is 5-coordinate. Microperoxidase is prepared by enzymatic digestion of cytochrome c, which leaves a short length of the peptide chain covalently attached to the heme at the three amino acids identified above. There are several microperoxidase species identified by the length of the attached peptide, e.g., MP8, MP9 and MP11. Generally, tryptic digestion of HHC leads to MP8 whereas peptic degradation typically gives rise to MP11 (shown in Figure 3.9). For example, one MP11 preparation reported by Adam's group [11] prescribes the incubation of 200 μg of cytochrome c with 5 μg pepsin at pH 2.6 and 25°C;

digestion is allowed to proceed for 14-18 h, at which time an additional 5 μg of pepsin is added and digestion continued for an additional 16 h.

In our study, however, digestion of cytochrome c by pepsin was of short duration, lasting only 5 min, which would result in less complete digestion compared with Adam's MP11 preparation method. The first 21 amino acids, therefore, are assumed to remain linked to the heme group under the experimental conditions used in our work. A Mascot database search was conducted in which an intact ligand mass value for the heme group was included, but this search failed to find any additional peptides. It is not known why peptide-heme complexes were not detected. One possible reason could be that the heme-linked peptide was not captured by the trap column and was washed off prior to the start of the gradient elution. In order to extend protein coverage to the heme-containing region, future work should address LC/MS analysis of MP11 and establish the experimental conditions needed for its determination.

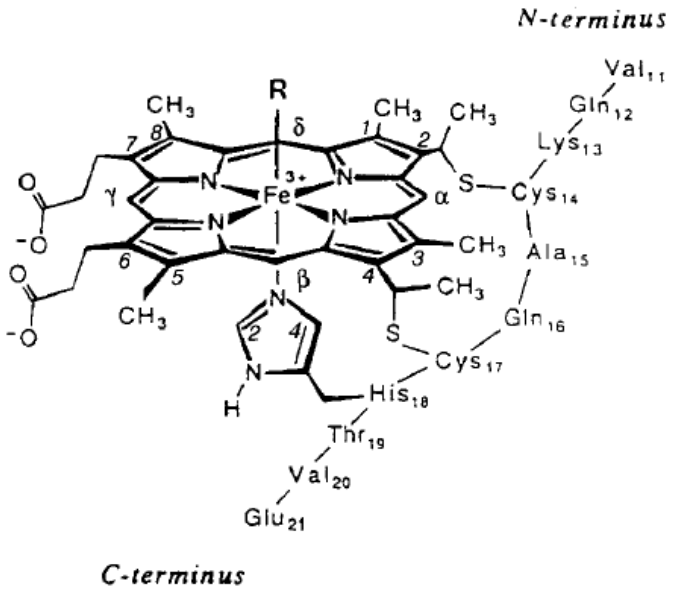

Figure 3.9 The structure of microperoxidase-11. [12]

3.4. Conclusions

Experimental results have shown that porcine pepsin A prefers to cleave cytochrome c at the carboxyl side of phenylalanine (F), leucine (L), and glutamic acid residues (E), although it can cleave at other sites as well. Cytochrome c was digested by pepsin for 5 min at pH 2.4 using an enzyme-to-protein ratio of 1:1. LC/MS/MS peptide analysis resulted in 78% sequence coverage of the 104 amino acid polypeptide chain of cytochrome c, namely, the peptide sequence from 22-104. The missing part of the sequence is believed to be a complex ligand consisting of the heme group covalently attached to the 1-21 peptide at Cys14, Cys17, and His18. Such a species would be similar to microperoxidase-11, which

has the same heme covalent attachment but with a shorter attached peptide sequence, namely, 11-21. A much shorter digestion was used in the present work compared to those typically used to prepare MP-11, and this difference could account for a longer peptide sequence remaining attached to the heme group. To extend peptide coverage to the heme-containing region of HHC, additional research on MP11 or other microperoxidases should be pursued.

3.5. References

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CHAPTER 4 HYDROGEN-DEUTERIUM EXCHANGE MASS SPECTROMETRY

4.1. Introduction

Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) has been widely applied to study the structure and dynamics of protein-protein and protein-ligand complexes [1]. The goal in HDX is to replace the covalently bound hydrogen atoms found in amide groups of the protein backbone by deuterium atoms [2]. Because HDX is acid catalyzed, the rate is greatly dependent on the pH and temperature. The reaction can be quenched by lowering the pH and/or temperature to inhibit back-exchange and allow sufficient time for MS measurements [3]. Characterization of protein-ligand interactions are made feasible by coupling HDX with electrospray ionization (ESI), a non-destructive ionization technique capable of transferring weakly associated complexes into the gas phase while preserving non-covalent interactions [4].

In the present chapter, we report results for both direct infusion analysis and peptide mapping analysis of horse heart cytochrome c (HHC). The goal is to study the binding interaction of HHC adsorbed on the COOH-SAM/Au surface from both analysis perspectives, i.e., direct infusion and peptide mapping. The ESI-MS direct infusion technique can provide insight into overall levels of HDX deuteration of proteins that are located in different environments and in particular for solution phase versus adsorbed HHC. Peptide mapping analysis can determine the location and relative amounts of deuterium uptake by performing protein digestion and detection of individual peptides by MS-MS [5]. In Chapter 3, we showed that peptic digestion of HHC in acidic solution allowed ~78% of the amino acid sequence to be identified under the experimental conditions that were employed.

Electrochemistry of adsorbed HHC on COOH-SAM/Au substrates was described in Chapter 2. Results showed that ~85% of adsorbed HHC (adsorbed at pH 7 in low ionic strength solution) underwent desorption by exposure to a 0.5% formic acid (pH~2.4) solution for 2 min. The formic acid simultaneously served to quench the D-to-H back reaction following the D_2O exposure. A suitable protein concentration range for intact protein direct infusion analysis is 5-20 μM. For a 10 μL injection volume, this corresponds to ~100 pmol of protein, which is 1-2 orders of magnitude greater than the ~4 pmol adsorbed on a single planar electrode, i.e., Γ of ~13 pmol/cm^2 for surface area = 0.32 cm^2. Thus, desorption of protein from an electrode surface would provide insufficient sample amounts for MS analysis. Previous work in Bowden's group [6], however, demonstrated that SAM-coated gold powder could be used in lieu of the planar gold film electrodes, providing a similar interface but with much higher surface area. The surface area of gold particle samples can be estimated using equation 4.1, which assumes spherical geometry. In this study, gold particles of <45 μm diameter were used, which gives rise to a minimum specific surface area (S) = 69 cm^2/ gram.

$$V_p = \frac{4}{3}\pi r^3 \qquad \text{Equation 4.1a}$$

$$S_p = 4\pi r^2 \qquad \text{Equation 4.1b}$$

$$S = \frac{m}{V_p \rho} \cdot S_p = \frac{3m}{r\rho} \qquad \text{Equation 4.1c}$$

Where V_p = volume of one particle, r = radius, S_p = surface area of one particle, ρ = density of gold, m = sample mass, and S = sample surface area.

4.2. Experimental Design

4.2.1. Materials

Deuterium oxide (99.9%), gold particles (<45 μm), horse heart cytochrome c and formic acid were purchased from Sigma Chemical Company. Porcine pepsin was purchased from VWR International. Water for all experiments was purified on a Milli-Q/Organex-Q system.

4.2.2. Au-SAM-HHC Preparation

Horse heart cytochrome c was purified via column chromatography as previously described in Chapter 2. Gold particles (diameter < 45 μm) were used in these experiments instead of gold films coated on glass slides. Procedures for cleaning, self-assembly, and adsorption of HCC were essentially the same as previously described for gold films [Section 2.2] except for the absence of any electrochemical treatment of the gold. Approximately 1 gram of gold particles was introduced into a 0.5 mL Eppendorf tube. For cleaning, the particles were sonicated in 1% Liqui-Nox detergent for 10 min followed by two additional 10-min sonications in Milli-Q water. Afterwards, the particles can be rapidly settled to the bottom of the tube by gently tapping on the side. For solvent or buffer exchange, the supernatant liquid was poured off and the particles were rinsed 1-2 times with the new solution. Self-assembled monolayers were formed by immersion of the gold particles for at least 12 h in a 95% ethanolic solution of 1 mM 11-mercaptoundecanoic acid at room temperature. After self-assembly, the gold particles were rinsed 3 times with 95% ethanol, followed by 3 times with Milli-Q water. HCC was adsorbed on the SAM/gold structures by

exposure to a 30 μM cytochrome c solution in 4.4 mM KPB (pH=7) at 4°C for at least 30 min. After the adsorption step, the gold particles were rinsed 3 times with Milli-Q water.

4.2.3. HDX of Adsorbed Cytochrome c and its Desorption

Gold particles carrying adsorbed cytochrome c were rinsed in D_2O to remove water. The HDX exchange time was commenced upon subsequent addition of D_2O. Exchange times of 30 min, 1 h, 2 h, 3 h and 4 h were evaluated. At the end of the HDX step, D_2O was poured off. Deuterated HHC molecules were then desorbed by immersion of the particles in 0.5% formic acid (pH~2.4) for 2 min (see Chapter 2 for further details), which also quenches the HDX reactions. A 1 gram quantity of gold particles can release ~700 pmol of HHC. After desorption, the final concentration of HHC was measured by UV-vis and further diluted with 0.5% formic acid solution to ~20 μM for mass spectrometry analysis.

4.2.4. HDX of Solution Cytochrome c

The exchange reaction was initiated by adding 200 μL of D_2O to 200 μL of 20 μM horse heart cytochrome c (in H_2O) at 25°C. The mixture was collected in a microcentrifuge tube (EMD Millipore, volume = 0.5 mL) and centrifuged for 5 min at 6500 rpm. Another 200 μL of D_2O was added to the sample retained by the centrifugal filter and the centrifuge step was repeated to fully exchange the buffer. The HDX exchange time was commenced upon addition of the initial 200 μL of D_2O. Exchange times of 30 min, 1 h, 2 h, 3 h and 4 h were evaluated. The HDX reaction was quenched by adjusting the pH to ~2.4 with 0.5% formic acid and lowering the temperature to ~0°C. To remove D_2O, 200 μL of 1% formic acid (in H_2O) was added to the HHC solution, which was then centrifuged for 5 min at 6500 rpm.

The final volume was adjusted to 200 μL using the HDX quenching buffer, maintaining the HHC concentration at ~20 μM.

4.2.5. Intact Protein Analysis

A time-of-flight mass spectrometer (Agilent Technologies 6210 LC-TOF) was used for intact protein analysis. Samples were directly injected into the LC-TOF via ESI without any LC separation. Native HHC in H_2O served as the control. Samples denoted as d-HHC_{soln} refer to samples of native solution cytochrome c that had been incubated in D_2O solution for various time durations, as described in Section 4.2.3. Samples denoted as d-HHC_{surf} refer to samples of adsorbed cytochrome c on SAM/Au particles that were immersed in D_2O for various times and then desorbed using 0.5% formic acid H_2O solution, as described in Section 4.2.2 and Chapter 2. To minimize back-exchange, the deuterium-modified HHC samples (d-HHC_{soln} and d-HHC_{surf}) were stored in an ice bath and analyzed as soon as possible. The temperature during the entire centrifugation procedure was controlled to ~0°C.

4.2.6. Peptide Mapping Analysis

An HPLC-MS/MS (Eksigent cHiPLC-Nanoflex System coupled with Thermo Fisher Scientific LTQ-Orbitrap XL) was used for peptide mapping analysis. The peptic digestion and mass spectrometry of HHC was described in Chapter 3. Samples of d-HHC_{soln} were incubated in D_2O for a defined period and then digested by porcine pepsin (HHC:pepsin = 1:1 mol/mol) for 5 min in 0.5% formic acid H_2O solution (pH~2.4). Solution samples of native HHC digested by pepsin under identical conditions acted as the control. The injection volume of each sample was 10 μL. The peptides were first separated on two sequential columns using a gradient mobile phase and then ionized via ESI for tandem MS/MS analysis

as described in Chapter 3. The Mascot database search was used to identify the peptides based on Orbitrap raw mass data.

4.3. Results and Discussion

4.3.1. Intact Protein Analysis

4.3.1.1 Native HHC

Native HHC (20 μM) in 100% H_2O was injected into the Agilent 6120 TOF-MS for intact protein analysis without LC separation. The masses were deconvoluted at the top of isotopic envelope centroid using Agilent MassHunter Workstation Software – Quantitative Analysis program. The measured molecular weight of native HHC was 12359.12 Da. The TIC direct infusion peak is shown in Figure 4.1 as 3 overlaid replicates. The entire time after injection was ~0.4 min. Deconvoluted masses from different parts of the peak were found to be consistent with each other. Figure 4.2 shows the deconvoluted mass spectrum at time after injection = 0.116 min, located within the first half of the peak.

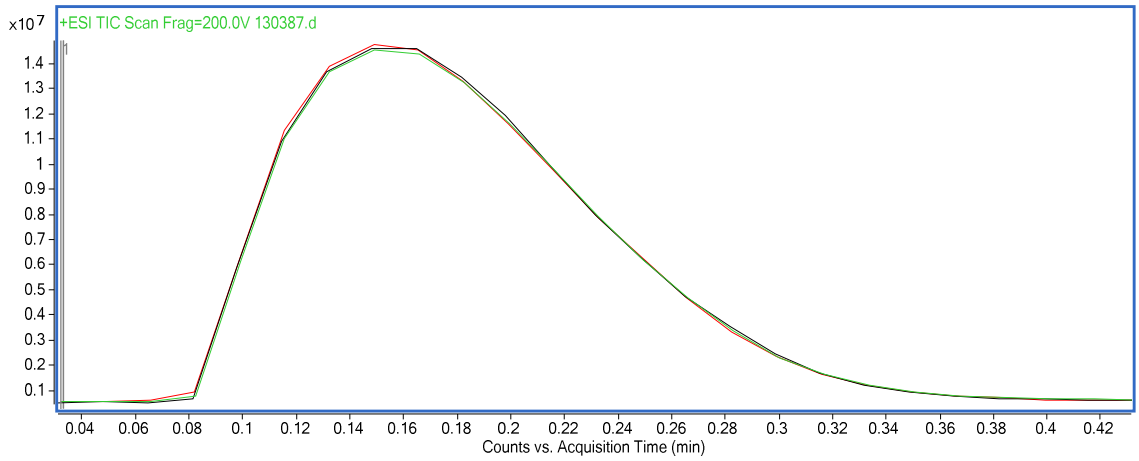

Figure 4.1 Direct infusion total ion currents for native cytochrome c in H_2O. Three replicate injections are overlaid.

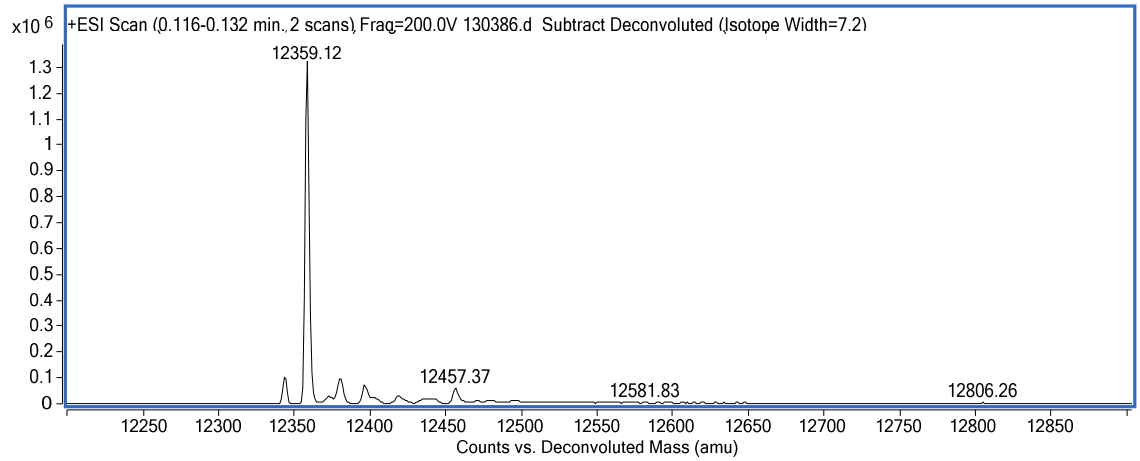

Figure 4.2 Deconvoluted mass spectrum of native cytochrome c in H_2O at a time after injection of 0.116 min.

4.3.1.2 HDX-Induced Mass Shifts

A significant mass shift for the intact protein was observed when samples of solution cytochrome c were deuterated. The deconvoluted masses of deuterated HHC (d-HHC_{soln}; 30

min exposure to D_2O) were integrated based on raw mass-over-charge data taken at different time after injection. It was observed that the intact protein mass was dependent on the time after injection at which the spectrum was acquired. For example, in Figure 4.3, the mass has shifted from 12409.26 Da at time after injection = 0.112 min to 12402.39 Da at time after injection = 0.229 min, a difference of 6.87 Da. This dependence of mass on time after injection was not observed when native HHC in 100% H_2O was analyzed. Figure 4.4 shows the dependence of the intact protein mass obtained from direct infusion as a function of time after injection for native and deuterated solution HHC samples. For deuterated samples, results for two different D_2O exposure times, 30 min and 2 h, are shown. In both cases, it was observed that the intact protein mass increased slightly at first with time after injection, but then fell off. We hypothesize that this phenomenon arises from two different factors involving H-D exchange reactions that took place during on-line sample processing. The first factor is the ionization of protein molecules by addition of some deuterons, which results in a 'virtual' increasing neutral protein mass (M). This reaction would be feasible because d-HHC_{soln} samples actually contained ~25% D_2O as a result of performing only a single buffer exchange following deuteration in order to keep sample handling time to an absolute minimum. The second factor is the back exchange of deuterium to hydrogen that occurs during sample transit between injection and detection. This reaction results in loss of isotopic labeling signals and brings about a declining mass. The observed mass is believed to reflect a combination of these two factors, as discussed next in more detail.

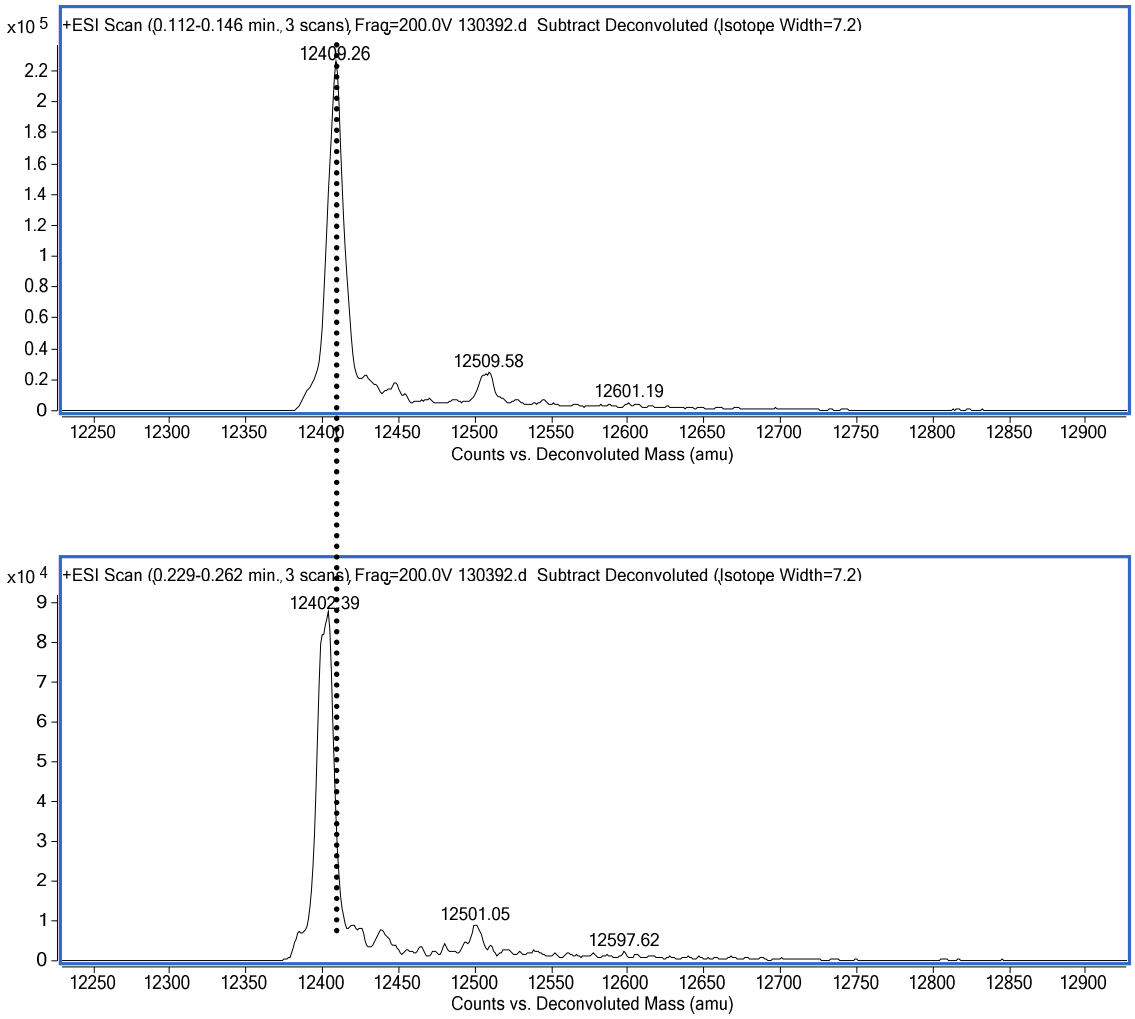

Figure 4.3 Mass spectra of deuterated solution HHC acquired at two time after injection: 0.112 min (top panel) and 0.229 min (bottom panel). D_2O exposure time was 30 min.

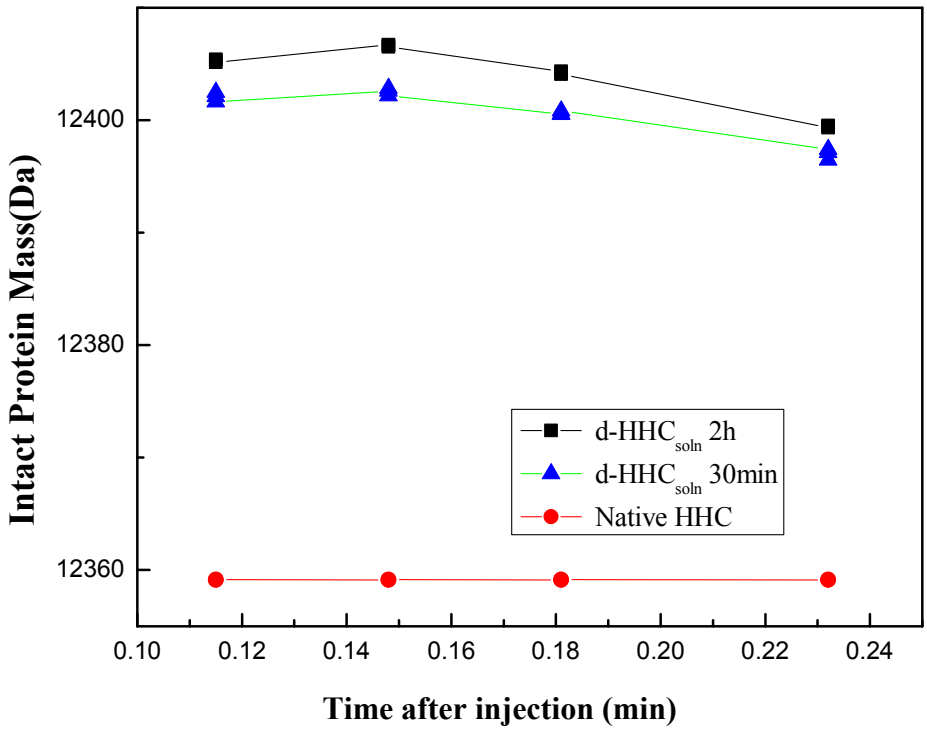

Figure 4.4 Intact protein masses of native and deuterated cytochrome c as a function of time after injection.

ESI ionization produces multiply-charged ions, $[M+nH]^{n+}$, where the protons originate from proton donor species in the ionization solutions. Neutral molecular mass (M) can be determined from equation 4.2. In our experiments, protons are assumed to

$$m/z = \frac{M+n^1H}{n} \qquad \text{(Equation 4.2)}$$

originate from hydroxyl groups on formic acid (HCOOH). Because of the ~25% D_2O content of samples, however, we would expect deuterium to exchange with hydroxyl hydrogen of formic acid, which would lead to ionization of proteins by ^2D as well as ^1H. Ions of the type $[M+nH+mD]^{(n+m)+}$ would accordingly be expected, with commensurately

increasing mass. Presumably, deuterium-for-hydrogen exchange on formic acid prevails initially over the H-D back reaction for the protein, discussed next, accounting for the initially increasing mass that was observed (Figure 4.4).

Back-exchange HDX would also take place on the labeled protein while the sample is undergoing analysis. This is believed to be the reason for the observed declining mass trend seen in Figure 4.4. As mentioned before, HDX has its lowest exchange rate at pH=2.4 and 0°C. Upon sample injection into the LC-TOF, however, the protein environment will change. For direct infusion, it takes at least 0.08 min for the sample to move through the injection loop and be detected. During that time, the sample is mixed with the ESI solution (0.2% formic acid in H_2O) and the temperature is uncontrolled. Although back-exchange can presumably occur both in solution-phase and gas-phase, the actual ESI process is fast, lasting only ~1 ms from droplet emission to the formation of gas-phase ions. The HDX back exchange that takes place in the solution phase is, therefore, presumed to be the major stage where deuterium labeling signals are lost.

The trend seen in Figure 4.4 can perhaps be rationalized on the basis of different reaction rates for the two processes described previously. During the first half of the direct infusion peak, H-to-D exchange on formic acid dominates, while during the second half, protein D–to-H has a greater effect. Under this scenario, more than 14.9% of the labeling signal is lost during solution-phase HD back-exchange.

Ultimately we seek to compare d-HHC_{soln} samples with d-HHC_{surf} samples and elucidate the nature of HHC/SAM binding interactions from the differential extents of deuteration. For this comparison, time after injection of 0.112 min was chosen. The impact

of on-line HDX processes is less during the first half of the peak, and, furthermore, the general trends in mass shift between different runs of d-HHC_{soln} were found to be similar at this time after injection.

4.3.1.3 Effect of Adsorption on Extent of Deuteration: Comparison of d-HHC_{surf} to d-HHC_{soln}.

For this comparison, deuterated solution cytochrome c (d-HHC_{soln}) serves as the reference sample for cytochrome c deuterated while adsorbed on SAM/gold particles (d-HHC_{surf}). Reference samples of d-HHC_{soln} were prepared for four different D_2O exposure times: 30 min, 1 h, 2 h and 3 h. Figure 4.5 shows the variation of intact protein mass over this time frame. All these data were acquired at time after injection = 0.112 min, where the impact of HDX artifacts on mass shift is minimized, as discussed above. The results show that the intact protein mass increases rapidly within the first 30 min of D_2O exposure, but at a much reduced rate thereafter. At 30 min, the HDX reaction has resulted in approximately 46 deuterons being added to the protein (mass increase of 46.19 Da), which corresponds to a deuteration extent of ~44% of the 104 amino acids in the polypeptide. A further 150 min of D_2O exposure resulted in the addition of another 8-9 deuterons (overall mass increase of 54.59 Da at 3 h), corresponding to a deuteration extent of ~52%.

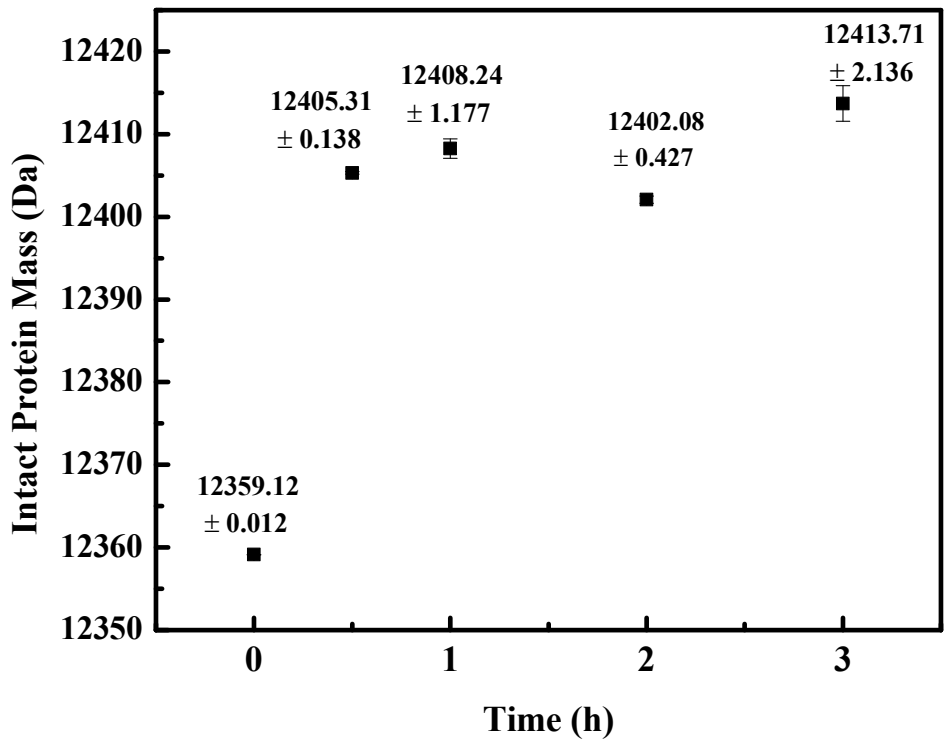

Figure 4.5 Intact protein mass of solution cytochrome c as a function of exposure time to D_2O. Masses were determined at time after injection = 0.112 min

Our working hypothesis is that cytochrome c adsorbed on a surface will be less prone to hydrogen-deuterium exchange than solution cytochrome c due to one side of the protein being more-or-less sequestered or protected from solution. To compare the extent of deuteration between solution and adsorbed cytochrome c, a D_2O exposure time of 30 min was selected based on results shown in Figure 4.5. To prepare samples of d-HHC_{surf}, cytochrome c was adsorbed on SAM/gold particles (see Section 4.2.2), exposed to D_2O for 30 minutes, and desorbed using formic acid, which also quenches the back exchange (see Section 4.2.3). A comparison of the intact protein masses is presented in Table 4.1, from which it can be discerned that adsorption does lead to a decreased extent of deuteration. The

average mass decrease of 10.85 Da indicates that 10-11 fewer amide hydrogen atoms underwent exchange during the 30 min of exposure to D_2O. The decrease in extent of deuteration in the adsorbed state is thus ~23%, i.e., (46.19-10.85)/46.19 x 100%. The important conclusion to be drawn from these results is that adsorption results in a significant decrease in extent of deuteration, presumably due to the sequestration of the contact domain side of cytochrome c from solution. This result therefore provides strong support for the validity of the overall approach being taken. Peptide mapping, however, will be needed to verify that it is the sequestered side of adsorbed cytochrome c that is the location where deuteration is less extensive.

Table 4.1 Comparison of intact protein masses of deuterated *solution* cytochrome c (d-HHC_{soln}) and deuterated *adsorbed* cytochrome c (d-HHC_{surf}).[a]

	d-HHC_{soln}	d-HHC_{surf}	Δm (Da)[b]
Replicate 1	12405.36	12394.79	–10.57
Replicate 2	12405.41	12394.43	–10.98
Replicate 3	12405.15	12394.16	–10.99
Average	12405.31	12394.46	–10.85
Std Dev	0.138	0.316	0.24
% deuteration	44.1% [c]	34.1% [c]	–23% [d]

[a]30 minute exposure to D_2O.

[b]Mass of d-HHC_{surf} minus the mass of d-HHC_{soln}.

[c]Relative to theoretical 100% deuteration of the polypeptide chain (104 amino acids)

[d]Percentage decrease due to adsorption: –10.85 / (12405.31 – 12359.12) x 100% = –23%

4.3.2. Peptide Mapping Analysis

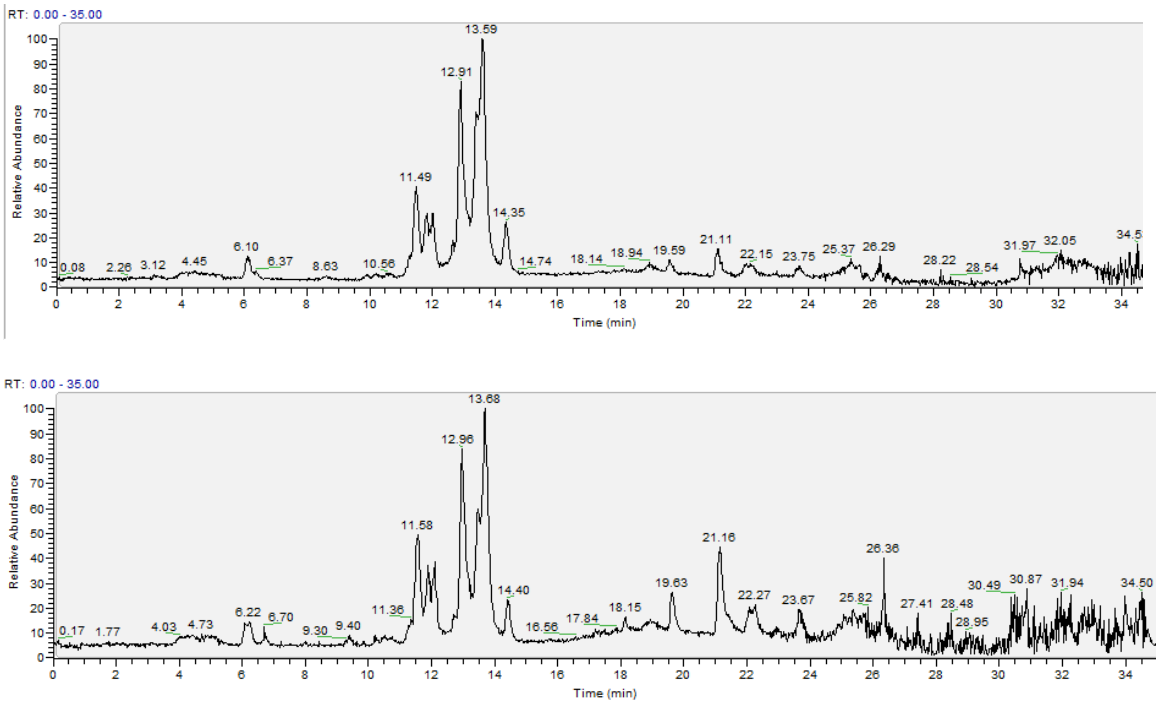

Figure 4.6 Liquid chromatography of peptic digestion mixtures of native HHC (top) and deuterated solution cytochrome c (d-HHC$_{soln}$) (bottom). The deuterated samples were incubated for 5 h in D_2O.

Our attempts to obtain useful data from peptide mapping analysis of deuterated cytochrome c were not very successful due to experimental limitations that precluded effective control of deuterium back exchange. Figure 4.6 displays typical liquid chromatograms of digested native cytochrome c (top panel) and deuterated solution cytochrome c (bottom panel). The shapes and times are very similar, as to be expected. Mass analysis successfully detected all 37 peptides listed in Table 3.2. Of these, however, no significant isotopic envelope mass shift was observed for 36 of them. A typical result is shown in figure 4.7 (for peptide 37-64). Figure 4.8 displays mass spectra for peptide 46-64, the only peptide that exhibited an isotopic mass shift due to deuterium atom uptake. The

isotopic signals are assumed to have been lost for nearly all the peptides because of back exchange during LC-MS analysis. The entire analytical time required for peptide mapping exceeded 20 min and was without pH and temperature control during this time. These results clearly demonstrate the need to conduct peptide mapping using state-of-the-art HDX/LC/MS instrumentation, as discussed in Chapter 6.

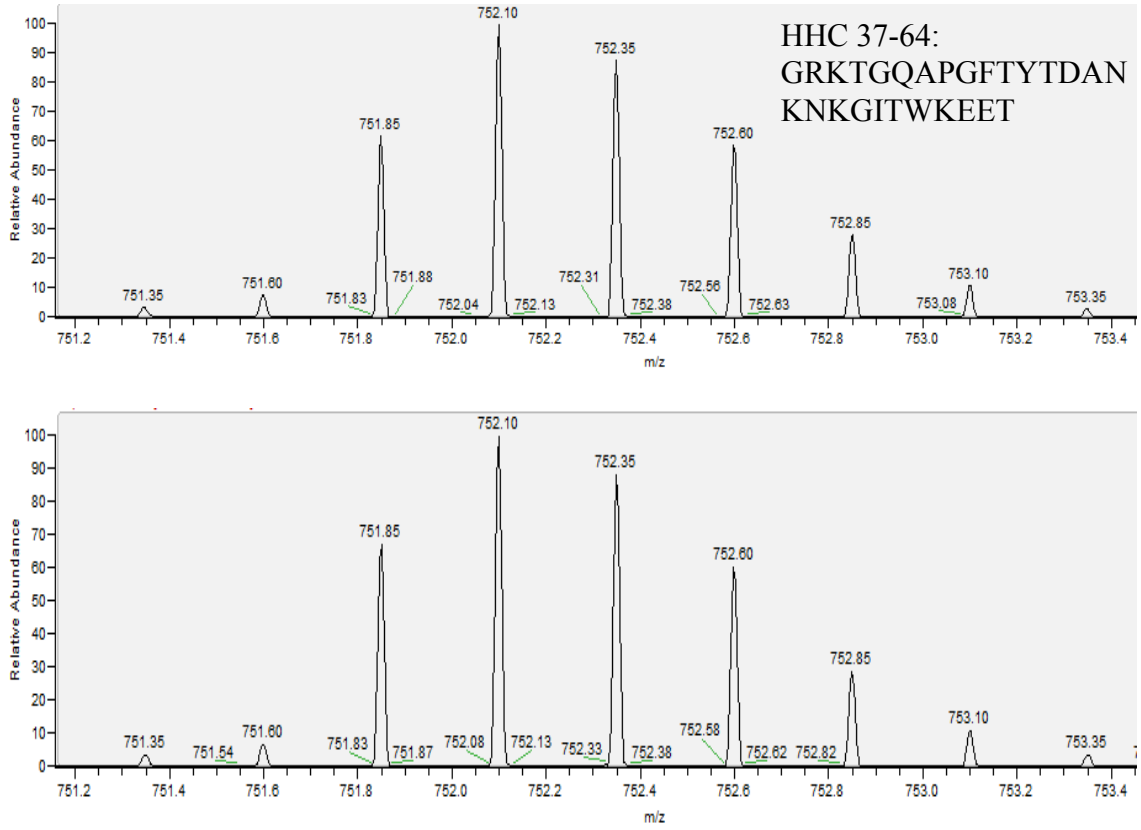

Figure 4.7 Mass spectra of HHC peptide 37-64 showing no mass shift between native and deuterated protein. Native HHC (Top) and deuterated solution HHC (5 h D_2O exposure) (Bottom).

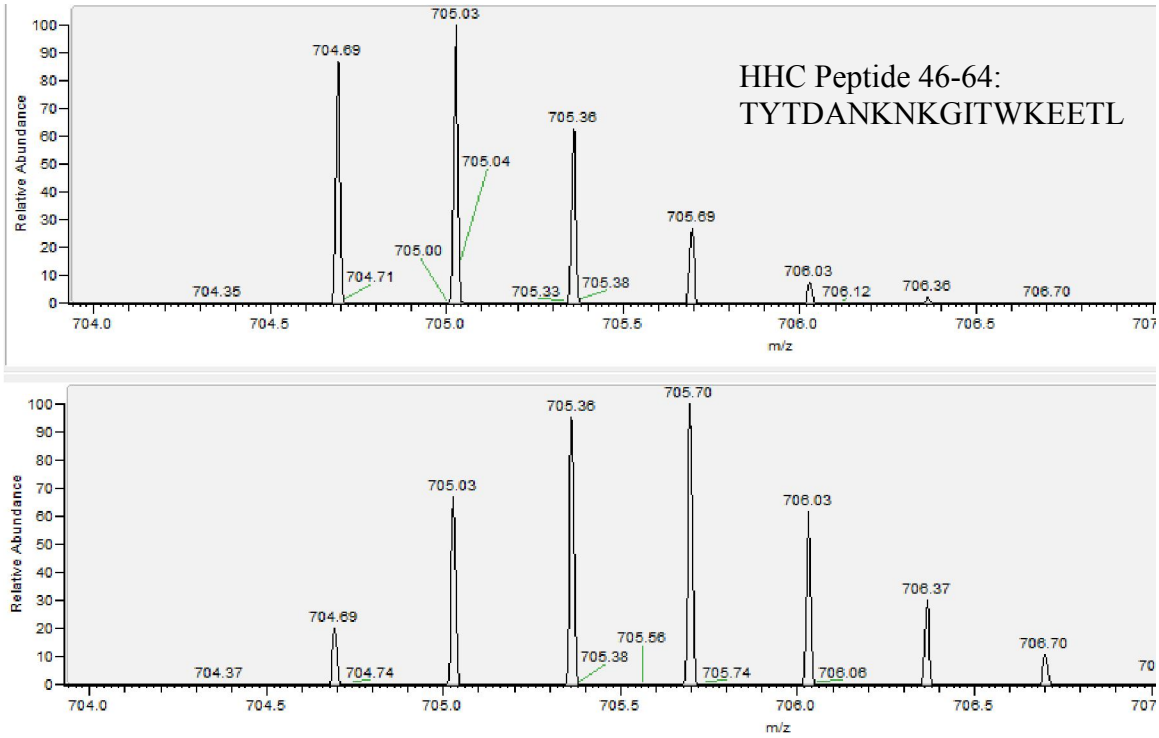

Figure 4.8 Mass Spectra of HHC peptide 46-64. Native HHC (Top) and deuterated solution HHC (5 h D_2O exposure) (Bottom).

4.4. Conclusions

Direct infusion of intact protein was examined to gain information on global extents of deuteration. Results showed that adsorption of cytochrome c on COOH-SAMs resulted in a 23% decrease in deuteration extent relative to solution protein under the conditions examined here. This significant finding appears to validate the premise underlying this research project, namely, that the protected side of an adsorbed protein will experience decreased rates of HD exchange. Unfortunately, peptide mapping analysis, which would have confirmed the premise, was not successful due to experimental limitations that precluded effective control over the back exchange reaction. Any differential modification information was subsequently lost during the separation and analysis time. The need for

automated HDX/LC/MS instrumentation in future peptide mapping experiments was made clearly evident.

4.5. References

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CHAPTER 5 FUTURE DIRECTIONS

5.1. Future Work

In this thesis work, key evidence was obtained that validates the proposed HDX/MS strategy for determining protein orientation at solid/aqueous interfaces. Also, progress towards the eventual characterization of cytochrome c orientation on COOH-terminated self-assembled monolayers was documented, and key obstacles that remain have been identified. Using intact protein analysis, it was shown that the extent of deuteration of cytochrome c decreases by ~20% in the adsorbed state relative to the solution state. This is an important result that presumably reflects our assertion that rates of backbone amide deuteration will be decreased on the sequestered, or protected, side of adsorbed cytochrome c. In order to prove this assertion and to delineate the surface binding site on cytochrome c, detailed peptide mapping is needed to measure differential rates of deuteration throughout the protein structure. Although progress was made towards this latter goal, experimental limitations precluded its attainment. A major limitation was our inability to adequately suppress the HDX back reaction, which resulted in loss of isotopic labeling information. Accordingly, future work should be directed towards optimization of HDX/MS to minimize sample handling/analysis time and to exert more rigorous control over temperature. Future analysis should be undertaken using state-of-the-art instrumentation that is fully automated for effective control of the back reaction [1]. Commercial HDX-LC-MS instrumentation such as the LEAP H/D-X PAL [2] is equipped with online digestion and integrated cooling to minimize back exchange of deuterium labels during protein digestion, chromatographic separation and sample injection.

Our evaluation of HDX reaction extent for cytochrome c was limited to times of 0.5 h, 1 h, 2 h, 3 h to 4 h. A fuller evaluation of the deuteration behavior of both solution and adsorbed cytochrome c would be valuable, covering a time range of seconds to days, in order to establish a complete dynamic response curve. This would provide insight into the role of interfacial protein conformation by fully characterizing localized hydrogen-deuterium exchange rates for each peptide. Commercial HDX-MS instruments feature integrated software that can typically control the duration of HDX labeling to times as low as 10 sec.

Another challenge for HDX-MS investigations is the data analysis component. In HDX studies, dozens to hundreds of peptic fragments are commonly analyzed and data analysis can be time-consuming [3]. In recent years, a number of HDX analysis software packages have emerged, such as HX-Express [4], HD-Benchtop [5], Mass Analyzer [6] and DynamX (Waters). Future work should take advantage of such resources, which reduce data analysis time and provide enhanced interpretation. However, these approaches may extract data for only well resolved, cleanly identifiable peptides. Manual analysis of peptides, therefore, especially those of low signal-to-noise or resolution, may still be required.

Due to time constraints, the missing sequence consisting of amino acids 1-21, presumably found covalently attached to the heme moiety, was not investigated. To gain further insight into this impediment to a full sequence analysis, HDX-LC-MS analysis of microperoxidase-11 (and perhaps other microperoxidase species) is recommended. This particular aspect of the study is unique to cytochrome c because of the covalent heme attachment and would not be a factor in comparable analyses of most other proteins. Extending digestion time for adsorbed cytochrome c beyond the 5 minutes used in the

present work should decrease the length of the peptide remaining attached to the heme and thus extend the sequence coverage. A thorough study of the heme end of the protein as a function of digestion time should be undertaken using automated HDX-LC-MS instrumentation.

To complement and independently confirm HDX-MS orientation determination of cytochrome c on SAMs and other surfaces, one could consider using other techniques as well such as scanning probe microscopy [7] and surface-enhanced infrared difference adsorption spectroscopy (SEI-DAS) [8].

Finally, the extension of HDX methodology developed for cytochrome c to the determination of orientations of other immobilized proteins is highly desirable for assessing the generality of the approach. A related protein/SAM system that has been recently studied in our group, namely, dehaloperoxidase (DHP) adsorbed on COOH-SAMs [9-10] would also make for an interesting comparison. DHP, a monomeric hemoglobin from the marine annelid *A. ornata*, has a tertiary and secondary structure that is very similar to mammalian myoglobins and hemoglobins. Unlike cytochrome c, globins lack a well-defined binding site on their heme edge side and, as a result, they do not form uniquely oriented protein/protein complexes with protein redox partners such as cytochrome b_5 [11]. Based on electrochemical results, we have similarly proposed that DHP does not adsorb on COOH-SAMs in a preferred orientation, but rather in a broad distribution of orientations. A comparison of differential HDX modification of DHP to cytochrome c would appear to be a valid test of this hypothesis.

5.2. References

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