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Sourav Chakraborty
University of New Orleans

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Molecular Probes for Biologically Important Molecules: A Study of Thiourea, Hydroxyl radical, Peroxynitrite and Hypochlorous acid

A Dissertation

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

Doctor of Philosophy
in
Chemistry

by

Sourav Chakraborty

B. Sc. Chemistry (Honors) University of Calcutta, 1999
M.Sc. Wood Science and Technology, Forest Research Institute Deemed University, 2001

May, 2010

To my family...

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List of Abbreviations

3-NT	3-Nitrotyrosine
4-HBA	4-Hydroxybenzoic acid
4-HNE	4-Hydroxynonenal
ABC	Ammonium Bicarbonate
ADA	American Dental Association
AHA	American Heart Association
Apo A-I	Apolipoprotein A-I
Apo B-100	Apolipoprotein B-100
Apo B-48	Apolipoprotein B-48
BLAST	Basic Local Alignment Search Tool
CID	Collision Induced Dissociation
Compound 1	1,8-bis(phenylthioureido)naphthalene
CVD	Cardiovascular Diseases
DMSO	Dimethyl sulfoxide
DOPA	Dihydroxyphenylalanine
DTT	Dithiothritol
EDTA	Ethylenediamine Tetracetic Acid
e-NOS	Endothelial Nitric Oxide Synthase
ESI-MS	Electrospray Ionization – Mass Spectrometry
EST	Express Sequence Tag
GC-MS	Gas Chromatography – Mass Spectrometry
HACA	6-Hydroxy-2-aminocaproic acid
HAVA	5-Hydroxy-2-aminovaleric acid
H-bond	Hydrogen bond
HDL	High Density Lipoprotein
HNE-His	4-Hydroxynonenal – histidine adduct
HPLC	High Performance Liquid Chromatography
IgM	Immunoglobulin M
ISE	Ion Selective Electrode

LC-MS/MS	Liquid Chromatography – Tandem Mass Spectrometry
LDL	Low Density Lipoproteins
LTQ	Linear Trapping Quadrupole
MALDI-TOF	Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry
MCO	Metal Catalyzed Oxidation
MPO	Myeloperoxidase
NFK	N-formylkynurenine
NHLBI	National Heart, Lung and Blood Institute
NMR	Nuclear Magnetic Resonance spectrometer
Ox-LDL	Oxidized Low Density Lipoprotein
PBS	Phosphate Buffer Saline
PET	Photon Induced Electron Transfer
PVDF	Polyvinylidene Fluoride
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SIN-1	3-morpholinopyridone hydrochloride
TBAF	Tetrabutylammonium fluoride
TLC	Thin Layer Chromatography
TMS	Tetramethyl silane
TNF- α	Tumor Necrosis Factor - α
WHO	World Health Organization
Xcorr	X correlation score

Abstract

Numerous chemical species are important to the health of biological systems. Some species can be beneficial at low doses and harmful at high doses. Other species are highly reactive and trigger serious cell damage. Improved methods to detect the presence and activity of such species are needed. In this work, several biologically important species were studied using appropriate analytical techniques.

Fluoride is an important species in human physiology. It strengthens teeth and gives protection against dental caries. However, elevated concentrations of fluoride in the body can lead to health problems such as dental and skeletal fluorosis. Reported fluoride sensors used fluorescence quenching methods in determining fluoride concentration. Our study explored synthesis and characterization of 1,8-bis(phenylthioureido) naphthalene (**compound 1**) as a fluoride sensing molecule. **Compound 1** showed a remarkable 40 fold enhancement in fluorescence with 5 eq of fluoride addition. **Compound 1** also showed possibility of visual colorimetric sensing with fluoride.

Free radical mediated oxidations of biomolecules are responsible for different pathological conditions in the human body. Superoxide is generated in cells and tissues during oxidative burst. Moderately reactive superoxide is converted to peroxy, alkoxy and hydroxyl radicals by various enzymatic, chemical, and biochemical processes. Hydroxyl radical imparts rapid, non specific oxidative damage to biomolecules such as proteins and lipids. Superoxide also reacts with nitric oxide in cells to yield peroxynitrite, which is highly reactive and damages biomolecules. Both hydroxyl radical and peroxynitrite readily react with amino acids containing aromatic side chains.

Low density lipoprotein (LDL) carries cholesterol in the human body. Elevated concentration of LDL is a potential risk factor for atherosclerosis. Previous research drew a strong correlation between oxidized low density lipoprotein (ox-LDL) and plaque formation in the arterial wall. More importantly, oxidative damage causes structural changes to the LDL protein (apo B-100) which might facilitate the uptake of LDL by macrophages. In this study LDL was exposed to various concentrations of hydroxyl radical peroxynitrite and hypochlorite. Thereafter oxidized amino acid residues in apo B-100 were mapped by LC-MS/MS methods. We found widely distributed oxidative modifications in the apo B-100 amino acid sequence.

Keywords: fluoride, fluorescence enhancement, thiourea, chromogenic sensing, low density lipoproteins (LDL), Fenton Chemistry, free radicals, hydroxyl radical, peroxynitrite, hypochlorous acid, surface mapping, proteomics, LC-MS/MS, natural variants

Chapter 1.

Introduction to fluorescence enhancement based fluoride sensing

1.1. Fluoride - friend or foe?

Fluoride is one of the most important elements of the periodic table. It belongs to the halogen family (Group 17) of elements and showed high electronegativity. American Dental Association (ADA) and World Health Organization (WHO) identified fluoride as one of the most important elements in maintaining dental health.¹ It helps in preventing dental caries. It also gives protection against several other teeth and gum related diseases. Extensive research was done on the ability of fluoride to prevent and combat against dental caries.² This protection can be achieved by multiple ways. Three chief mechanisms were previously reported in the literature.²⁻⁶

- **Inhibiting bacterial metabolism:** Bacterial infection in the plaque region reduces the pH of the plaque significantly. Fluoride from the plaque fluid readily combines with H^+ and yields HF. HF diffuses through the bacterial cell wall, acidifies the cell and releases fluoride ion which inhibits enzymatic activities (enolase) inside the cell. The process becomes cumulative due to the trapped fluoride.^{5,7}
- **Inhibiting demineralization of the surface:** Dentin or enamel crystals markedly adsorb fluoride in their crystal surfaces thereby prevent dissolution of the teeth by acid. Supplemental fluoride plays a critical role in this process. Incorporated fluoride is not sufficient to prevent dental caries and fluoride supplement all through out the life span is necessary^{2,4} for maintaining dental health.

- **Remineralization of the surface:** In the cary lesion, fluoride from topical sources helps in nucleating and remineralizing the partially demineralized surface by forming fluorapatite [FAP: $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$] which is less soluble than carbonated hydroxyapatite.⁶

However, elevated concentrations of fluoride can lead to several physiological symptoms. Long term exposure to fluoride may cause dental, skeletal and crippling skeletal fluorosis. In severe cases it leads to mottled and disfigured teeth. Also it increases bone density in case of skeletal fluorosis. Therefore it makes the bone fragile and susceptible to fracture particularly at the joints. One of the major problems with fluoride in the physiological system is the inability of the system to remove fluoride by biochemical pathways.

Also, high concentrations of fluoride can initiate multiple problems in plants, aquatic and marine environment. Aluminum fluoride can also play critical role in cellular biochemistry of plants and animals.⁸

Additional resources provided results that were even more alarming and uncomfortable. Ayoob & Gupta's most informative review⁹ on fluoride contaminated ground water revealed that long term exposure to low level of fluoride (0.5, 0.7, 2.8 ppm) can cause wide geochemical disease such as fluorosis. An interesting recent review on a book was published in *Chemical and Engineering News*¹⁰ regarding fluoridation of water.¹¹ Unfortunately, the book could not remain unbiased. It was coined that the book instead of revealing a clear picture took sides in favor of fluoridation.

In recently published notes, the World Health Organization (WHO) recommended the fluoride level of drinking water should be 1.5 mg/L (ppm).¹ Interestingly, United States Environmental Protection Agency (EPA) does not regulate the addition of fluoride to water. EPA's Safe Drinking Water Information System (SDWIS) actively tracks fluoride concentration

only in water systems with naturally occurring fluoride level above the established regulatory limits (≥ 2.0 ppm).¹²

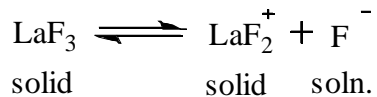
The objective of this research, in this regard, is not to raise a new debate on fluoride, but rather to emphasize the fact that the concentration of fluoride is extremely important in physiological and environmental systems. Improved analytical methodology is necessary to address some of the related problems in fluoride detection.

1.2. Fluoride Detection

Anions are important in physiological and environmental systems¹³⁻¹⁷. Various methods exist for determination of fluoride and other anions in solutions.^{13, 18, 19} Most commonly used methods are ion selective electrodes (ISE)^{20, 21} and photoluminescence. Photoluminescence methods are based on absorbance and fluorescence. In this chapter we will mostly discuss fluorescence based methodologies over other methods.

1.2.1. Ion selective electrode based detection of fluoride

Lanthanum fluoride, LaF_3 is widely used as a crystalline membrane electrode for determining the fluoride ion.²² Europium fluoride (EuF_2) can enhance its semiconductor property. The mechanism of the development of fluoride sensitive response (potential) are very similar to glass or pH sensitive membranes. In short, in the membrane interface ionization creates a charge on the membrane surface as shown by the following scheme (Scheme 1.1). The magnitude of the charge depends on the activity (commonly termed as concentration) of the fluoride ion in solution.



Scheme 1.1. Dissociation of LaF₃ in membrane electrode.

Commercially available LaF₃ electrodes are rugged and can be operated in the range of 0 – 80 °C. The response is linear down to micromolar (10⁻⁶ M ~ 0.02 ppm) concentration.²² The anionic interferences come from hydroxide ion. Other polyvalent cations can interfere in the measurements too by disrupting the chemical equilibria for the fluoride in solution. Basicity of F⁻ can cause problems at lower pH. Thus the measurements for ISE's are best within the pH range of 5 – 9. Also, ISE theoretically measures activity of the ions in solutions not the actual concentration.

Fluorescence based anion detection were a popular alternative to ISE in determining anions in solutions. Both fluorescence and absorbance methods are extensively employed to determine fluoride and other anion affinity towards various organic chromophores.

1.2.2. Fluorescence basics

Matter can absorb and re-emit light by the phenomenon called luminescence. Fluorescence and phosphorescence are the two different types of luminescence. Fluorescence involves emission from a singlet excited state to a singlet ground state. In phosphorescence, the emission involves a transition from a triplet excited state to a singlet ground state. Because the spin flip results in a forbidden transition, the lifetime of phosphorescence is considerably longer than that for fluorescence. Fluorescence lifetimes are around 10⁻⁸ sec, while those for phosphorescence are 10⁻³ to 1 sec or sometimes even longer. Most interestingly, in fluorescence, the energy of the emitted photons is at longer wavelength than that of the absorbed photons.

Some of the absorbed energy of the photons was channeled into other processes (vibrational level, phonons, thermal energy). The resulting emission wavelength is therefore longer (lower energy). The process is detailed in the energy diagram below called Jablonski diagram (Figure 1.1).^{22, 23}

A photon with frequency ν and energy $h\nu_a$ was absorbed by the ground state (S_0) fluorophore (fluorescing moiety) (typically 10^{-15} sec) and reached its singlet excited state S_1 or S_2 . At higher energy levels internal conversion (10^{-12} sec) occurs when the photons passes from higher vibronic level to lower vibronic levels (blue dotted arrows). Also non radiative (red dotted arrows) processes are associated with transfer of photons from high energy singlet state S_2 to lower energy singlet state S_1 . In short, the molecule will partially relax and reach the excited state singlet state S_1 . It can further relax back to the ground state by emitting a photon of energy $h\nu_f$ (which is of longer wavelength and lower energy than $h\nu_a$) and show fluorescence (represented by solid green arrow). Stokes shift is described as a shift to longer wavelength due to vibrational relaxation and internal conversion. Fluorescence lifetime typically is 10^{-5} seconds or shorter.

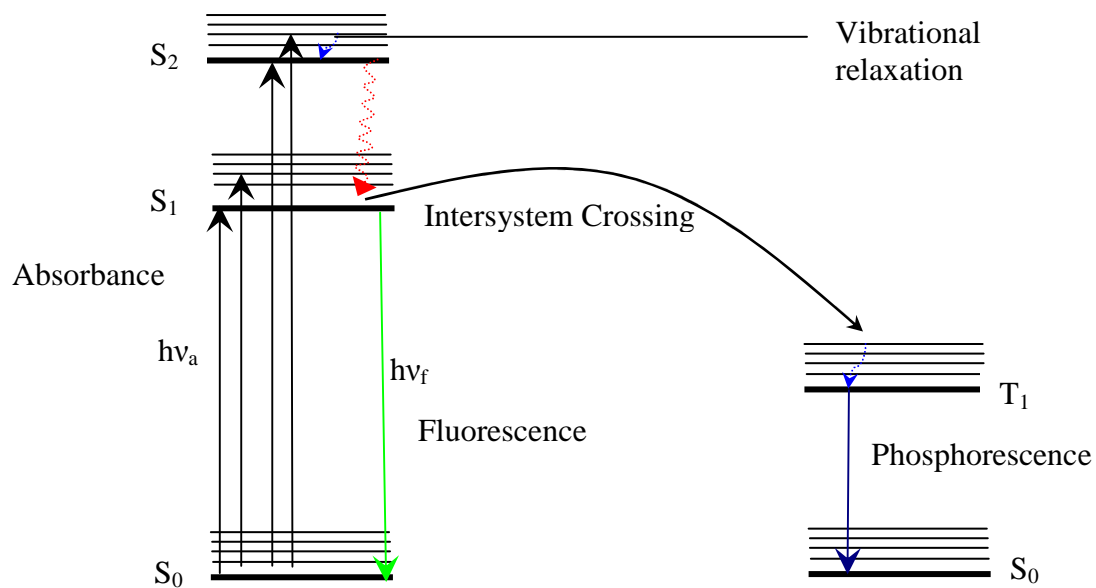


Figure 1.1 Jablonski diagram.

In phosphorescence, through an inter system crossing (ISC), a spin conversion of the molecule from excited state singlet S_1 to its triplet state (T_1). This transfer represents a spin forbidden transition. Due to spin-forbidden transition, phosphorescence (represented by a navy blue arrow) lifetime is much longer than that of fluorescence life time. Chemiluminescence is a related emission process in which the excited state is achieved through a chemical reaction instead of a photon absorbance. Once formed, the electronic excited state in chemiluminescence behaves the same as those observed in fluorescence.

Fluorescence has several different advantages over other analytical techniques.²⁰⁻²³ It is a non-invasive technique in the first place. The detection limit can easily reach nanomolar to sub-nanomolar levels. Fluorescence gives at least one to three orders of magnitude better detection limits than that of absorbance.²³ Also fluorescence has longer linear dynamic range compare to absorbance spectroscopy. Fluorescence is also more selective because not all compounds fluoresce. The compounds which show fluorescence have characteristic emission wavelength.

1.2.3. Example of typical fluorescence spectra

A fluorescence emission spectrum is obtained by scanning emission wavelength with a constant excitation wavelength (often at the absorbance maximum). An excitation spectrum can be obtained by scanning the excitation wavelength while keeping the emission wavelength constant. An idealized depiction of the excitation and emission spectrum is shown above. The peak-to-peak distance between excitation and emission maximum was considered as Stokes Shift (Figure 1.1).

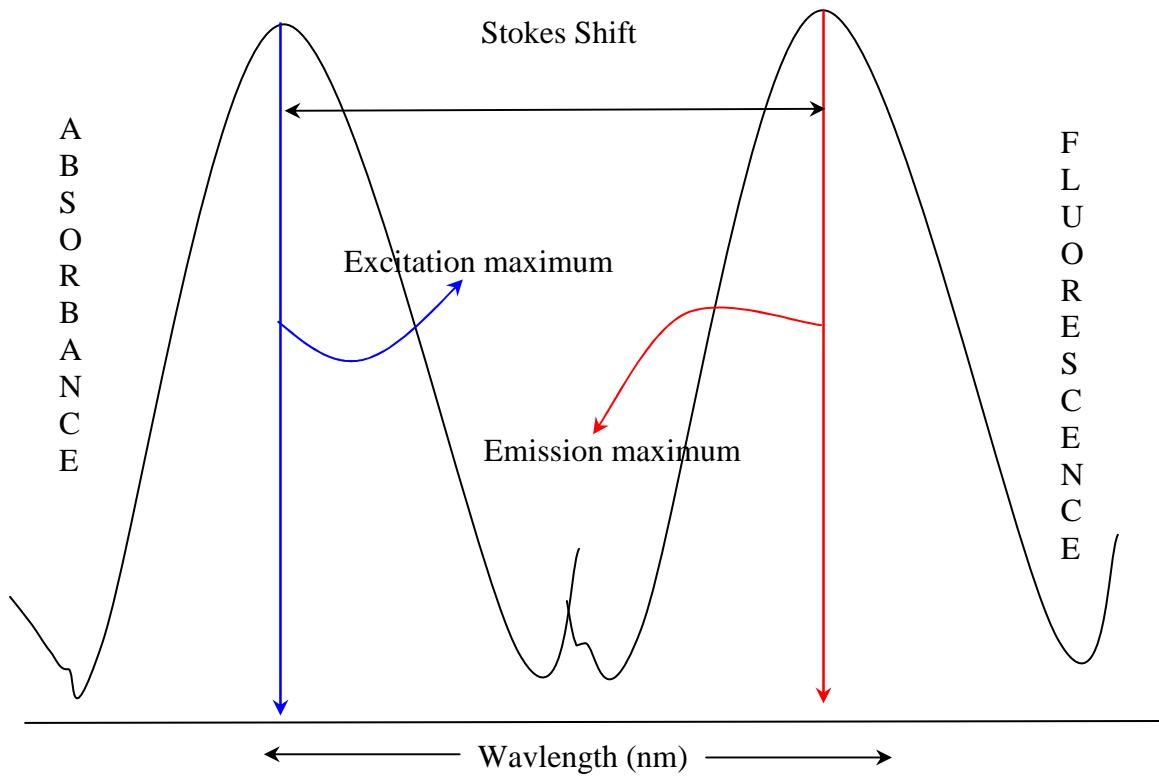


Figure 1.2 An idealized fluorescence spectrum was shown. Both excitation and emission scans were depicted. Stokes shift was also pointed out.

1.2.4. Benefits of fluorescence measurements

Fluorescence measurement exhibits some outstanding benefits over absorbance techniques, primarily because it is based on a dark background. In a typical fluorescence spectrophotometer the detector is placed at a 90° angle relative to the light source (Figure 1.3), while in absorbance the setup is 180°. A 90° configuration minimizes the transmission and reflection of non-fluorescent light reaching the detector. In other words scattered light does not interfere with the experiment.²² The signal to noise (S/N) ratio is substantially enhanced, and an approximately 10,000 times better detection limit can be achieved with a 90° setup.^{22, 23} The excitation and emission monochromators are wavelength selectors which allow specific wavelength to pass before and after the fluorescence events. Most interestingly, fluorescence detection is highly sensitive due to a cyclic process which gives the instrument a better detection. The same fluorophore when excited in a cyclic way can release thousands of photons during detection. For absorbance the cyclic method does not yield any improvements since the detector is in the same line with the light source.

Several different factors contribute to the fluorescence of a particular moiety. In general, a rigid structure, extended conjugation and planarity in the fluorescing moiety play a crucial role for a fluorophore to fluoresce.²⁴ In addition; excitation and emission spectra also depend upon the micro-environment and type of solvent used.¹⁹

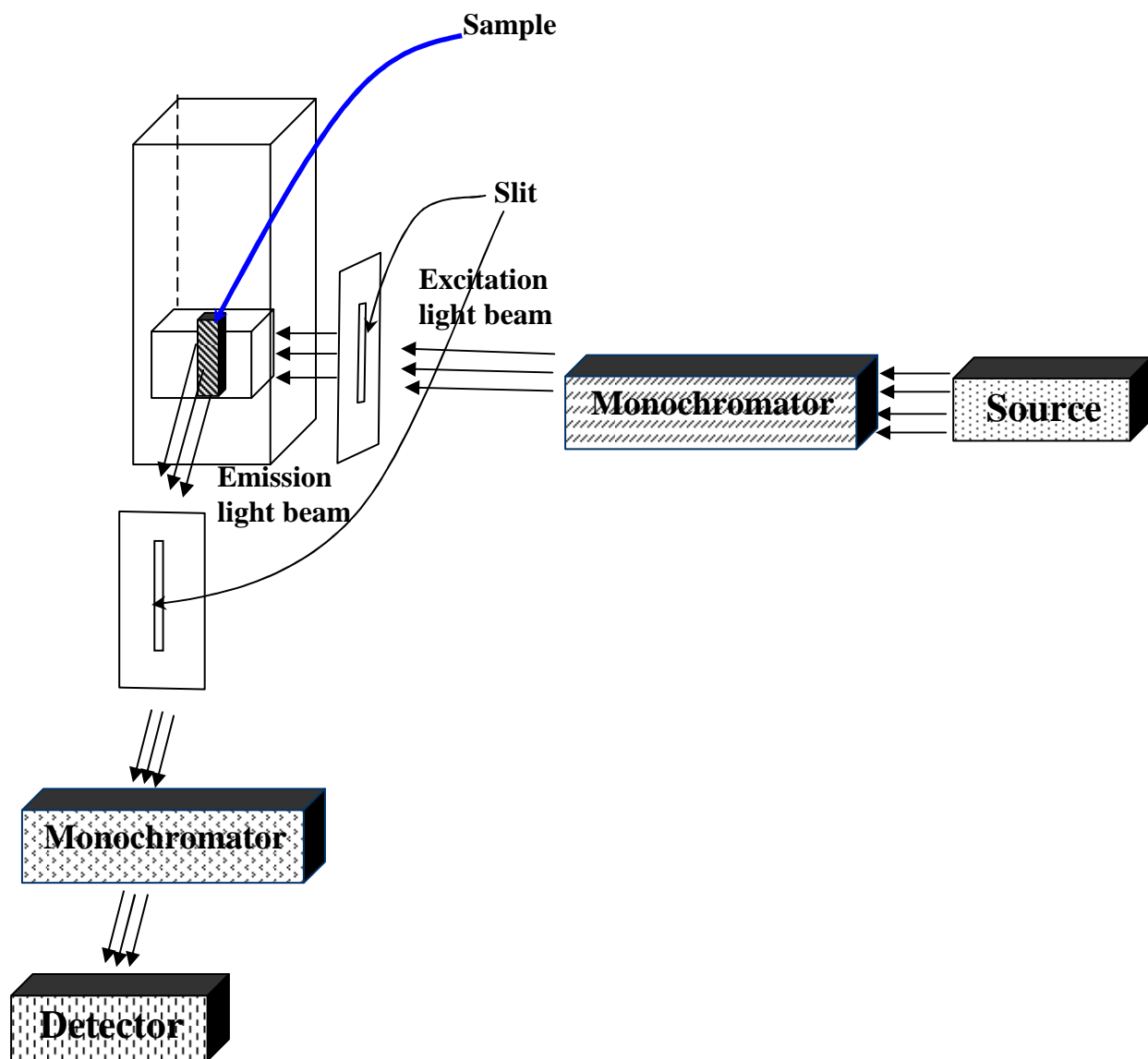


Figure 1.3 Schematic of a fluorescence spectrophotometer. A 90° angle between incident light and the emitted light was depicted in the picture. Monochromators are wavelength selectors for excitation and emission beams.²²

1.2.5. Fluorescence quenching and enhancement

The Term fluorescence quenching is related to the decrease in fluorescence of a fluorescing moiety by any process. Several different processes including excited state reaction, molecular rearrangements, energy-transfer, ground state complex formation and collisional quenching can cause quenching of fluorescence signal.²³

Collisional quenching is often termed as dynamic quenching as it involves the direct encounter of the excited state fluorophore to the quencher in a dynamic way. When the fluorophore interacts very strongly with the quencher and forms a non-fluorescent complex, it is known as a static quencher.²³ However, increase in fluorescence after complexation between the fluorophore and the interacting ligand can also occur and has advantages. Such interactions improve the signal to noise ratio. A lower limit of detection can also be achieved in the case of fluorescence enhancement compared to quenching. Fluorescence reveals pertinent information about the change in the electronic and molecular configuration during the fluorophore-ligand interaction.

1.3. Urea and thiourea in anion sensing

Hydrogen bonding was depicted as one of the most crucial events in anion sensing with urea and thiourea.^{18, 19, 25-31} Highly electronegative atoms such as oxygen, nitrogen and fluorine interact strongly with hydrogen forming a stronger interaction than dipole-dipole interactions. The interaction is however weaker than the regular ionic or covalent bond. Previous researches revealed the potential hydrogen-bond (H-bond) donor capability of urea and thiourea.^{19, 26, 27, 30-32} Both Urea and thiourea make strong H-bonds with dicarboxylates and hydrogen phosphates. Urea based receptors were exploited for various other applications including anion sensing.

Stephan and co workers³³ incorporated urea bond in lipophilic dendrimers. These unique dendrimers showed classical host-guest chemistry in separating anions from aqueous solutions³³. Xie and co-workers³⁴ reported a tripodal naphthylurea based moiety which fluoresces selectively in the presence of dihydrogen phosphate (H_2PO_4^-). Nam *et. al.*³⁵ explored a calix[4]diquinone compound where urea is appended in the structure of the compound. Interestingly, this compound showed selective affinity towards HSO_4^- over H_2PO_4^- , Cl^- and CH_3CO_2^- . In a related but slightly different subject, a complex calix[4]arene based thiourea compound was synthesized which showed better efficiency in transporting CsCl and KCl than other cation or anion receptors.³⁶

1.4. Fluoride Sensing

Over the past few decades, several different fluoride sensing molecules were reported in the literature.^{13, 17-19, 25, 37, 38} Boron, aluminum and calixarene based compounds showed promising results. Yamaguchi and coworkers reported a boron based anthracene compound where the boron π -orbital accepts electrons from fluoride³⁹ (Figure 1.4). The extensively conjugated boron anthracene compounds were highly colored. With these compounds, fluorescence quenching was observed with fluoride. Also a dramatic color change indicated the possibility of colorimetric anion sensing. Chiu *et. al.* reported another cationic borane which showed interaction between boron and fluoride via H-bonding.⁴¹

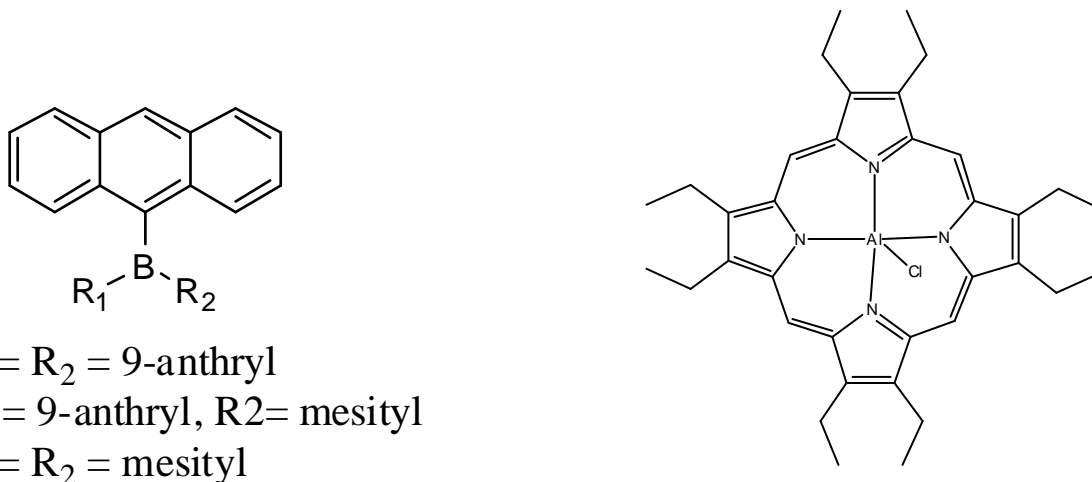


Figure 1.4 Boron based analogue by Yamaguchi *et. al.*³⁹ and aluminum based analogue reported by Badr *et. al.*⁴⁰

Aluminum binds strongly to fluoride. Aluminum porphyrin based compounds can be useful in sensing fluoride in solution. A thin polymer based sensor using aluminum (III) octaethylporphyrin was reported (Figure 1.4) that can detect fluoride in the micromolar to submicromolar range. This methodology, however, was based on absorbance detection.⁴⁰

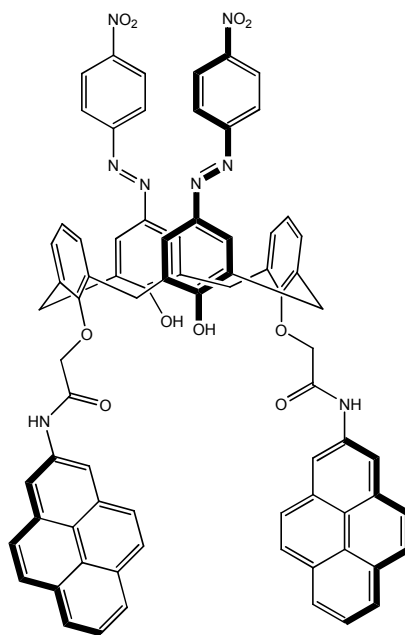


Figure 1.5 Calixarene based fluoride sensing molecule reported by Kim *et. al.*⁴²

Several different calixarene based molecules functionalized by urea and thiourea moieties were reported in the last two decades. Kim *et. al.* reported more than one calix[4]arene molecule which can behave as cation as well as anion sensors.⁴³ In 2006, a urea functionalized calix[4]arene based molecule was synthesized (Figure 1.5) which showed excimer emission as well as an increase in fluorescence in the presence of fluoride.⁴⁴ However, it was apparent that the amount of fluoride added was significantly high. Esteban-Gomez *et. al.* reported urea based supramolecular compounds which showed color development in the presence of various anions.²⁶

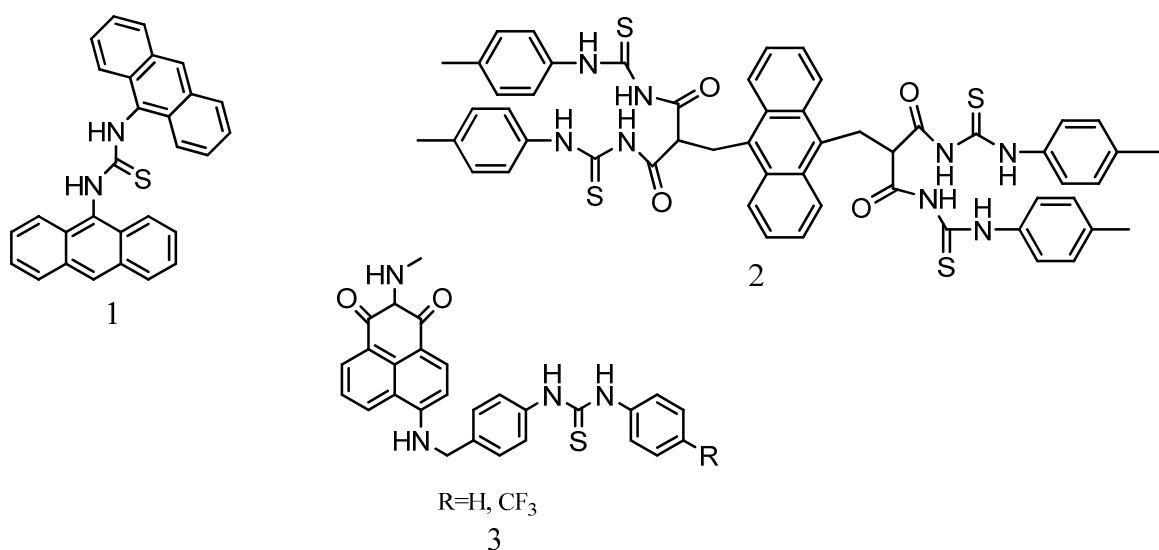


Figure 1.6 Various thioureido analogues reported by Gunnlaugsson *et. al.*^{19, 27, 45}. Both anthracene based receptors and thalimide based receptors were reported.

Ureido protons are somewhat acidic in nature. Highly basic anions such as fluoride or acetate can easily abstract two hydrogen atoms from the urea bond. Although absorbance represented poor detection limit compare to fluorescence, the possibility of chromogenic sensing had revealed some interesting new sights in urea based sensing. Gunnlaugsson's pioneering work in the area of anion sensing^{19, 27, 45} incorporating urea and thiourea in supramolecular structures (Figure 1.6) revealed several interesting facts. Most of these urea and thiourea based compounds

interact strongly with fluoride by strong hydrogen bond formation involving the ureido or thioureido hydrogens. In the same context these groups also reported several different naphthalimide based receptors which can be used for fluoride sensing based on absorbance as well as fluorescence.¹⁹ These receptors work following the photoinduced electron transfer (PET) mechanism.⁴⁵ Several anthracene and naphthalimide based urea and thiourea moieties (Figure 1.6) were studied for fluoride, acetate, hydrogen phosphate and other halides. Both absorbance and fluorescence results were presented for ureido and thioureido compounds. The majority of these compounds showed PET based quenching which was originated from fluoride binding leading to conformational changes in the respective compounds.¹⁹

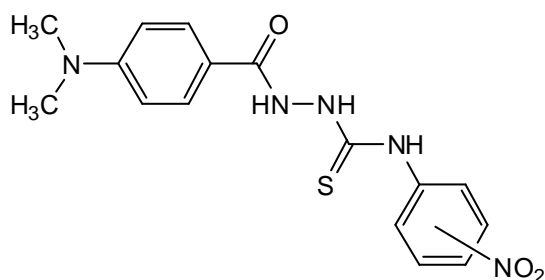
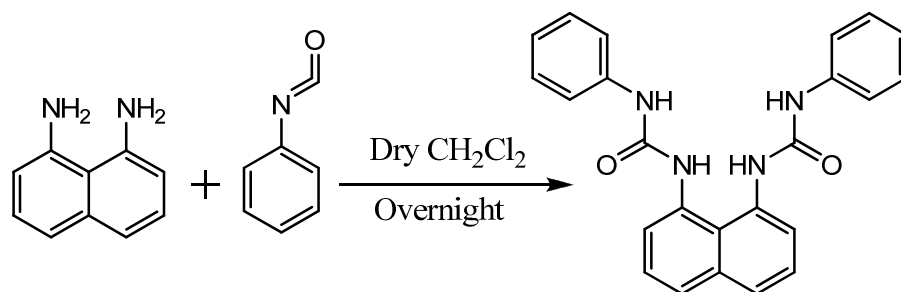
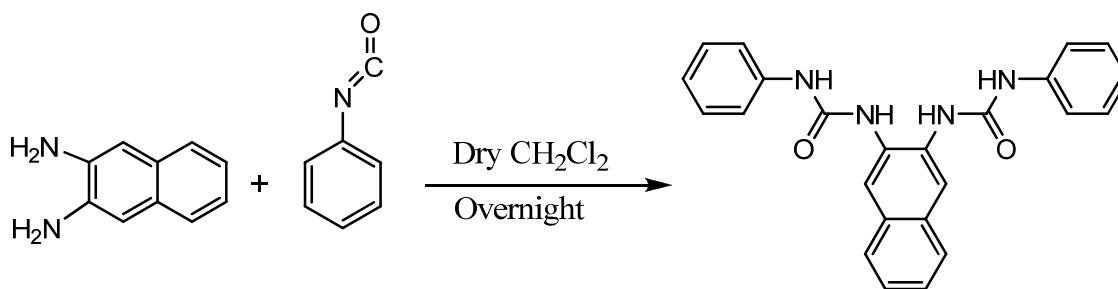


Figure 1.7 Thiourea compound reported by Nie and co-workers³¹. The compound showed fluorescence enhancement with acetate and fluoride.

Nie and coworkers³¹ over the years reported several phenyl thiourea based compounds (Figure 1.7) which showed fluorescence enhancement or quenching in the presence of acetate even over fluoride.^{28, 30, 31} In most cases a clearly defined isosbestic point²³ was observed after addition of the host. This indicated the change in conformational structure after binding to the host. Some of these studies showed fluorescence enhancement in presence of the analyte. Detection limit for various hosts lie in the range of micromolar to submicromolar range.



Scheme 1.2 Synthesis of 1,8 bis-(phenylureido) naphthalene analogue as presented by Xu *et. al.*⁴⁶ and Cho *et. al.*⁴⁷



Scheme 1.3 Synthesis of 2,3 bis-(phenylureido) naphthalene analogue reported by Xu *et. al.*⁴⁶

Naphthalene is a substantially rigid molecule with extended conjugation. Cho *et. al.*⁴⁷ studied phenylurea immobilization on diaminonaphthalene based systems. Their work was focused on chromogenic sensing of anions. Therefore the research was mostly emphasized on absorbance measurement while the whole new vista of fluorescence remained unexplored. Xu *et. al.*⁴⁶ synthesized phenylurea analogues of 1,8-diaminonaphthalene (Scheme 1.2) and 2,3-diaminonaphthalene (Scheme 1.3). 1,8-bis(phenylureido)naphthalene showed fluorescence enhancement with fluoride while the 2,3-analogue did not reveal any promising results. It was indicated that the presence of fluoride could induce planarity to the 1,8-analogue to yield extended conjugation and fluorescence enhancement. Jose and coworkers⁴⁸ reported urea and thiourea based anthraquinone compounds (Figure 1.8) which can act as a fluoride sensing molecule. A pronounced variation in the absorbance spectra was observed after addition of

fluoride to the respective ureido and thioureido analogues. However fluorescence behavior of the mentioned ureido and thioureido analogues were not explored extensively in Jose's study.

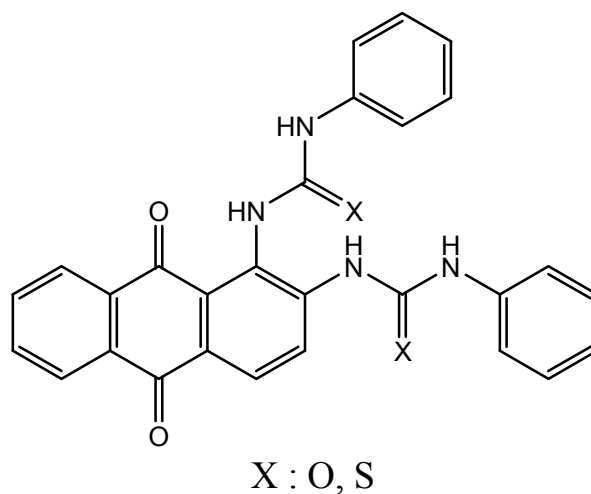


Figure 1.8 Anthraquinone ureido and thioureido analogue reported by Jose *et. al.*⁴⁸

Considering the above mentioned approaches we have synthesized 1,8-(bisphenylthioureido)naphthalene⁴⁹ (**Compound 1**) as a potent fluoride sensing molecule (Figure 1.9). The compound was characterized by ¹H, ¹³C NMR and MALDI-Mass spectrometry. Fluorescence experiments were performed on the compound in the presence of fluoride and other halides. Acetate and hydrogen phosphate were also added. A remarkable 40 fold enhancement in fluorescence signal was observed in the presence of 5 equivalence of fluoride in acetonitrile.⁴⁹ It was selective to fluoride over other halides. Hydrogenphosphate and acetate did show enhancement in fluorescence, but the increase in fluorescence signal was small compared to fluoride. From the structure it is evident that four thioureido protons can interact strongly with fluoride, which is small and has a high charge density.

In addition, **Compound 1** (Figure 1.9) showed promising results (Chapter 2) in the area of visual colorimetric chromogenic sensing.⁴⁹ The solution of **compound 1** changed color (a bathochromic shift was observed in the absorbance spectra) upon addition of 10 equivalent of

fluoride in acetonitrile. The change in color was visually perceptible at high concentration of fluoride.

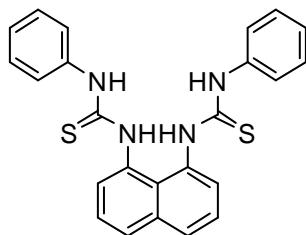


Figure 1.9 1,8-bis(phenylthioureido)naphthalene (Compound 1).

Binding affinity of fluoride to the thioureido protons were discussed in previously published articles.^{19, 44} Plausibly, the twisted structure of naphthalene, particularly after isothiocyanate immobilization, could achieve higher degree of planarity in the presence of fluoride ion. Fluoride being small could gain a high degree of accessibility to the thioureido protons thereby enhancing the ability to form strong H-bond with the molecule. Remarkable fluorescence enhancement after addition of fluoride to **compound 1** suggests that interaction with fluoride was forcing the twisted structure to become planar. In ureido compounds energy minimization calculation (AM 1) supports the conformational changes around the naphthalene ring for better planarity.⁴⁶ In anthraquinone based thioureido analogues Jose *et. al.*⁴⁸ calculated relevant energy minimization data (AM 1). Jose's calculation revealed the insight that fluoride, being the smallest among the halides, will reach closer to the thioureido protons. The other halides being bigger could not fit themselves properly in the space near thioureido protons.

Compound 1 gives several advantages over the other reported fluoride sensing molecules. While a large number of fluoride sensing molecule were based upon fluorescence quenching^{19, 50} or absorbance,^{47, 51} **compound 1** showed fluorescence enhancement in the presence of fluoride. No other halides interfere in this detection. Very high concentrations of

acetate or hydrogen phosphate can interfere slightly, although it can not completely undermine the detection limit of **compound 1**.

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Chapter 2.

Fluoride detection based on fluorescence enhancement of naphthalene thioureido derivative

2.1. Abstract

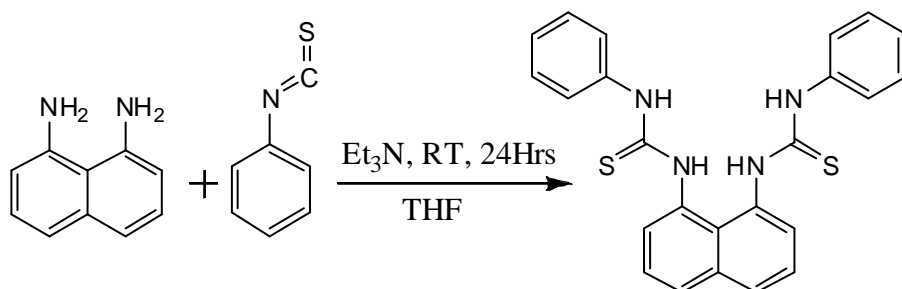
A novel thioureido naphthalene derivative was synthesized and characterized. The compound proved itself as an effective fluoride sensor with respect to selectivity and sensitivity. In acetonitrile, the fluorescence intensity increased 40 fold with addition of 5 equivalents of fluoride. Fluorescence intensity did not substantially change with other halides, suggesting that the thioureido protons interact strongly with fluoride but not with other halides. The enhanced fluorescence is due to increased quantum efficiency of the fluoride complex.

Keywords: Fluoride sensing; thioureido compound, fluorescence

2.2. Introduction

Anions play various important roles in biological and environmental processes in nature.¹⁻⁶ Though considerable attention has focused on the selective detection of anions using visible colorimetric sensors,⁷⁻¹⁹ less progress has been made with the more powerful technique of fluorescence. Fluoride sensing is important since fluoride plays crucial roles in dental caries²⁰ and other medical issues such as osteoporosis.²¹ Excess fluoride however causes fluorosis,²²⁻²⁵ a type of fluoride toxicity that is typically related to an increase in bone density. The important consequences of elevated and depleted fluoride concentration make effective fluoride sensing an important tool. Though a series of fluoride sensor compounds have already been reported^{7, 9, 12, 13,}

^{15-17, 26-41} by various researchers, there remains a strong practical impetus to improve the detection at micromolar to submicromolar³⁰ levels. In addition past research mainly focused on urea based sensors^{27-29, 31} while the whole new vista of novel thiourea based sensors^{12, 15, 16, 36, 40, 41} was largely unexplored (Chapter 1). We therefore synthesized (Scheme 2.1) and characterized 1,8-bis(phenylthioureido)naphthalene as a fluorescent probe for fluoride detection as well as a chromogenic fluoride sensor. The structure of compound 1 was confirmed by ¹H (Figure 2.1) and ¹³C NMR (Figure 2.2) and mass spectral analysis (Figure 2.3). In the presence of fluoride, this compound shows a new fluorescence peak (with $\lambda_{\text{max}} = 447.5 \text{ nm}$), as shown in Figure 2.4. Chloride (Figure 2.5), bromide (Figure 2.6) or iodide (Figure 2.7) addition did not cause any change in the fluorescence intensity or shape.



Scheme 2.1 Synthesis of 1,8-bis(phenylthioureido)naphthalene (Compound 1).

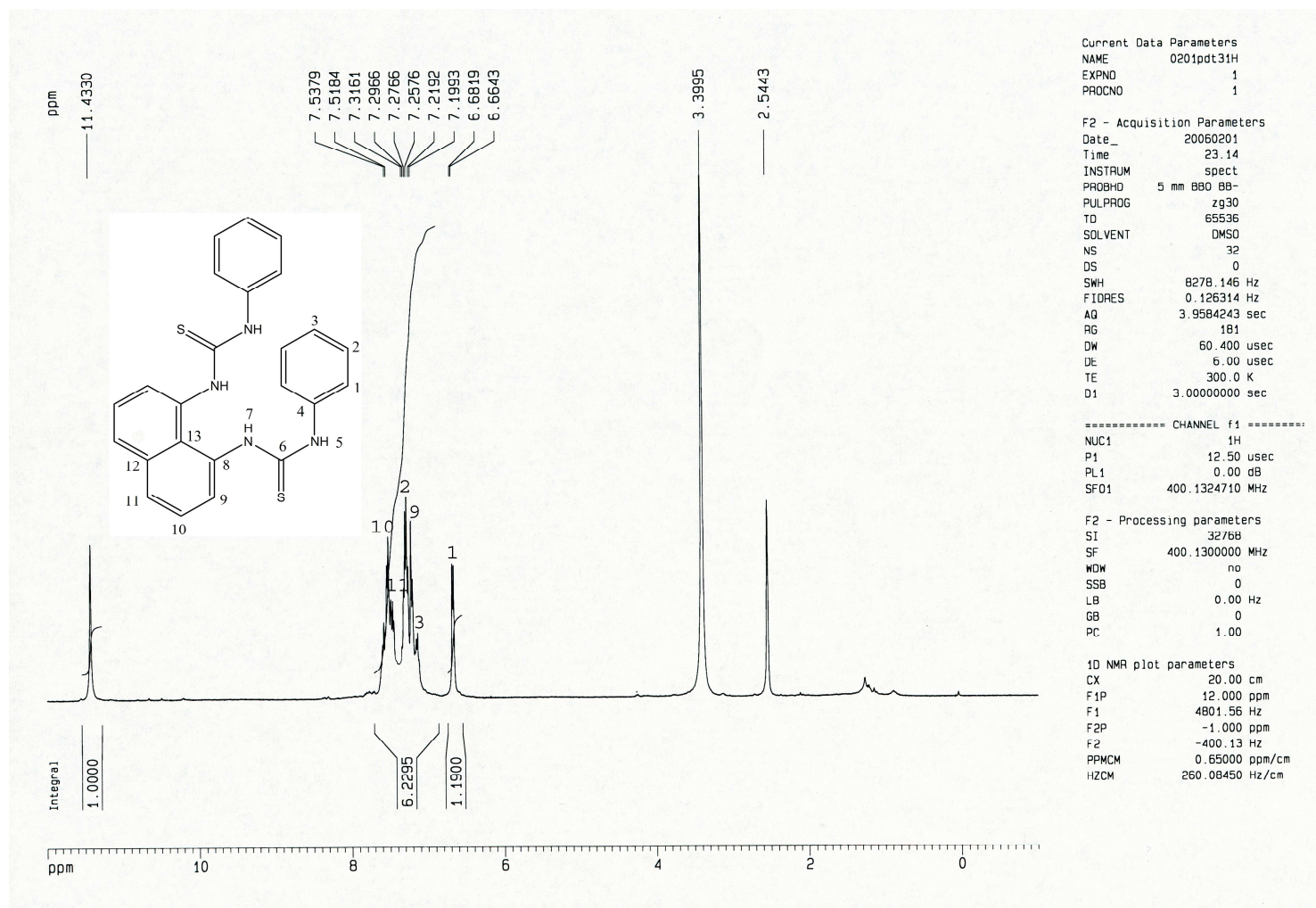


Figure 2.1 ^1H NMR spectra for compound 1. Frequency calculation data can be viewed at the end of the chapter in selective data for compound 1 section. DMSO was used as a NMR solvent. Two thioureido protons were missing from the spectra due to rapid exchange with the solvent.

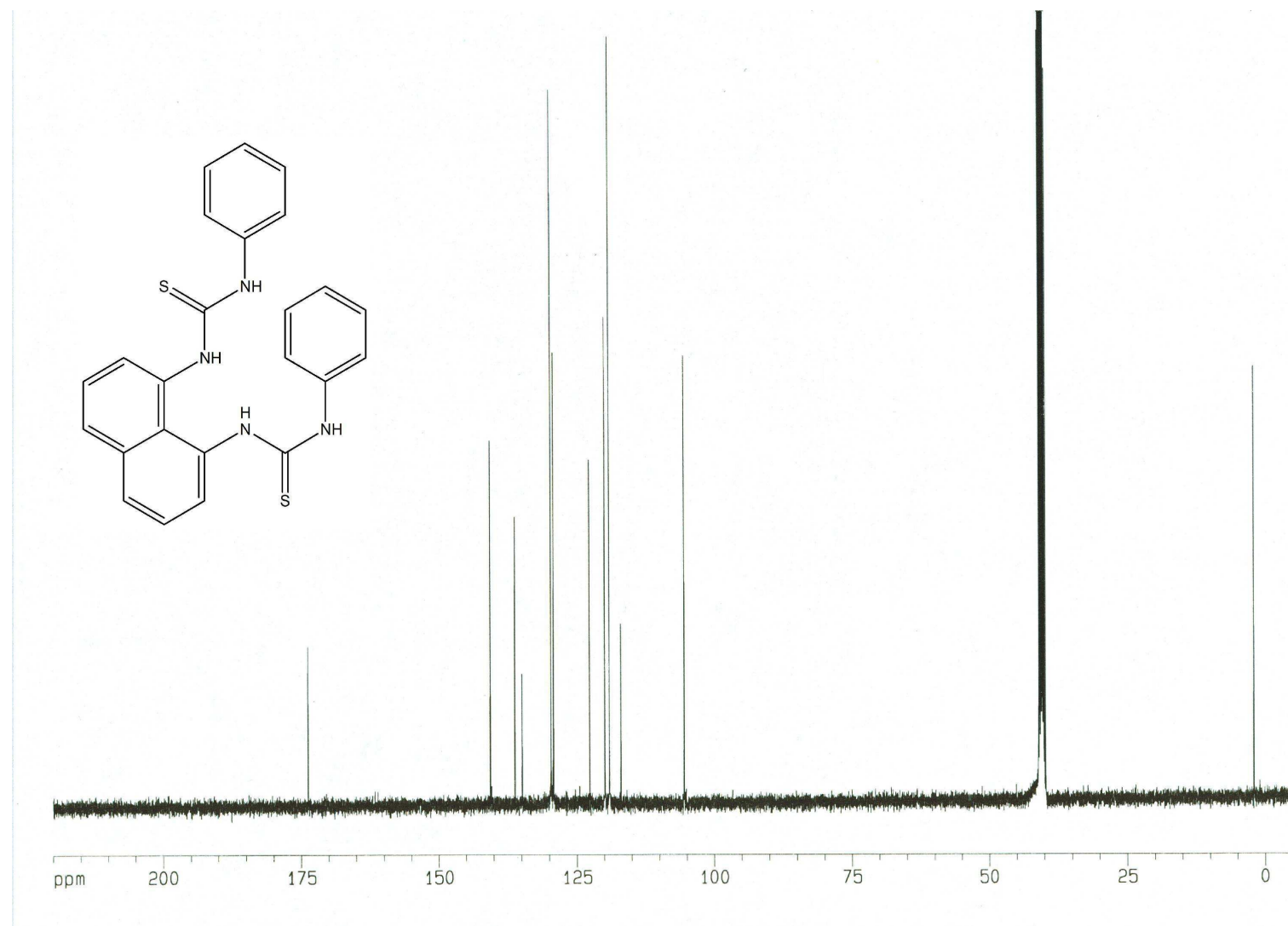


Figure 2.2 ^{13}C NMR data for compound 1. Frequency calculation data can be viewed at the end of the chapter in selective data for compound 1 section. DMSO was used as NMR solvent.

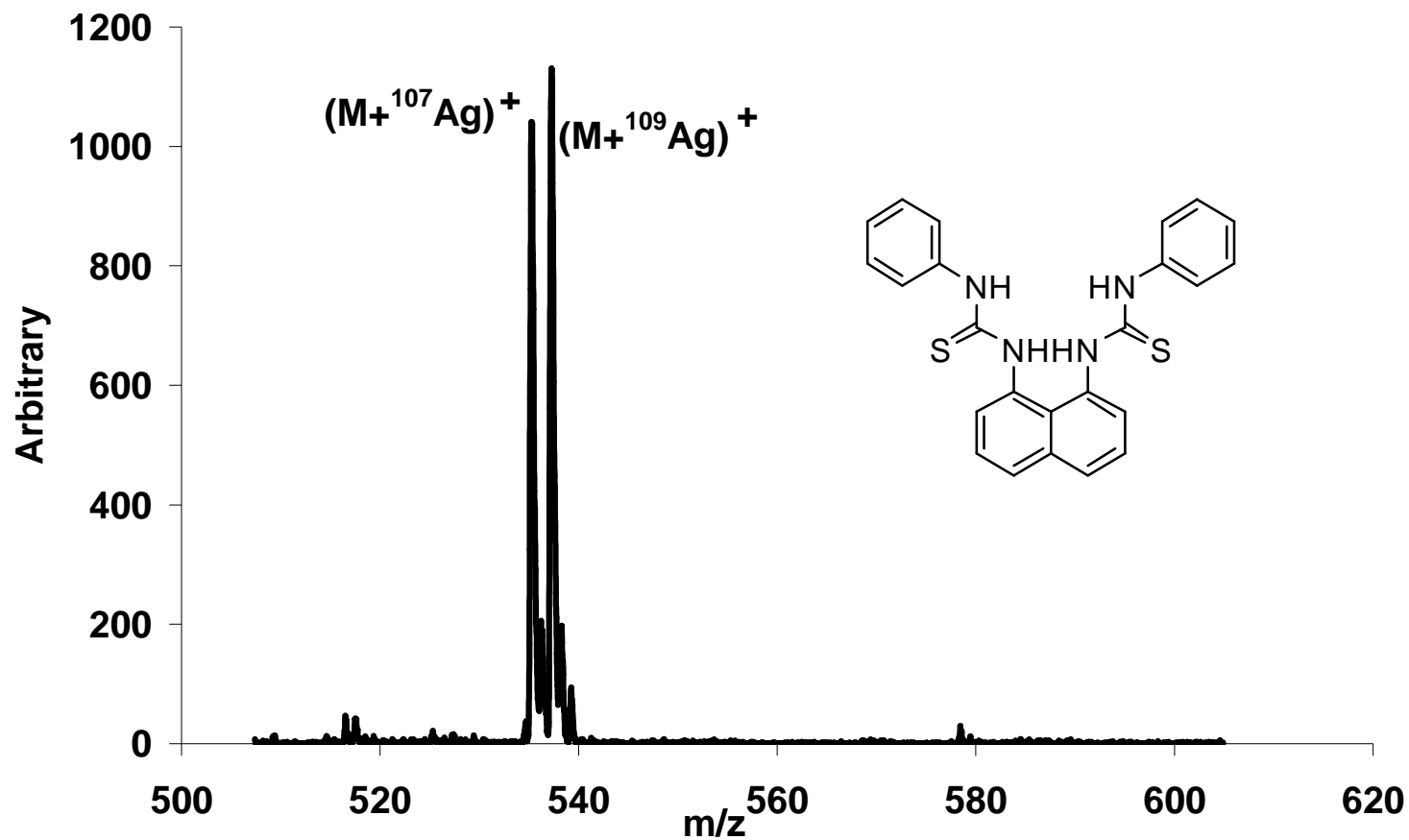


Figure 2.3 MALDI-Mass spectrum of compound 1 in presence of 0.1 (M) AgNO_3 solution. Mass of compound 1 is 428 amu. 535 amu and 537 amu masses were observed indicating the silver adduct formation.

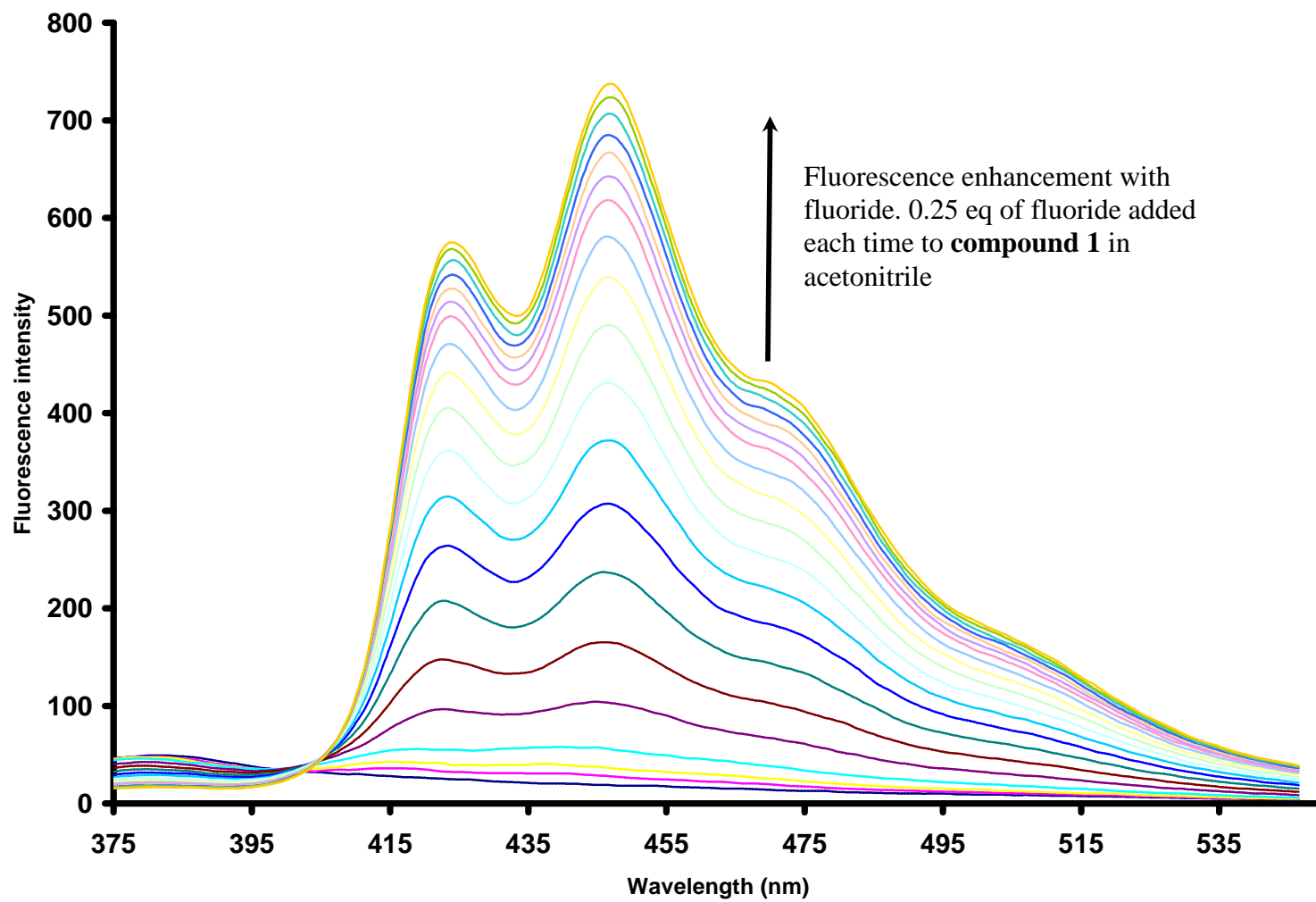


Figure 2.4 Fluorescence spectra on fluoride addition to compound 1 in acetonitrile. Initial concentration of compound is 50 μM . Fluoride was added in 12.5 μM increments. Excitation wavelength = 345 nm; emission maximum = 447.5 nm.

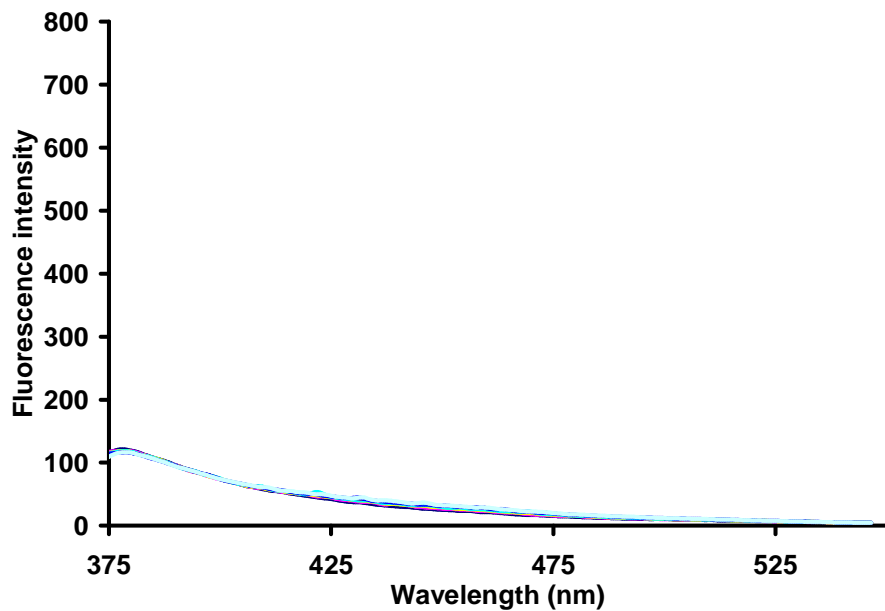


Figure 2.5 Effect of chloride on compound 1 (50×10^{-6} M). 1 eq chloride per addition. A total of 9 eq of chloride was added. No perceptible change in the signal was observed.

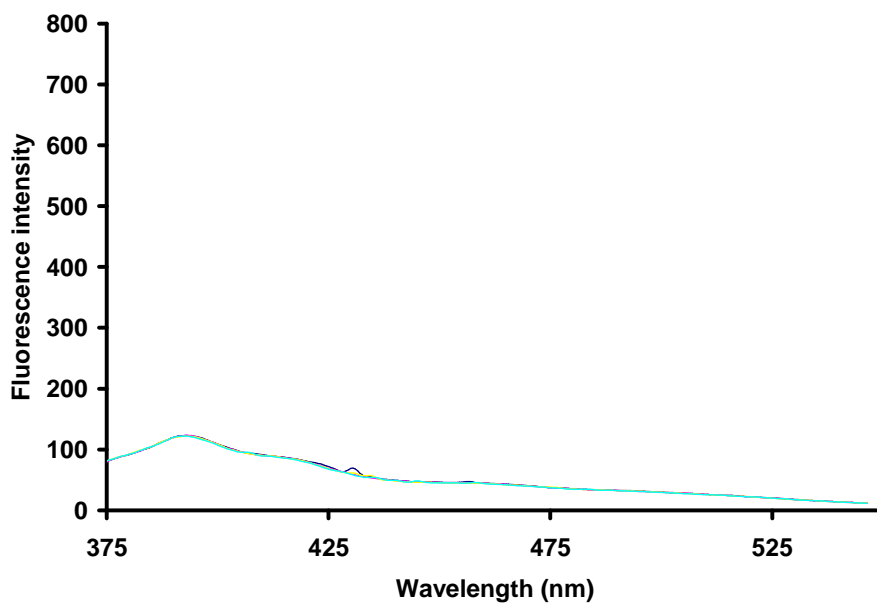


Figure 2.6 Effect of bromide on compound 1 (50×10^{-6} M). 1 eq bromide per addition. A total of three eq of chloride was added.

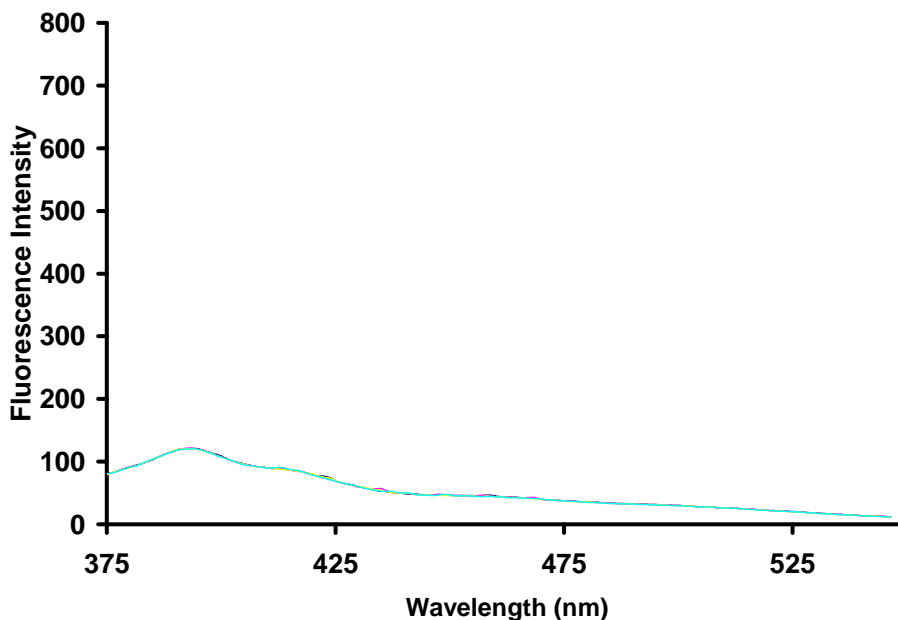


Figure 2.7 Effect of iodide on compound 1 (50×10^{-6} M). 1 eq iodide per addition. A total of three eq of chloride was added.

However, a fluorescence response to dihydrogenphosphate (Figure 2.8) and acetate (Figure 2.9) has been observed, although the response to fluoride is substantially higher than that for these other anions. The fluorescence response to nine equivalents of dihydrogenphosphate is approximately equal to that observed for one equivalent of fluoride. Similarly, nine equivalents of acetate yielded a fluorescence signal that was approximately equal to addition of two equivalents of fluoride¹. Separate experiments showed that even in the presence of 1 mM dihydrogenphosphate (Figure 2.10) or 1 mM, acetate (Figure 2.11) **compound 1** still showed enhancement in fluorescence with addition of 0.25 equivalent of fluoride. While some interfering signal may result from the presence of these anions, the response to fluoride is not inhibited by their presence.

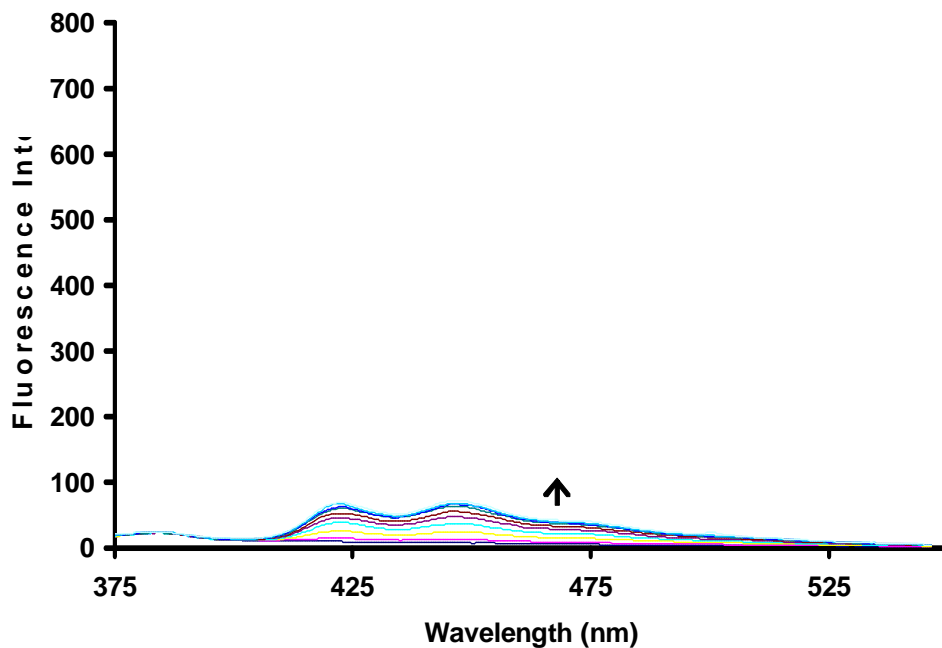


Figure 2.8 Effect of dihydrogenphosphate (H_2PO_4^-) on compound 1 (50×10^{-6} M). 1 eq H_2PO_4^- per addition. A total of ten eq of H_2PO_4^- was added. Slight increase in the signal was observed.

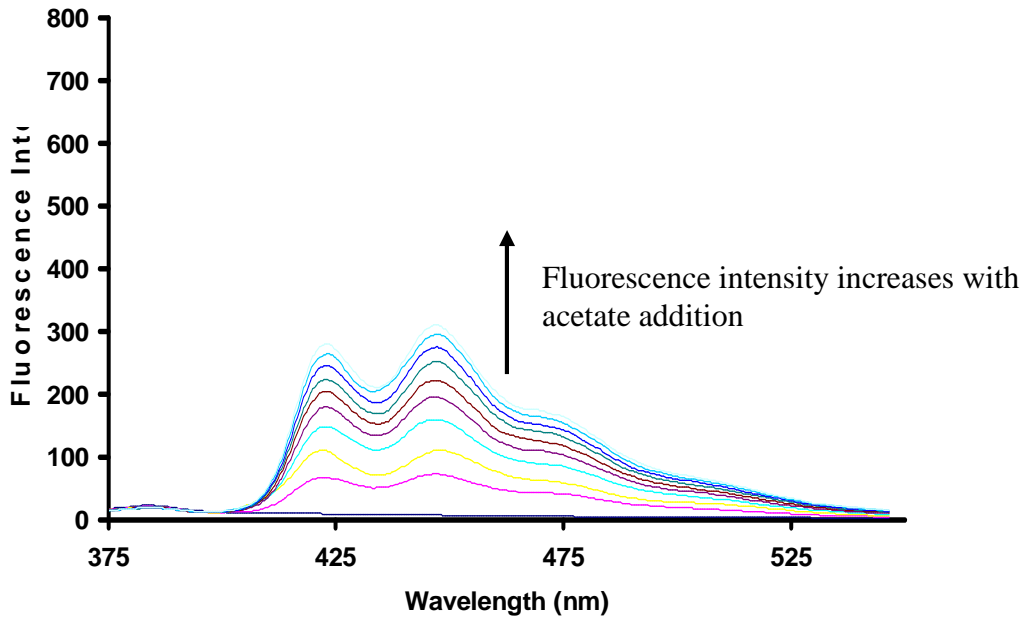


Figure 2.9 Effect of acetate on compound 1 (50×10^{-6} M). 1 eq acetate per addition. A total of ten eq of acetate was added.

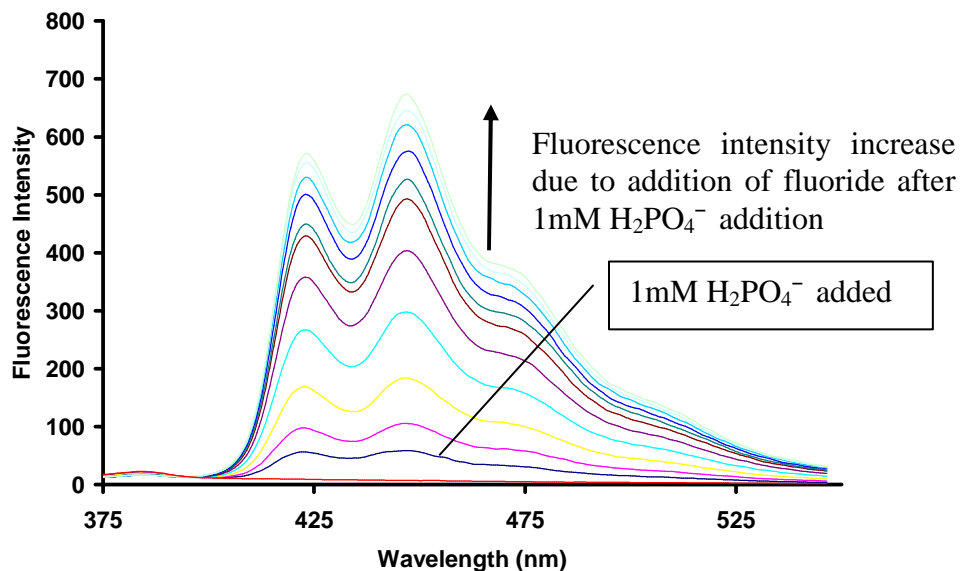


Figure 2.10 Fluorescence enhancement with fluoride in presence of 1mM dihydrogenphosphate (H_2PO_4^-) on compound 1 (50×10^{-6} M). 0.25 eq of fluoride per addition. A total of 2.5 eq of fluoride was added. Significant fluorescence enhancement after fluoride addition was observed.

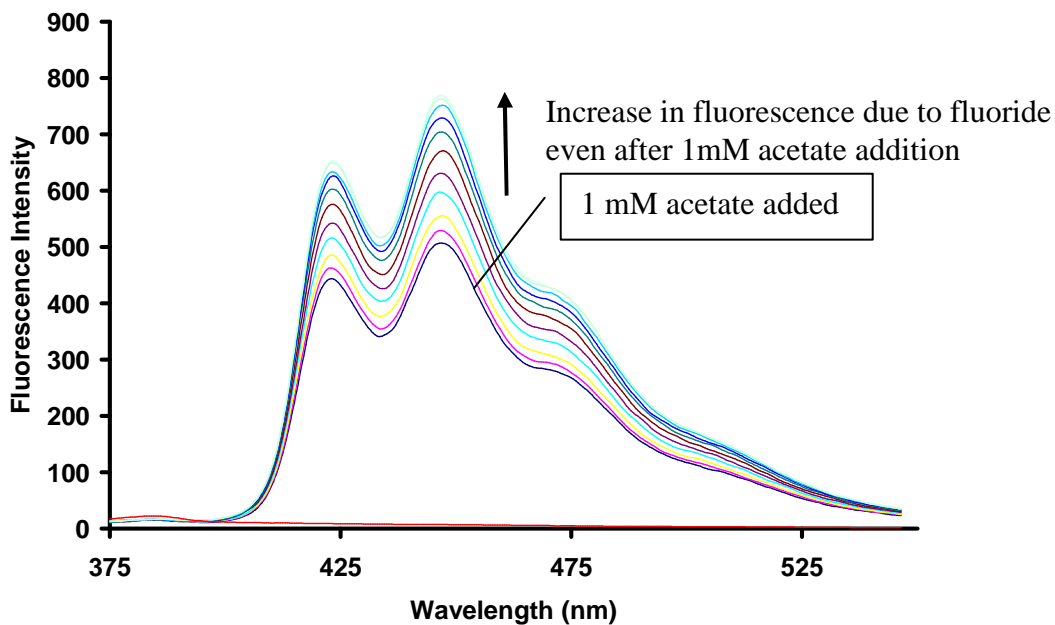


Figure 2.11 Fluorescence enhancement with fluoride in presence of 1mM acetate (CH_3COO^-) on compound 1 (50×10^{-6} M). 0.25 eq of fluoride per addition. A total of 2.5 eq of fluoride was added. Significant fluorescence enhancement after fluoride addition was observed.

A titration experiment (^1H NMR) showed substantial changes upon addition of fluoride. The thioureido proton signal decreased rapidly and broadened with addition of fluoride (Figure 2.12). No significant changes in the aromatic proton signals were apparent. The observed changes in the thioureido proton NMR signal suggest strong H-bonding between thioureido protons and fluoride ions with exchange on the NMR time scale. Due to its small size and high charge density, the fluoride ion had a much stronger interaction with the thioureido protons than the other halides, resulting in the selective enhancement of fluorescence by fluoride.

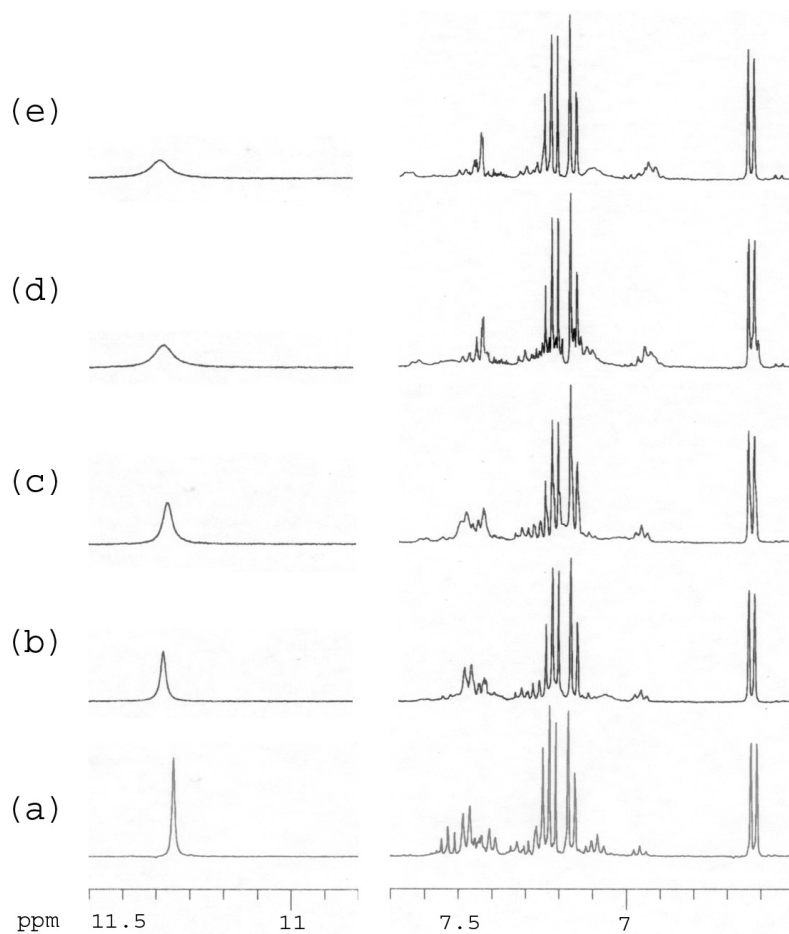


Figure 2.12 : Partial ^1H NMR (400 MHz) of compound 1 (5 mM) in DMSO-d_6 upon the addition of tetrabutylammonium fluoride: (a) 0 μM , (b) 10 μM , (c) 20 μM , (d) 30 μM , and (e) 40 μM .

Fluorescence intensity increased significantly with addition of fluoride, while other halides showed little or no change. An approximate 40 fold enhancement in fluorescence intensity at 447.5 nm was observed in the presence of 5 equivalents of fluoride (50 μM compound **1**, 250 μM F^-). Furthermore, addition of only 0.25 equivalent of fluoride (12.5 μM F^-) resulted in an approximate 1.5 fold increase in fluorescence intensity at 447.5 nm, indicating that detection limits in the low to sub micromolar range are possible. UV-Vis absorbance spectra (Figure 2.13) were acquired to determine if **compound 1** can also behave as a chromogenic sensor. After addition of 10 equivalents of tetrabutylammonium fluoride, a bathochromic shift in the absorbance spectrum was clearly observable. With fluoride present, the absorbance extended into the visible region, indicating that detection of fluoride could be carried out colorimetrically, perhaps even with the naked eye. However, superior detection limits exist using fluorescence methods.

The data in Figure 2.13 indicate an approximate doubling in the absorbance at 345 nm upon the addition of excess fluoride. Fluorescence data (Figure 2.4) illustrate an approximate 40 fold enhancement near saturation (the point where all **compound 1** molecules are bound to fluoride). Since fluorescence at low concentration is proportional to absorbance, these data indicate that only a small portion of the fluorescence enhancement can be accounted for by increased absorbance. Consequently, we can conclude that the fluoride complex has a larger quantum yield than unbound **1**. Based on the absorbance and fluorescence data, the enhancement in quantum yield is estimated to be 20 fold. This observation is in agreement with previous reports which suggested improved planarity²⁹ and lack of relaxation via twisted conformations^{42, 43} as possible mechanisms for enhanced fluorescence in ureido derivatives.

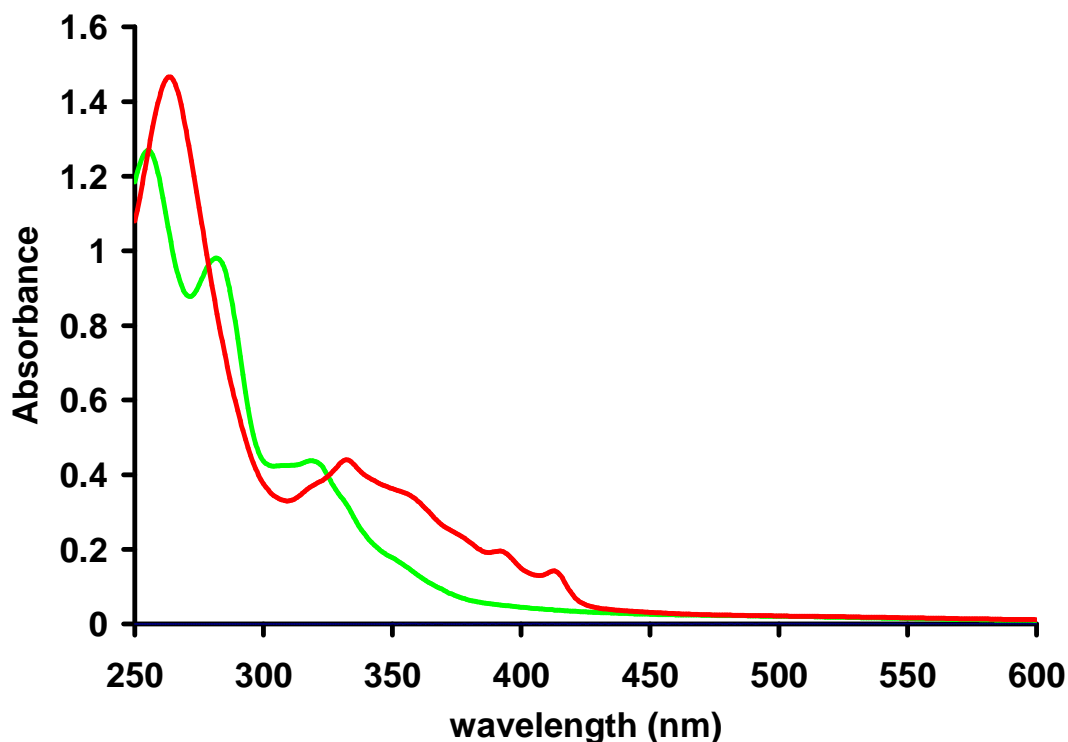


Figure 2.13 Absorbance of compound **1** (50 μM) before (green trace) and after 500 μM fluoride addition (red trace).

A novel thiourea based sensor molecule, 1,8-bis(phenylthioureido)naphthalene, was synthesized and characterized. The compound showed fluorescent enhancement in the presence of fluoride. This response was selective for fluoride, with chloride, bromide, and iodide producing little or no response. The large enhancements in fluorescence (40 fold observed in this study) suggest that this molecule may provide improved analytical characteristics over previously described molecules, especially since many other studies have reported molecules that exhibit either fluorescence quenching in the presence of fluoride¹⁴ or show only changes in absorbance.^{15, 27, 31, 32} **Compound 1** may also be advantageous since its fluoride complex can be excited in the visible region.

The potential practical applications of **compound 1** appear to be versatile and include fiber optic and nanometric sensors for fluoride. Current work focuses on immobilizing

compound 1 on a solid support (such as a fiber optic or nanoparticles) for detection of fluoride in aqueous media.

2.3. Experimental section

All starting materials were purchased and used without further purification. ^1H and ^{13}C NMR were recorded on a 400 MHz spectrometer operating at 9.4 T. NMR experiments were carried out in $\text{DMSO-}d_6$ with 0.05% v/v TMS. Mass spectra were acquired with a MALDI-TOF mass spectrometer. Fluorescence experiments were carried out in acetonitrile solution with excitation wavelength of 345 nm and emission range of 375-550 nm with 7.5 nm slit widths. UV-Vis absorbance experiments were carried out with quartz cuvettes (path length 1 cm).

2.39 mmol of 1,8-diaminonaphthalene was dissolved in 20 ml dry tetrahydrofuran. Two drops of triethylamine was added. This mixture was then added drop wise over one hour to 32.5 mmol of phenylisothiocyanate which was dissolved in 20 mL tetrahydrofuran. The system was kept in a nitrogen atmosphere. The mixture was stirred at room temperature for 24 hours. The progress of the reaction was monitored by TLC (eluent: 4% acetonitrile in chloroform). The mixture of the compound was purified by column chromatography (eluent: 4% ACN in chloroform). The yield was approximately 20-22%.

2.3.1. Selected data for compound 1

MP: 135-137 EC decomposed; ^1H NMR ($\text{DMSO-}d_6$) δ 11.43 (s, 2H), 7.53 (d, $J = 7.8$ Hz, 2H), 7.46 (t, $J = 7.8$ Hz, 2H), 7.28 (t, $J = 7.8$ Hz, 4H), 7.21 (d, $J = 7.9$ Hz, 4H), 7.13 (t, $J = 7.9$ Hz, 2H), 6.67 (d, $J = 7.8$ Hz, 2H), two additional thioureido protons were not observed due to exchange; ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 173.7 (q), 140.6 (q), 136.1 (q), 134.8 (q), 129.6 (2 X CH), 129.1 (CH), 122.6 (CH), 119.9 (CH), 119.0 (2 X CH), 116.9 (q), 105.4 (CH); matrix

assisted laser desorption ionization mass spectrometer (MALDI-MS) showed the silver adducts (^{107}Ag , ^{109}Ag) at $m/z = 535, 537$ amu, corresponding to the expected molar mass of 428 amu.

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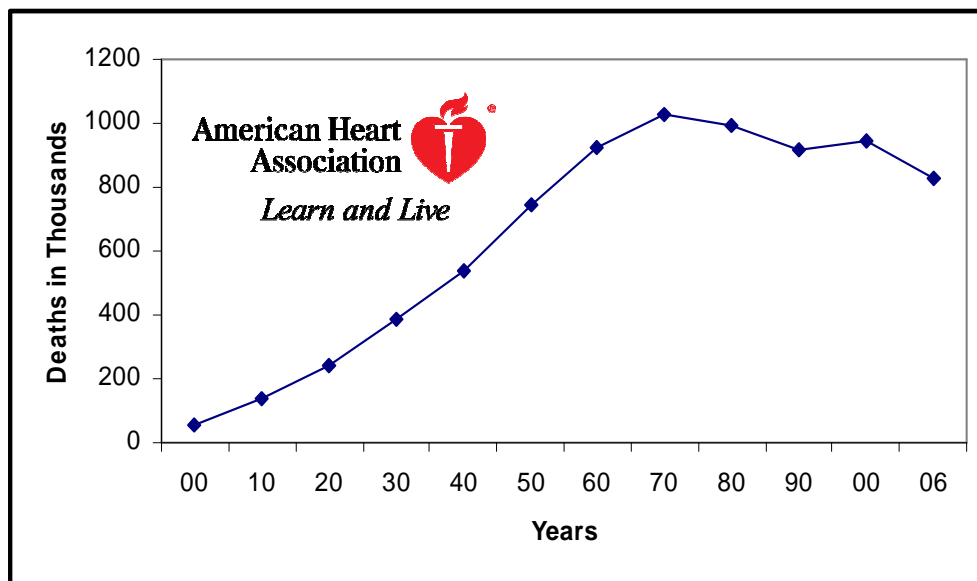
Chapter 3.

Mapping amino acid oxidations of apolipoprotein B-100 in LDL by LC-MS/MS and analysis of natural variants: An introduction

3.1. Cardiovascular diseases (CVD) – epidemiology and risk factors

Cardiovascular diseases (CVD) are among the leading causes of death in western countries. Millions of people die from these diseases every year in the United States (Figure 3.1). Each year the American Heart Association, in conjunction with several other agencies, publishes a variety of literature, notes and safe practices for healthy living. Some interesting statistical updates on CVD are furnished below.

- The 2006 overall death rate from CVD (was 262.5 per 100,000. The rates were 306.6 per 100,000 for white males, 422.8 per 100,000 for black males, 215.5 per 100,000 for white females, and 298.2 per 100 000 for black females. From 1996 to 2006, death rates from CVD declined 29.2%. Mortality data for 2006 show that CVD accounted for 34.3% (831 272) of all 2 426 264 deaths in 2006, or 1 of every 2.9 deaths in the United States.¹
- On the basis of 2006 mortality rate data, nearly 2300 Americans die of CVD each day, an average of 1 death every 38 seconds. The 2007 overall preliminary death rate from CVD was 250.4. More than 151,000 Americans who succumbed to death by CVD in 2006 were <65 years of age. In 2006, nearly 33% of deaths due to CVD occurred before the age of 75 years, which is well before the average life expectancy of 77.7 years.¹



Source: United States, 1900 – 2006 American Heart Association, NCHS, NHLBI

Figure 3.1 Deaths from Cardiovascular diseases chart. A decreasing trend after 1970 could be noted.

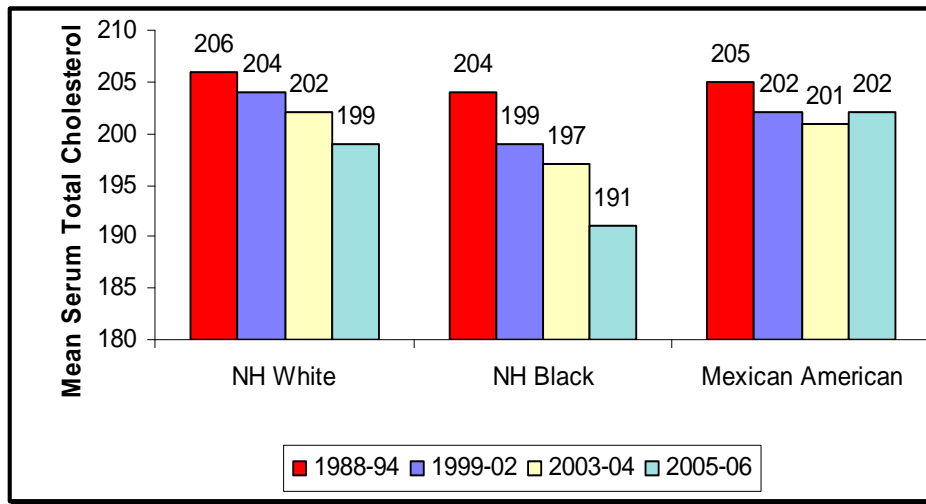
Undoubtedly a general mass awareness of cardiovascular diseases is playing a critical role in decreasing the number of deaths from this disease. The data, however, seems to indicate that it is still a severe threat to public health in developing and developed countries. According to the World Health Organizations fact sheet² on cardiovascular diseases, the annual death toll from this disease is highest compared to any other disease.

An estimated 17.1 million people died from CVD in 2004, representing 29% of all global deaths. Of these deaths, an estimated 7.2 million were due to coronary heart disease and 5.7 million were due to stroke.² Most alarmingly, by 2030, almost 23.6 million people will die from CVD per year, mainly from heart disease and stroke. These are projected to remain the single leading causes of death. The largest percentage increase will occur in the Eastern Mediterranean Region. The largest increase in number of deaths will occur in the Southeast Asia Region.²

In general, CVD are considered as a group of disorders including coronary heart diseases, cerebrovascular diseases, peripheral arterial diseases, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. Several different behavioral factors

contribute to the cardiovascular disease risk factors. Unhealthy diet, physical inactivity and tobacco use are the chief risk factors.^{1,3}

It has been understood for many years that diet is an essential component to avoid CVD. Elevated blood cholesterol was implicated as one of the major risk factors for cardiovascular diseases.^{1,3} Although a recent survey indicated a downward trend in serum cholesterol levels (Figure 3.2) in people of age 20 and older, elevated cholesterol still poses a considerable threat to the community health.



Source: NCHS and NHLBI. NH – non-Hispanic.

Figure 3.2 Trends in mean total serum cholesterol among adults of age 20 and older, by race/ethnicity survey. (NHANES: 1988-94, 1999-02, 2003-04, 2005-06)

3.2. Low density lipoproteins (LDL)

3.2.1. Lipoproteins

A lipoprotein is an assembly of lipid and protein held together (Figure 3.3) by non-covalent forces.^{4,5} Low density lipoprotein (LDL) is one major lipoprotein which is synthesized in the liver. LDL plays a critical role in cholesterol transport in physiological systems (Figure 3.4). The other lipoproteins are chylomicrons, very low density lipoprotein, intermediate density lipoprotein and high density lipoproteins (HDL).^{6,7} Higher level of HDL helps preventing cardiovascular diseases.^{3,8,9} LDL serves as a representative of serum blood cholesterol in medical conditions and is often termed as “bad” cholesterol.³

3.2.2. LDL function

Low density lipoproteins consist of three components. The core is made up of cholesteryl ester and triacylglycerol, the outer layer free cholesterol and phospholipids. One single monomeric protein apolipoprotein B-100 (apo B-100) encircles the LDL particle^{6,7} (Figure 3.3). The average molecular weight of LDL particle is approximately 3 million Dalton¹⁰. Brown and Goldstein’s legendary work^{11,12} on LDL and LDL receptors earned them a Nobel Prize in the 1980’s. LDL transports cholesterol through circulation. In general, when a cell needs cholesterol, LDL is transported to the cell (Figure 3.4). There, LDL binds to the LDL receptors (LDL-R) which are positioned in the cell surface. After binding, the assembly is taken inside the cell by endocytosis, forming a coated pit followed by a coated vesicle. In the endosome, the assembly becomes protonated, and LDL receptors break free from LDL. The LDL enters lysosome and is degraded to release the cholesterol. The protein breaks down to amino acids. The LDL receptors go through the recycling vesicles and return back to the surface.¹¹

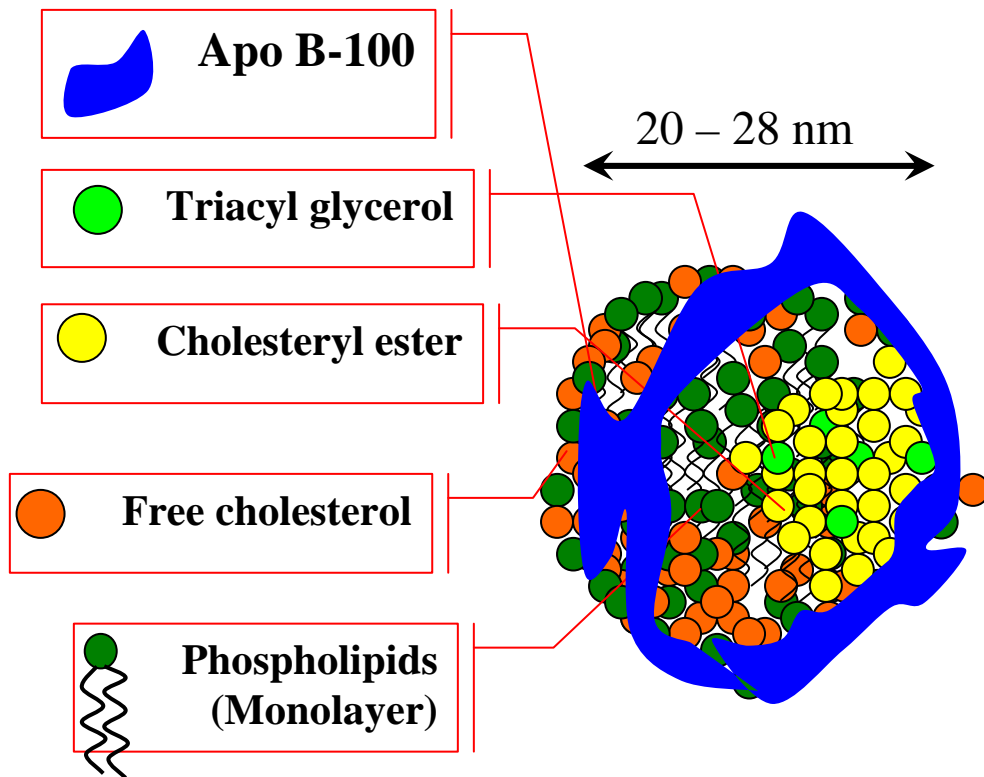


Figure 3.3 Schematic representation of an LDL particle.

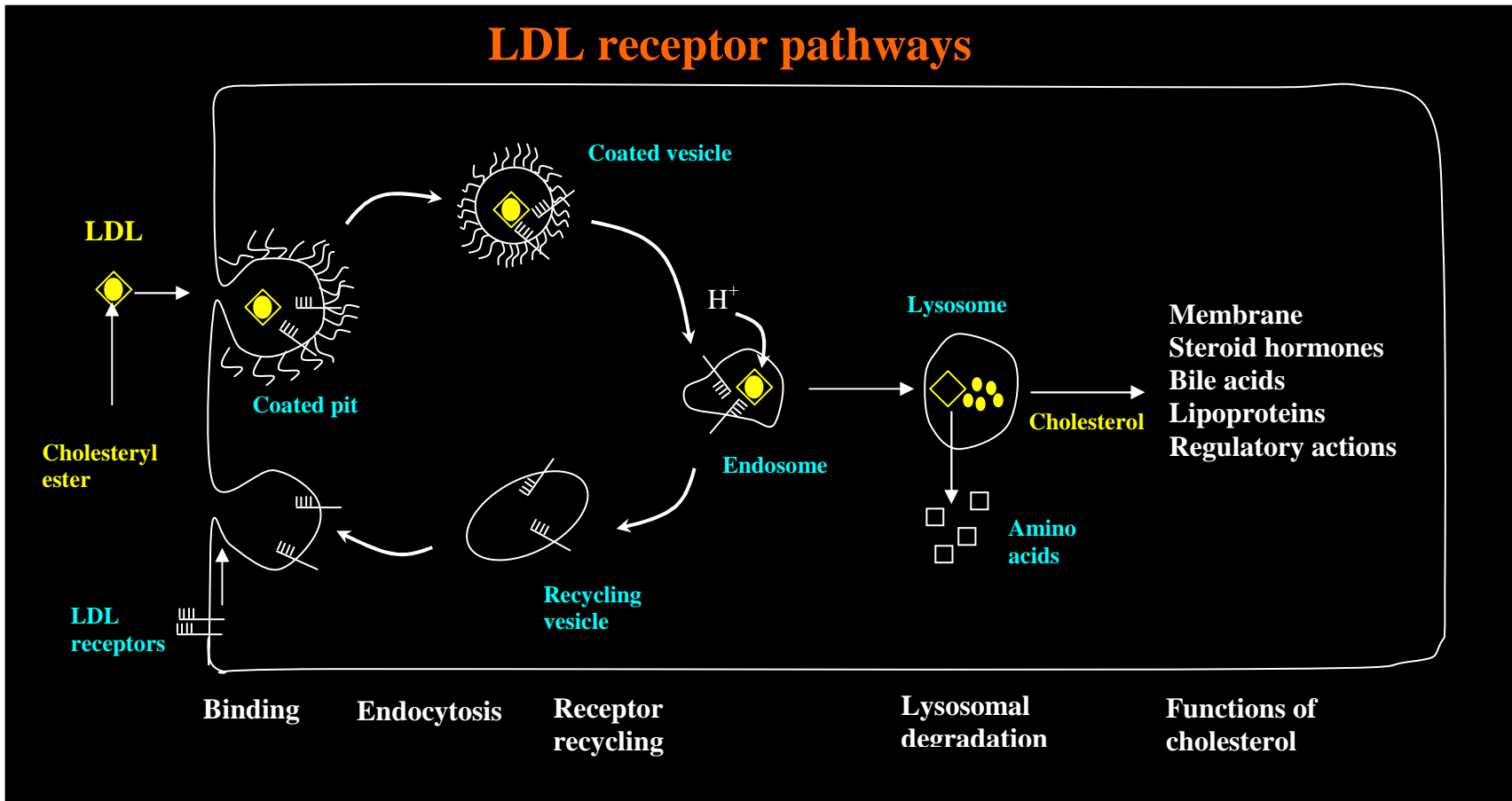


Figure 3.4 Mechanism of LDL uptake by cells in the physiological systems. LDL was included in the cells after binding to LDL receptors. After going through Lysosomal degradation, cholesterol is released and used in cellular functions.

3.2.3. Structure of apolipoprotein B-100 in LDL

Apolipoprotein B-100 (apo B-100) stays in the outermost side of LDL (Figure 3.3). This protein is secreted from the liver and is one of the largest monomeric proteins in existence (molecular weight = 550 kDa).¹³⁻¹⁵ It consists of 4563 amino acids (accession # P04114).¹⁶ The structure of apo B-100 is still poorly understood. Due to the enormous size of this protein, it is rather difficult to determine the crystal structure of this protein. Also, the protein is lipid bound hence there could be a possibility of structural change if the protein is delipidated during the structural studies and not represent a true account of the structure. It should be noted that Apo B-48 which is an isoform of apo B-100 was found in chylomicrons (stop codon at amino acid AA2180) and are synthesized in the small intestine.⁵

Several attempts were made to elucidate the structure of apo B-100 in LDL.^{5, 14, 17} Despite some useful information published in the literature over the last two decades, the structure of the protein is still not lucid. Molecular modeling method, although showing promise, were limited and speculative in this case particularly due to the size of the protein.^{10, 18, 19} In homology based molecular modeling researchers used a putative ancestor of apo B-100 known as lipovitellin found in egg yolk.^{10, 20} This protein showed N-terminal matching up to 20.5% and basic local alignment search tool (BLAST) matching up to 20.8%.²¹

Apo B-100 is amphipathic (contains both hydrophobic and hydrophilic regions) in nature. It consists of 3 α -helices and two β -strands in between the α -helices. Generally, the structure is represented by $\text{NH}_2 - \beta\alpha1 - \beta1 - \alpha2 - \beta2 - \alpha3 - \text{COOH}$.^{10, 19, 20} Most interestingly, β -domains are associated with the neutral lipid core imparting stability to the LDL structure while the α -helices are flexible and are associated with flexibility of the molecule and their intravascular

mechanism.²² The amphipathic nature of apo B-100 helps it maintain strong lipid binding during transport through the blood.

LDL lipid oxidations and their implication in atherosclerosis were studied by various research groups around the world.²³⁻³⁰ However, limited data exists on protein oxidation and the resulting changes in LDL.³¹⁻³⁷ It was thus apparent that oxidation of protein are associated with the structural changes in apo B-100. These structural changes can be crucial for deposition of LDL in atherosclerotic plaque formations.

3.3. Free radicals and other oxidants in physiological systems

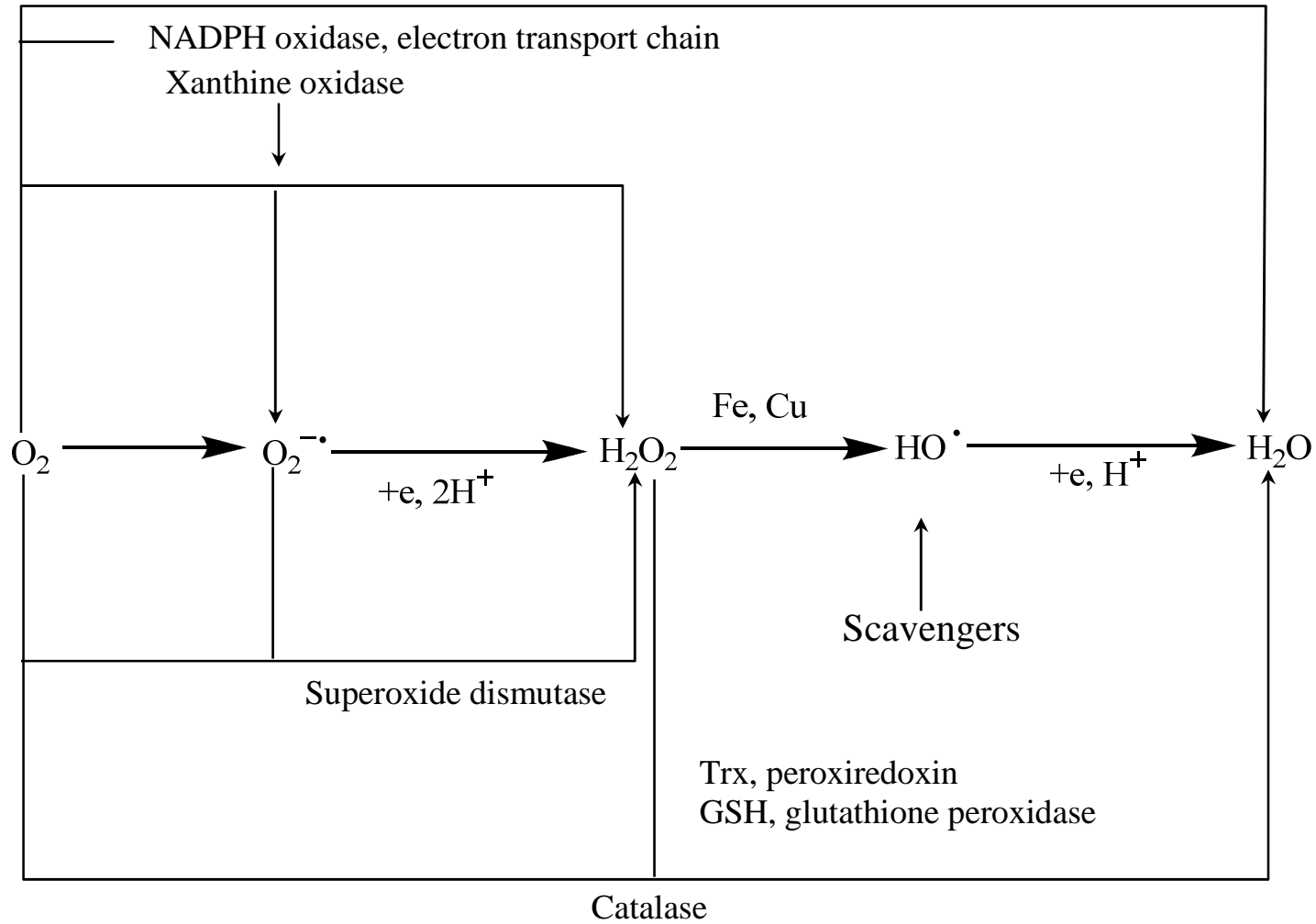
3.3.1. Reactive oxygen species (ROS): Fenton Chemistry and hydroxyl radical

Free radicals are defined as any atom or molecule bearing unpaired electrons.³⁸ The presence of unpaired electrons makes free radicals chemically reactive. They can be cationic, anionic or neutral. In biological systems, oxygen radicals are well known for their broad spectrum reactivity. Ground state molecular oxygen has two unpaired electrons. Although it behaves as an oxidant it is relatively unreactive. However, reduction of molecular oxygen (addition of electrons) yields a broad spectrum of intermediates which showed high reactivity towards biomolecules.³⁹

Although several parts of the human body could be a source of free radical formation, mitochondria are the major source of free radical production in cells. Leakage from electron transport chain or oxidative burst can produce superoxide anion radical.³⁹ Also, partially reduced mitochondrial components and oxygen tension play important roles in generating superoxide and other reactive oxygen species.⁴⁰ Superoxide stays in equilibrium with hydroperoxyl radical. It can be converted to peroxy, alkoxy and hydroxyl radical by various pathophysiological

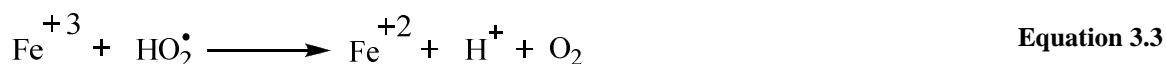
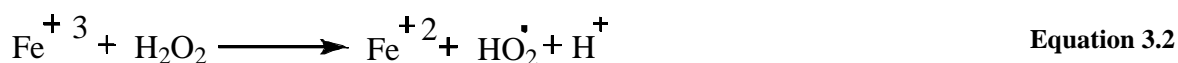
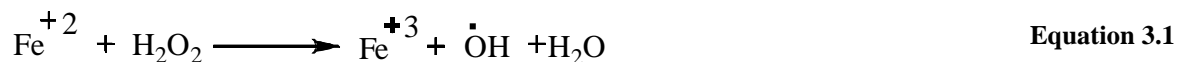
processes.⁴⁰ Cytochrome C oxidase converts oxygen to water thereby eliminating the possibility of ROS formation in cells. NADPH oxidase, however, promotes the formation of superoxide from oxygen (Scheme 3.1). Superoxide dismutase disproportionates superoxide to hydrogen peroxide and oxygen. Also, one electron reduction of superoxide followed by protonation can lead to hydrogen peroxide formation. Imbalance of hydrogen peroxide can be counter balanced by catalase and peroxidases. However metal ions can play a critical role in decomposing hydrogen peroxide to yield hydroxyl radical.⁴¹ For example Haber-Weiss reaction is a catalytic cycle for iron through which hydroxyl radical can appear from superoxide and hydrogen peroxide.^{42, 43} All of these radicals, particularly hydroxyl radical, are extremely reactive and can cause non specific damage to biomacromolecules. The half life of hydroxyl radical is fairly low (nanosecond range).^{41, 43-45}

Cytochrome C oxidase



Scheme 3.1 Mechanism of formation and detoxification of reactive oxygen species (ROS) in cells.

In a 1894 article, Henry J. Fenton first described a unique reaction between iron [Fe(II)] and hydrogen peroxide.⁴⁶ Haber-Weiss elaborated his method and described formation of hydroxyl radical from hydrogen peroxide and superoxide anion radical^{42, 47} (Equation 3.1). In short it is a catalytic cycle for iron by which iron generates hydroxyl radical. Fenton processes are described as a set of reactions noted below^{42, 48, 49} (Equation 3.1–Equation 3.6).



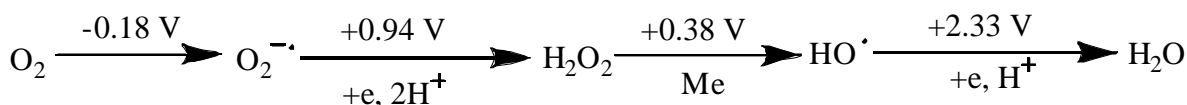
Equation 3.2 and Equation 3.3 emphasize the catalytic cycle for Fe. The last two reactions (Equation 3.5 and Equation 3.6) reveal scavenging ability of Fe (II) and hydrogen peroxide. In physiological systems, sources of “free” iron are fairly limited. Iron storage proteins such as transferrin, ferritin and hemoglobin have bound iron. However, non-transferrin bound iron can be considered as “free” iron. These iron species (conc. 1~10 μM) can bind to citrate or albumin in blood. Also iron-sulfur clusters can release iron in pathophysiological conditions.⁴³ This released iron can play important roles in generating hydroxyl radical in cells.

A negative free energy change of a system results in the spontaneity of a reaction. Free energy is directly proportional to the redox potential of the reactant(s). Hence mathematically we can write Equation 3.7. For spontaneity ΔG must be negative, therefore E should be positive.⁵⁰ If

we delve into the redox chemistry of cells we will see that after superoxide is formed all top down processes were favorable from free energy point of view (Scheme 3.2). Particularly, water formation from hydroxyl radical is thermodynamically favorable.⁵¹

$$\Delta G = - nFE \quad \text{Equation 3.7}$$

Where ΔG : Free energy change
 n: # of moles electrons
 F: Faraday
 E: reduction potential of the cell



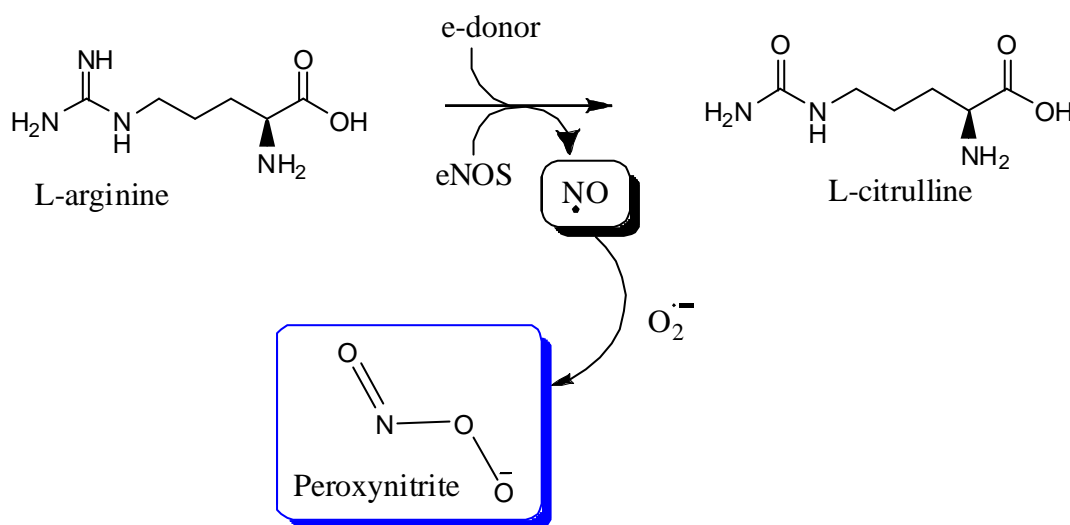
Scheme 3.2 Electrode potentials depicting the reactivity of different ROS. “Me” represents metal cation. More positive E depicts enhanced spontaneity in the reaction.

3.3.2. Reactive Nitrogen Species (RNS)

Since the discovery of nitric oxide radical as a cellular messenger, vasodilator and antiatherogenic molecule⁵²⁻⁵⁴, its reaction with superoxide anion radical yielding peroxynitrite became important to explain the cellular toxicity (Scheme 3.3). Together nitric oxide and peroxynitrite are known as reactive nitrogen species (RNS).⁴⁰ Reactive nitrogen species in conjunction with ROS induce cell damage, proliferation and apoptosis.⁵⁵ Indeed, peroxynitrite is much more reactive than its predecessor nitric oxide and superoxide in physiological systems.⁵⁶⁻⁵⁹ It has been implicated that peroxynitrite plays a pronounced role in atherosclerosis⁶⁰⁻⁶³, chronic inflammation⁶⁴ and neurodegenerative diseases.^{65, 66} Due to its powerful oxidizing properties it reacts readily with biomacromolecules in cells. In particular the damage alters structure and functions of proteins.

3.3.3. Peroxynitrite formation and reactions with proteins

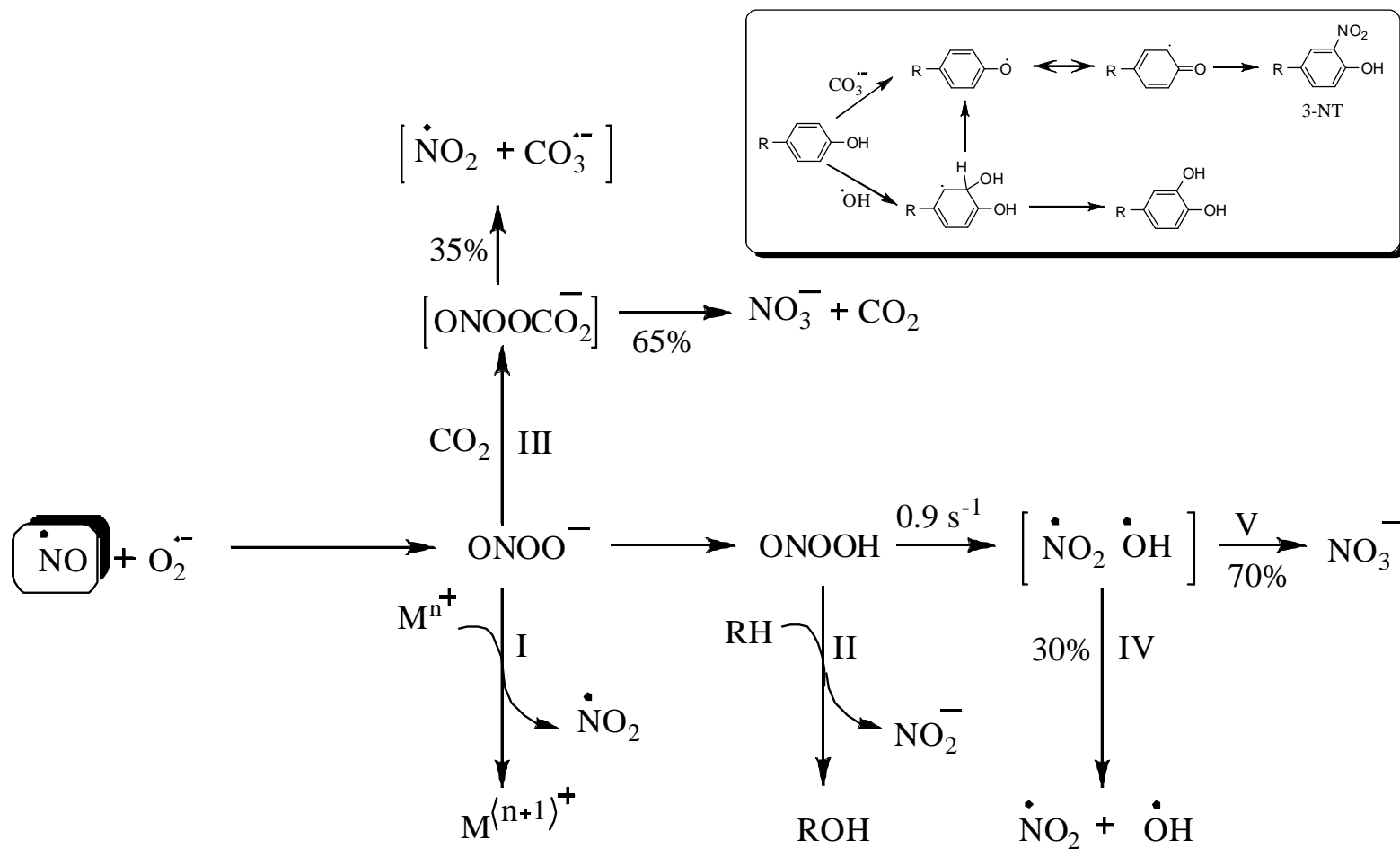
Although peroxynitrite can appear in different organs of the body, it is particularly important in vascular biology for endothelial dysfunction. In endothelial cells, nitric oxide is synthesized by enzymatic activity of the enzyme endothelial nitric oxide synthase (eNOS). Endothelial NOS converts L-arginine to L-citrulline and release nitric oxide in the process (Scheme 3.3). Nitric oxide reacts with endothelial superoxide almost at a diffusion controlled rate^{56, 59} ($10^{10} \text{ M}^{-1}\text{s}^{-1}$) (Scheme 3.3). Under physiological conditions, peroxynitrite stays in equilibrium with its conjugate acid form peroxynitrous acid⁵⁶ (ONOOH, pK_a : 6.8). ONOOH is relatively unstable and yields nitrite radical and hydroxyl radical by a homolytic fission pathway.



Scheme 3.3 Synthesis of nitric oxide from L-arginine in vascular endothelial cells and its reaction with superoxide to yield reactive peroxynitrite.

One of the most interesting and biologically relevant reactions of peroxynitrite in physiological system is its combination with carbon dioxide or bicarbonate to yield nitrosoperoxy-carboxylate (Scheme 3.4). Carbon dioxide reacts with peroxynitrite with a second order rate constant $4.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 and $37 \text{ }^\circ\text{C}$ ⁵⁷ leading to the formation of the adduct ONOOCO_2^- . This adduct, nitrosoperoxy-carboxylate, dissociates into nitrite radical and carbonate

radical.⁶⁷ Carbonate radical reacts readily with the side chain of tyrosine residues in proteins to yield a tyrosyl radical. Reaction between tyrosyl radical and nitrite radical is fast and yields 3-nitrotyrosine.^{63, 68} It is therefore conceivable that sulfur containing amino acids (cysteine) and aromatic amino acid side chains will react with peroxynitrite by various mechanisms.^{58, 59, 69, 70} Peroxynitrite decomposition yields radicals such as hydroxyl, nitrite and carbonate. The latter has high reactivity towards the amino acid side chains as well as the protein back bone.^{56, 59} Homolytic fission probability of peroxynitrite to yield nitrite and hydroxyl radical is <1% (Scheme 3.4). Therefore peroxynitrite can react directly with biomolecules before homolytic fission. Although several reactions are feasible, specific markers for peroxynitrite mediated oxidations are scarce. In fact, 3-NT and nitrotryptophans are the only definitive markers for oxidations of proteins by peroxynitrite.



Scheme 3.4 Various reactions of peroxynitrite were depicted in the above scheme. Reaction of dissociation products of nitrosoperoxydicarbonate (a reaction product of peroxynitrite and carbon-di-oxide) to tyrosine was shown in the inset. Carbonate radical plays important role in the formation of 3-nitrotyrosine (3-NT).

3.3.4. Hypochlorous acid (HOCl)

Similar to peroxytrite, hypochlorous acid is not a free radical. However it is a powerful oxidant that can act on macromolecules. Activated phagocytes release hydrogen peroxide (H_2O_2) and superoxide via a respiratory burst along with the heme enzyme myeloperoxidase (MPO).⁷¹ MPO catalyzes the reaction between H_2O_2 and physiologically available chloride to yields hypochlorous acid.⁶² Physiologically hypochlorous acid is used as a bacterioside.^{71, 72} However, excessive and/or off site generation of hypochlorous acid causes serious tissue damage⁷³ and is believed to be one of the key factors in progression of diseases such as atherosclerosis and chronic inflammation.^{32, 33, 68, 74}

3.3.5. Biomacromolecule oxidation with HOCl

DNA bases are a major target for HOCl mediated oxidation.⁷⁵ Unstable chloramines are formed during this reaction which can initiate further damage. Cholesterol and unsaturated fatty acids generate chlorohydrin upon treatment with hypochlorous acid which could easily be detected by GC/MS methods.⁷³ These products, however, were minor since the rate of formation is relatively slow.⁷⁶

Proteins are one of the major targets of hypochlorous acid.^{34, 77, 78} Although oxidation of side chains of amino acids prevail, peptide backbone fragmentation and aggregation in proteins were also observed⁷⁸. Hypochlorite anion is by far more reactive than its protonated form. Hypochlorous acid targets the protonated amine groups⁷⁹ in amino acid side chains. Second order rate constants were reported⁷⁹ for Cys and Met on the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$. Cystein forms sulfonic acid (cysteic acid) and methionine forms methionine sulfoxide during reaction with hypochlorous acid.⁷⁸ Oxidation of Trp (indolic residue) and chlorination of Tyr (phenolic residue) are also

common products.⁸⁰ Histidine has a slightly higher rate constant ($1.0 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$)⁷⁹ than its other hydrophobic amino acids counterparts. By using a kinetic modeling technique, Pattison *et. al.*, reported that the major targets with a slight excess of hypochlorous acid are the side chain residues of the proteins and not the peptide backbone.^{78, 79} The $\epsilon\text{-NH}_2$ group of lysine is also one of the major targets for hypochlorous acid. Monochloramines and dichloramines (with high excess of HOCl) are formed during the reaction of hypochlorous acid and lysine residues. Chloramines which are mildly oxidizing can transfer chlorine to the other amino acid side chains (such as tyrosine) and regenerate the parent amine. In Alzheimer's disease, a particular tyrosine residue was found to be chlorinated more than the other six Tyr residues in apo A-I. This phenomenon was attributed to a chlorine transfer mechanism from a nearby lysine residue through a chloramine intermediate.⁸¹ Recently, Pattison *et. al.* researched on proteins such as insulin and lysozyme to study chloramine transfer reactions more extensively. These proteins have low levels of Cys, Met and Trp residues thereby the major kinetic targets for HOCl were eliminated and chloramine pathway for His and Lys was promoted.⁸²

3.4. Apo B-100 oxidation in LDL

3.4.1. Hydroxyl radical mediated direct oxidation of apo B-100

Free radical mediated protein oxidations were established although not quite well understood. Since the early 1980's, Stadtman and coworkers have published pioneering work in the area of free radical mediated metal catalyzed oxidations (MCO) of amino acids, peptides and proteins.⁸³⁻⁸⁶ These reactions included side chain modifications in amino acids, sometimes polypeptide backbone fragmentations and cross linking.⁸⁷⁻⁸⁹ Depending upon the degree of oxidation, the protein can unfold, lose its reactivity and show altered behavior towards

proteolytic enzymes. Radical mediated damage was always associated with pathophysiological events such as aging, cancer, diabetes, cataract and atherosclerosis.

Oxidized LDL was implicated as a crucial factor for atherosclerotic plaque formation in the arterial wall^{28, 36, 89-97}. LDL can be oxidized by two different ways (Figure 3.5). If the radical site is generated near the protein the radical once formed could easily access the side chain of the amino acids. It will therefore modify any amino acids that are in the vicinity. On the other hand, apo B-100 constitutes 20% of LDL particles and the remaining 80% of LDL is comprised of lipids.⁶ If the free radical is generated near the lipids, it will oxidize the lipids first (Figure 3.5). During lipid oxidation, several different lipid peroxides could form which can be converted to reactive aldehydes such as acrolein or 4-hydroxynonenal (4-HNE).^{26, 98} These aldehydes are unsaturated and are nucleophilic in nature. Moreover, the radical such as hydroxyl radical might be short lived, but secondary reaction products such as aldehydes have fairly long life time and can damage several different amino acids in proteins.^{22, 26, 99, 100} Our main interests, however, lie in the direct damage caused by the radicals.

Recent studies on oxidation of apo B-100 showed that aberrant apo B-100 can interact with the arterial proteoglycans and initiate plaque formation in the arterial wall.^{94, 101-105} Moreover, we strongly believe that the site of the oxidation of apo B-100 is one of the key factors in atherosclerotic plaque formation due to the structure-function relationship for the protein.

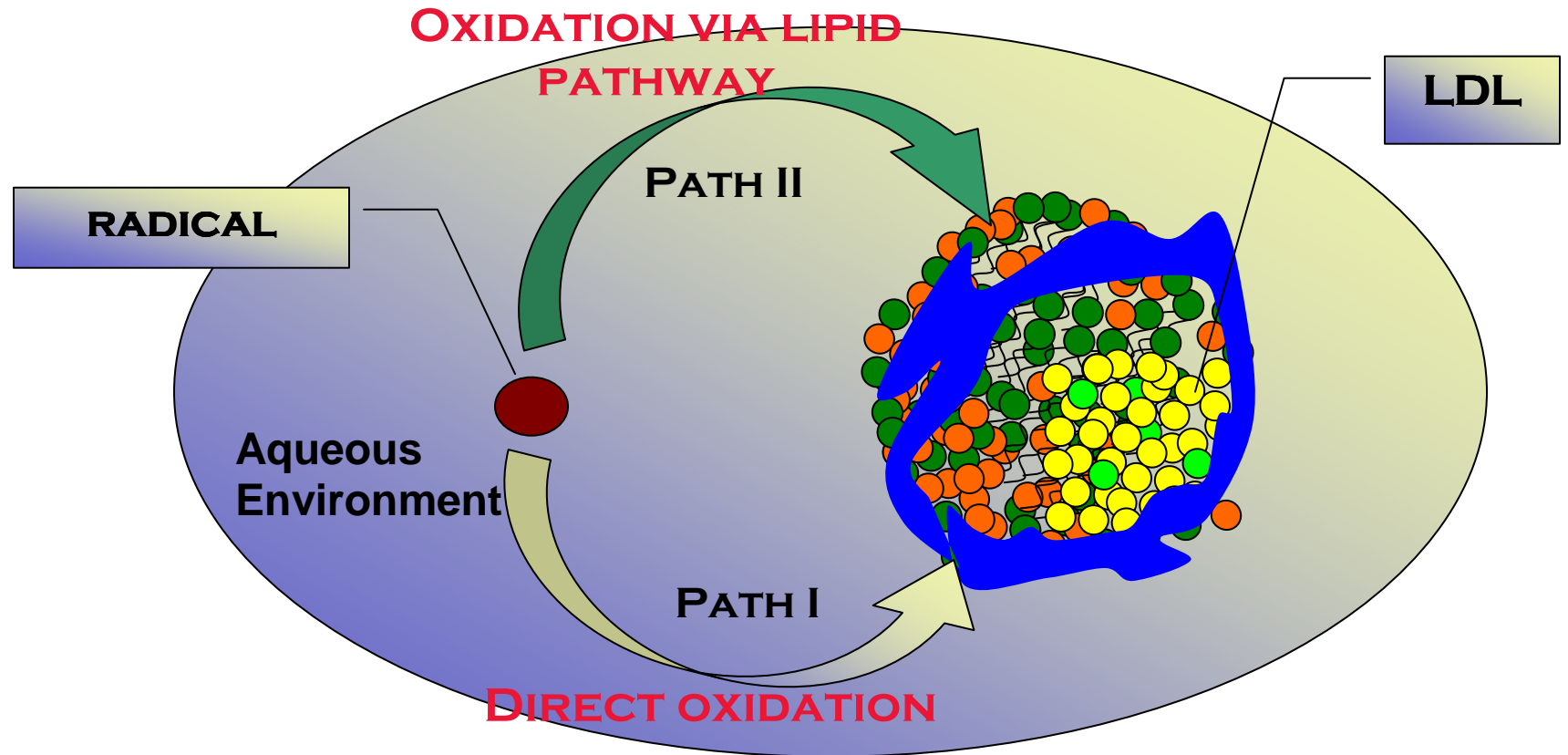
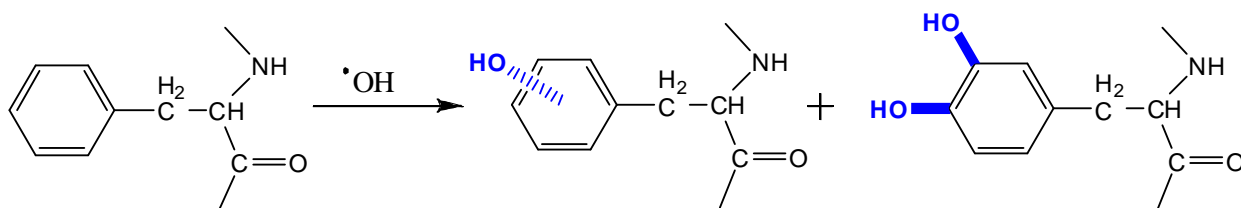
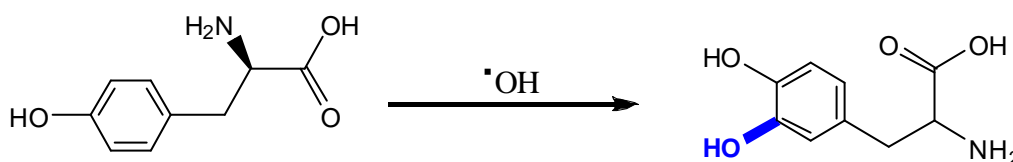


Figure 3.5 Free radical mediated oxidations of apo B-100 in LDL is shown in the above figure. Protein damage by both direct radical attack and lipid mediated intermediates were depicted.

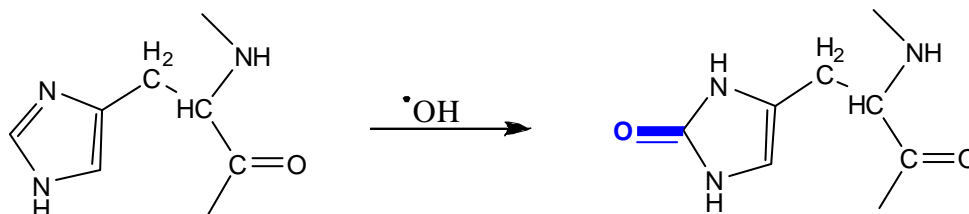
Aromatic amino acids such as phenylalanine (Phe) and tyrosine (Tyr) were considered as a sink for hydroxyl radical due to their high reactivity towards the radical.¹⁰⁶ One simple characteristic reaction of hydroxyl radical is hydroxylation of the phenyl ring. Products of Phe oxidation are therefore o-, m-, p- tyrosine and DOPA (Scheme 3.5).



Scheme 3.5 Oxidation of side chain of phenylalanine residue by hydroxyl radical yielding o-, m-, p- tyrosine (+16 Da) and dihydroxyphenylalanine (DOPA) (+32 Da).



Scheme 3.6 Oxidation of the side chain tyrosine residue by hydroxyl radical yielding dihydroxyphenylalanine (DOPA) (+16 Da).

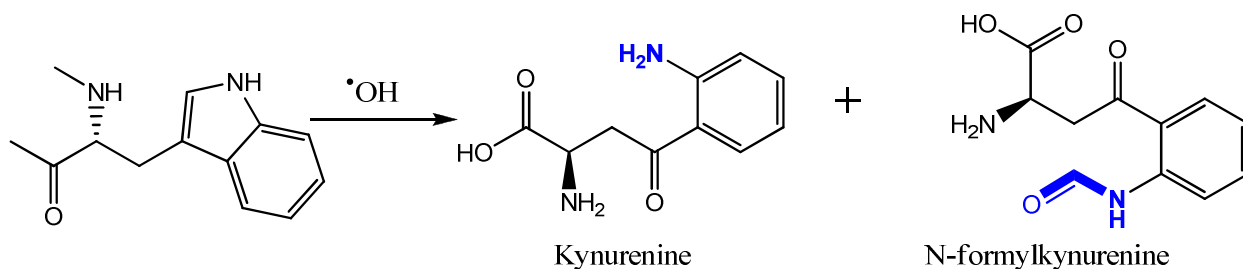


Scheme 3.7 Histidine side chain oxidation product after reacting with hydroxyl radical (+16 Da). Corresponding ketone is more stable and shown in the scheme.

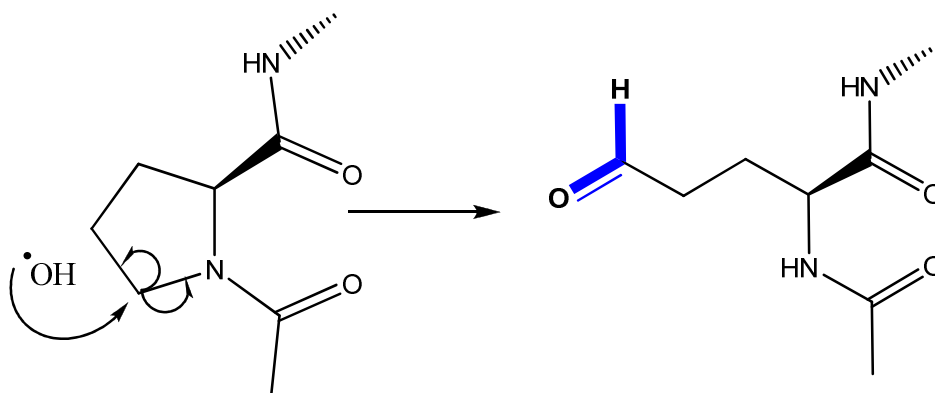
Similarly, Tyrosine predominantly generates 3, 4 dihydroxyphenylalanine (DOPA) (Scheme 3.6) both in aerobic and in non-aerobic conditions. Direct oxidation product of histidine (His) yields a complex mixture of products including hydroxylated (Scheme 3.7), carbonyl and

ring opening products. Tryptophan (Trp) oxidizes to form hydroxytryptophan (not shown) and the ring opening products kynurenine and N-formylkynurenine (Scheme 3.8).

Reactions of lysine (Lys), arginine (Arg) and proline (Pro) with hydroxyl radical is considerably fast. In all cases a semialdehyde is the initial product. Oxidation of proline and arginine leads to glutamic semialdehyde formation^{107, 108} (Scheme 3.9).



Scheme 3.8 Oxidation of tryptophan to Kynurenine (+4 Da) and N-formylkynurenine (NFK) (+32 Da). These products appear both in hydroxyl radical mediated oxidation as well as in presence of hypochlorous acid.



Scheme 3.9 Reaction of proline residue (in a generic peptide) with hydroxyl radical and formation of glutamic semialdehyde (+16 Da).

Lysine oxidation yields amino adipic semialdehyde^{85, 109} (not shown). Oxidation of these residues was detected by a sodium borohydride reduction followed by a hydrolysis with hydrochloric acid. This treatment yielded 5-hydroxy-2-aminovaleric acid (HAVA) (for Arg and Pro) and 6-hydroxy-2-aminocaproic acid (HACA) (for Lys) which could be detected by GC/MS methodology.¹⁰⁹⁻¹¹¹ Also as depicted by Stadtman and coworkers, carbonyl assay could be a sensitive technique for detecting glutamic and amino adipic semialdehydes.⁸⁵ In carbonyl assays,

classic spectrophotometric methods were used to quantitate the 2,4-dinitrophenylhydrazine derivatives of carbonyl compounds. Pietzsch detected high levels of HAVA during MCO of LDL oxidation *in vitro*.¹¹⁰ Also, sensitive and improved GC/MS based methodologies can identify the increased level of these carbonyls in cells and tissues in case of pathological conditions such as aging.^{109, 111, 112} Unfortunately, neither GC/MS nor other assay based methods can detect the position of the amino acids in a protein that was oxidized by a free radical.

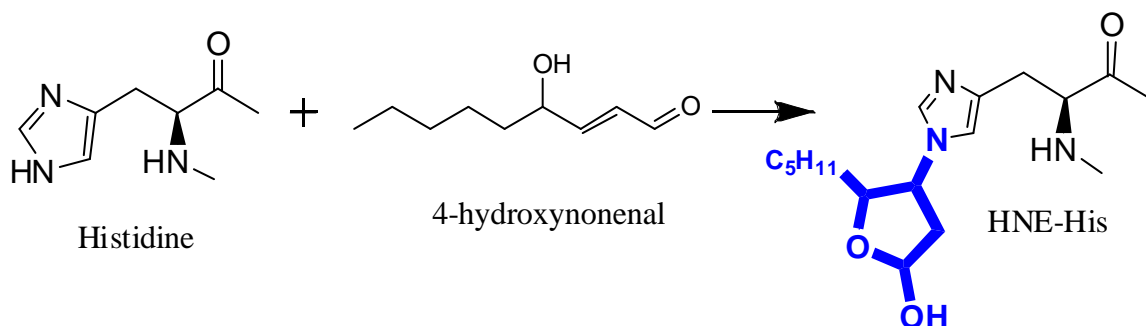
Most importantly all of these chemical changes are associated with characteristic mass changes. These mass changes leave specific footprint on the proteins. By using suitable mass spectrometric based methodologies (LC-MS/MS) the specific oxidation sites could be determined on the proteins.

3.4.2. Oxidation of amino acid via lipid intermediate

As mentioned earlier in these chapter lipids can be easily oxidized by hydroxyl radical to form aldehydes such as malondialdehyde, acrolein or 4-hydroxynonenal (HNE). All of these species are highly reactive electrophiles that can target specific amino acids. Uchida's pioneering work on covalent HNE adduct formation with Lys, His and Cys residues provides ample evidence in support of modification of amino acids in LDL.^{26, 98} These modifications are associated with structural changes in apo B-100.

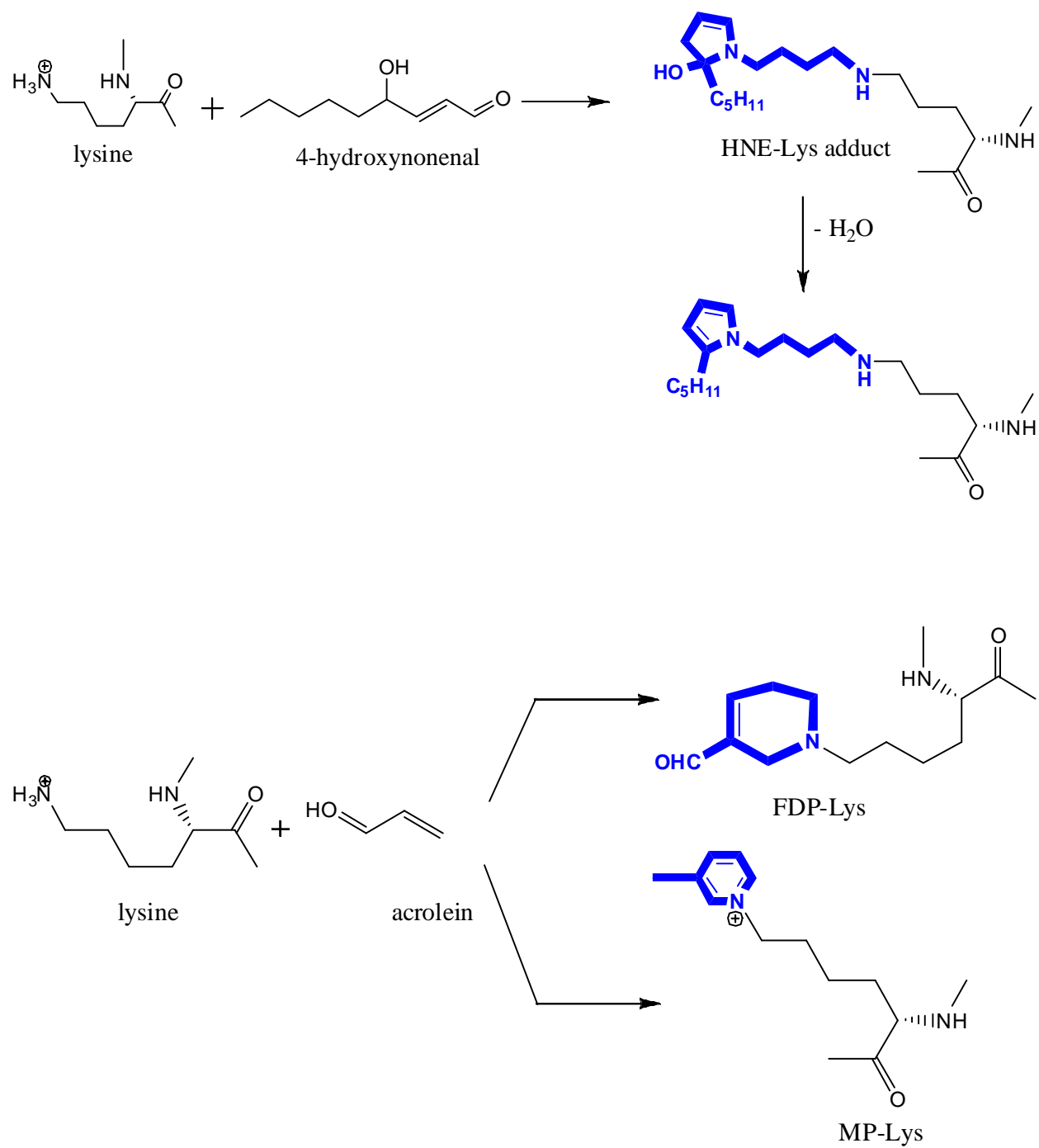
Michael addition type products (1, 4 addition) are typical in covalent attachment of HNE to His residues (+156 Da) (Scheme 3.10). Lysine which has a reactive ϵ -amine group was a specific target for acrolein. Acrolein forms heterocyclic adducts such as N $^{\epsilon}$ -(3-formyl-3,4-dehydropiperidino) lysine (FDP-Lys) (+94 Da) or N $^{\epsilon}$ -(3-methyl pyridinium) lysine (MP-Lys) (+76 Da)^{26, 98} (Scheme 3.11). Sayre discussed 2-pentylpyrrole adduct formation in Lys residues through a hemiaminal intermediate formation during reaction with HNE.¹¹³ HNE attaches to

lysine via a Schiff base adduct (1, 2 addition) formation. The Schiff base adduct formation is associated with characteristic mass increase of +138 Da and +120 Da (Scheme 3.11). HNE-Lys adducts were sometimes labile.¹¹⁴ Previous attempts to identify the HNE-Lys adduct in proteins were mostly unsuccessful when trypsin was used as the proteolytic enzymes.¹¹⁵ It is however apparent that chymotrypsin digestion gives better result in detecting HNE-Lys residues in protein oxidation.¹¹⁶



Scheme 3.10 4-Hydroxynonenal adduct (4-HNE) adduct on histidine residue (+156 Da).

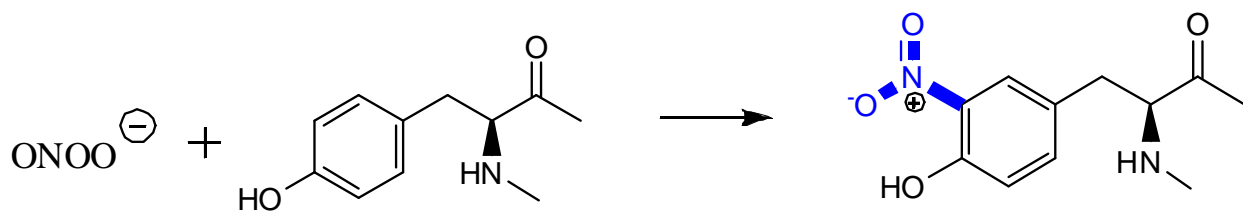
Interestingly, Cu (II) mediated oxidations of LDL initially yield lipid hydroperoxides which are converted to reactive aldehydes eventually causing damage in the amino acids of the protein. Cu (II) can bind to His residues in apo B-100 in LDL¹¹⁷ and initiate oxidation of the LDL. Other studies also indicated that Trp residue can act as a binding site for Cu (II) ions. Trp residues have a tight-binding spot as well as a weak binding spot. However, the position of those Trp residues in apo B-100 was not discussed in detail.^{117, 118} It seems therefore plausible that mechanism of Cu (II) mediated oxidations of apo B-100 vary significantly from hydroxyl radical mediated oxidation of LDL. Hydroxyl radical can react rapidly with the amino acids that are radical accessible and damage the protein directly. On the other hand in Cu (II) mediated oxidations predominantly secondary lipid oxidation products such as aldehydes are generated and subsequently modify amino acids (Lys and His).



Scheme 3.11 Lysine (Lys) reacts with α, β -unsaturated aldehydes (acrolein and 4-HNE) to yield various products including MP-Lys (+76 Da), FDP-Lys (+94 Da) and 2-petylpyrrole-Lys (+138 Da and +120 Da).

3.4.3. Peroxynitrite mediated oxidation of apo B-100 in LDL

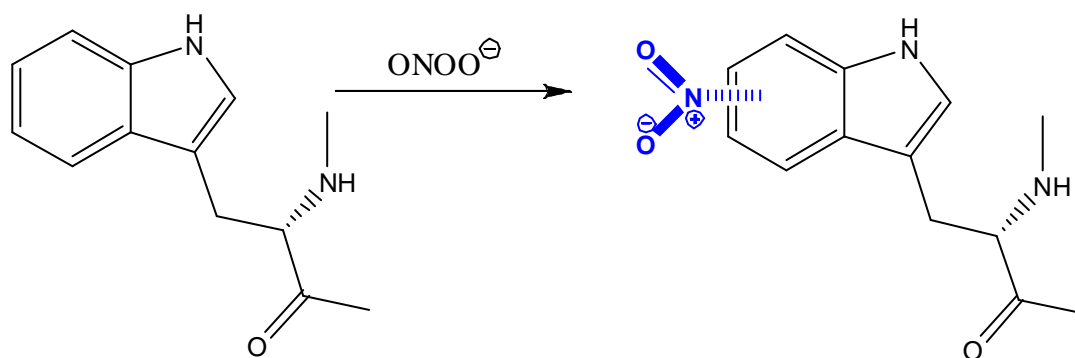
Peroxynitrite is not a free radical. The unpaired electron of nitric oxide is paired with the unpaired electron of superoxide to produce a covalent bond. Nonetheless it is highly reactive. Peroxynitrite leaves a specific stable footprint on side chains of amino acids. It directly modifies three different amino acids: cysteine, methionine and tryptophan.^{56, 59} Oxidation was also observed for other aromatic amino acids such as tyrosine, phenylalanine and histidine through secondary mechanisms (involving carbonate and hydroxyl radical). Peroxynitrite mediated cysteine oxidation in albumin was previously reported with a second order rate constant⁷⁰ on the order of $10^3 \text{ M}^{-1}\text{s}^{-1}$. Cysteine has reactive thiol groups which were converted to the corresponding sulfonic acids (+48 Da). Methionine contains thiol groups and is fairly susceptible to oxidation to yield methionine sulfoxide (+16 Da). Tryptophan residues are oxidized to form nitrotryptophan^{69, 119, 120} (Scheme 3.13) and N-formylkynurenine (+32 Da) (Scheme 3.8). Interestingly tryptophan can be nitrated (+45 Da) (Scheme 3.13) as well as hydroxylated (+16 Da) at the same time.¹²¹



Scheme 3.12 3-Nitrotyrosine (+45 Da) formation on peptides from peroxynitrite reaction on tyrosine side chain.

Tyrosine nitration (+45 Da)^{122, 123} (Scheme 3.12) in proteins requires special discussion. As nitric oxide cannot directly promote the nitration reactions in physiological systems, peroxynitrite appears to be the sole initiator for nitration. It has become apparent that other mechanisms of protein nitration exist. For example hemeperoxidase, hydrogen peroxide and

nitrite can cause nitration of tyrosine.^{68, 124, 125} However, it should be noted that protein tyrosine nitration could happen following this pathway only if the enzyme is immediately accessible to oxygen radical and nitric oxide radical. Direct oxidation by nitrite radical was minor particularly since low oxygen tension in tissues made the conversion of nitric oxide radical to nitrite radical a slow process and also nitrite radical will preferentially attack the thiols due to their higher reactivity. Alvarez *et. al.* provided interesting evidence on oxidation of Cys using human serum albumin and peroxynitrite.¹²⁶ In the presence of carbon dioxide, cysteine oxidation decreased and an increase in tyrosine nitration was observed.



Scheme 3.13 Tryptophan nitration by peroxynitrite (+45 Da), 4, 5, 6-nitrotryptophan are all possible.

Therefore nitration of biomolecules indicates the involvement of nitric oxide radical derived oxidant species during oxidative damage. Consequently, peroxynitrite plays a central part in protein tyrosine nitration.

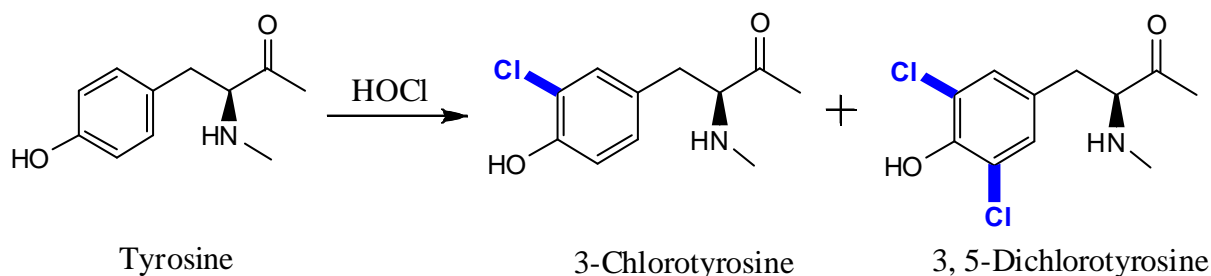
Since hydroxyl radical generation from peroxynitrite is not unlikely though less probable, markers for hydroxyl radical mediated oxidation products of Tyr and Phe were observed in peroxynitrite mediated oxidation pathways.¹²¹

Peroxynitrite mediated oxidation induces changes in the amino acid residue and primary structure of the proteins by changing their chemical environment. It oxidizes LDL and initiates unfolding in LDL protein.¹²¹

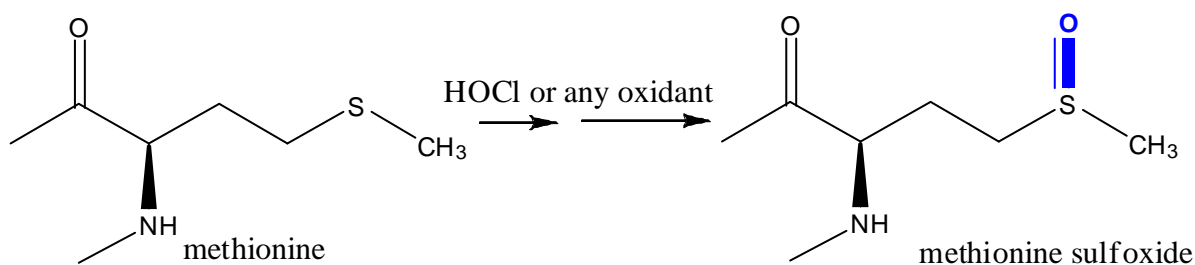
3.4.4. Apo B-100 oxidation with hypochlorous acid

LDL oxidation with hypochlorous acid was observed both *in vitro*^{127, 128} and *in vivo*.^{31-33,}
¹²⁹ In the late 1990's chlorotyrosine (+35 Da) (Scheme 3.14) was proved as one of the specific markers for HOCl mediated oxidation in apo B-100. By a stable isotope dilution gas chromatographic-mass spectrometric method, high levels of 3-chlorotyrosine deposition in atherosclerotic plaque was measured.^{27, 32} 3,5-Dichlorotyrosine formation was also observed in apo A-I when treated with high concentrations of HOCl.¹³⁰ Lysine chloramines were deemed to be unstable and difficult to detect in protein samples due to cross-linking and carbonyl formation. By studying model peptides related to the apo A-I sequence, Bergt *et. al.* was able to detect monochloramine (+34 Da) and dichloramine (+68 Da)¹³⁰ in Lys residues. Most interestingly, these peptides did not have any other reactive amino acids besides Lys and Tyr, indicating secondary pathways originated from chloramine hydrolysis.^{78, 131} Chloramines on Lys residues hydrolyze to form aldehydes (aminoadipic semialdehyde) which were previously detected by carbonyl method. Methionine is easily oxidizable and could form methionine sulfonic acid (+ 16 Da) (Scheme 3.15). Reactive thiol of cysteine goes to corresponding sulfonic acids (+48 Da)⁷⁸ (Scheme 3.16). Upon oxidation with HOCl, tryptophan hydroxylation (+16 Da) occurs in the indole ring (Scheme 3.17). Also, through intramolecular radical reaction Trp can yield the ring breaking products kynurenine (+4 Da) and N-formylkynurenine (+32 Da) (Scheme 3.8). Histidine forms an unstable chloramine on the side chain which can readily be donated to other neighboring amino acids (such as Lys) to regenerate parent His residue.^{78, 82} Guanidine moiety of Arg is susceptible to chloramine (+34 Da) formation upon incubation with hypochlorous acid. The stability of this chloramine on Arg residues are not extensively studied or known. Unfortunately, only chlorotyrosine appears to be a definitive marker of hypochlorous acid

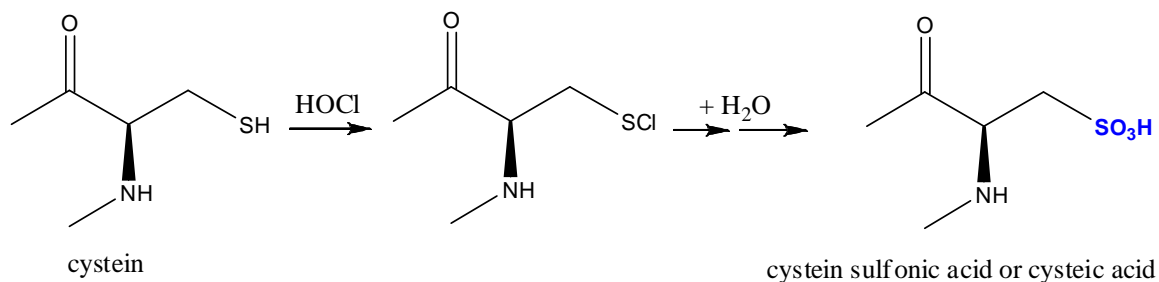
mediated oxidations. Other products were unable to provide specific indication of HOCl oxidation and could be originated from other oxidants.¹³²



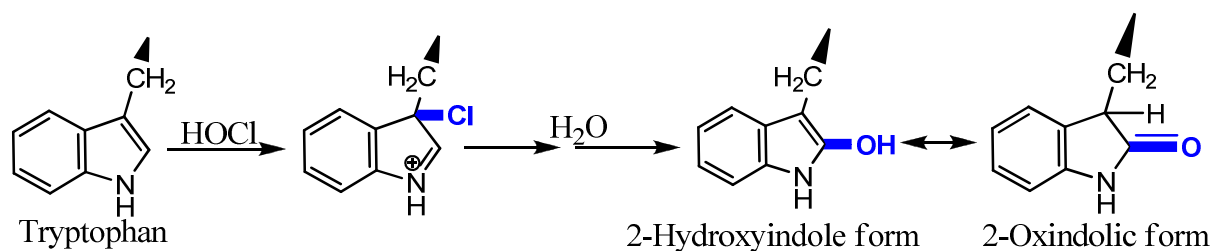
Scheme 3.14 Reactions of Tyrosine and hypochlorous acid to form of 3-chlorotyrosine (+34 Da) and 3, 5-dichlorotyrosine (+68 Da) in peptides and proteins.



Scheme 3.15 Methionine oxidation to methionine sulfoxide (+16 Da) is most common and can happen even with ambient oxygen.



Scheme 3.16 Oxidation of cystein (Cys) to cysteine sulfonic acid (+48 Da) in presence of HOCl. This product is also observed previously with peroxynitrite treated samples.



Scheme 3.17 Oxidation of Tryptophan (Trp) to 2-Oxindolic form by HOCl.

3.5. Atherosclerosis and oxidized LDL (ox-LDL)

Atherosclerosis is a complex cardiovascular disorder which was correlated with high levels ox-LDL formation. Although controversy exists on whether circulating plasma ox-LDL (detected in low concentration in blood) is responsible for atherosclerosis, recent prospective reports were more inclined towards a positive correlation.^{25, 133, 134} Specific oxidized epitopes on ox-LDL in blood may trigger atherosclerosis and other manifestations.⁴⁰ LDL can permeate through vascular endothelial cells and smooth muscle cells. During this infiltration to arterial intima, LDL can be oxidized by several reactive oxygen species (Figure 3.6). Ox-LDL was removed by immunoglobulin M (IgM) antibodies in the form of immune complex.¹³⁵ However, some of the ox-LDL particle can escape this removal pathway. The escaped ox-LDL particles will be taken up by macrophages through scavenger receptor CD-36.¹³⁶⁻¹⁴⁰ Inclusion of ox-LDL inside the macrophage could initiate lipid laden foam cell formation. Several signaling pathways such as pro-inflammatory cytokines, TNF- α and interleukins¹⁴¹ become activated. Foam cells release more ROS and myeloperoxidase (MPO) which could be responsible for generating more ox-LDL.⁹¹ Foam cells can also bind to T-cells circulating in blood^{40, 142} (Figure 3.6). Foam cells adhere strongly to endothelial cells and initiate fatty streak formation and thereby induce inelasticity in the arterial wall. This is major early event of plaque formation.

Interaction of apo B-100 with arterial proteoglycans increases the retention of the LDL in the arterial intima.^{94, 96, 102, 103, 105, 143} Proteoglycan obtained from atherosclerotic patients showed higher uptake of LDL than normal patients. Also ox-LDL particles are cytotoxic and induce cell apoptosis.^{55, 144, 145}

Ox-LDL formation in the sub-endothelial cells can facilitate atherogenic progression. Oxidized LDL limits the biological activity of the endothelium derived nitric oxide.⁴⁰ It was

apparent that this phenomenon increases entry of the inflammatory cells into the arterial wall. In addition it was discovered that ox-LDL stimulate macrophage expression of peroxisome proliferator activated receptor- γ (PPAR- γ). Scavenger receptor (CD36) expression and expression of pro-inflammatory genes were shown to be altered by macrophage expression of PPAR- γ .¹⁴⁶

Several studies described peroxynitrite mediated oxidation of LDL as an important event in atherosclerotic plaque formation. Leeuwenbergh *et. al.* emphasized enzyme involvement (MPO) and formation of tyrosyl radical in oxidation of LDL.⁶⁸ 3-nitrotyrosine (3-NT) was detected immunocytochemically in macrophages, foam cells and vascular smooth cells¹⁴⁷. Interestingly, 3-NT accumulated even in the presence of extracellular superoxide dismutase (SOD), indicating that the formation of peroxynitrite can outcompete superoxide dismutation.¹⁴⁸ Controversial data were observed on the level of 3-NT in LDL obtained from human plasma and arterial intima.^{68, 149} Vascular hemodynamics could play a crucial role in accumulating 3-NT through peroxynitrite mediated pathways. Immunohistostaining of coronary arterial bifurcations reveal the importance of fluid shear stress on peroxynitrite formation and accumulation in the arterial intima.¹⁵⁰

3-Chlorotyrosine is a known marker of atherogenic condition.^{27, 31, 32, 132} An increase in scavenger receptor CD36 uptake of HOCl treated ox-LDL was reported indicating high probability of lipid laden foam cell formation.^{151, 152} Chloramine formation on Arg residues inhibited endothelial cell nitric oxide synthase activity. Therefore vascular endothelial nitric oxide production mechanism was altered which may attribute to endothelial dysfunction.¹⁵³

Antioxidants play an obvious role in inhibiting the progression of atherogenesis. Ascorbate^{154, 155}, urate and α -Tocopherol reduce LDL oxidation. However, α -tocopherol is

associated with LDL and played the most important role in preventing LDL oxidation¹⁵⁶ in circulation as well as in sub-intima.

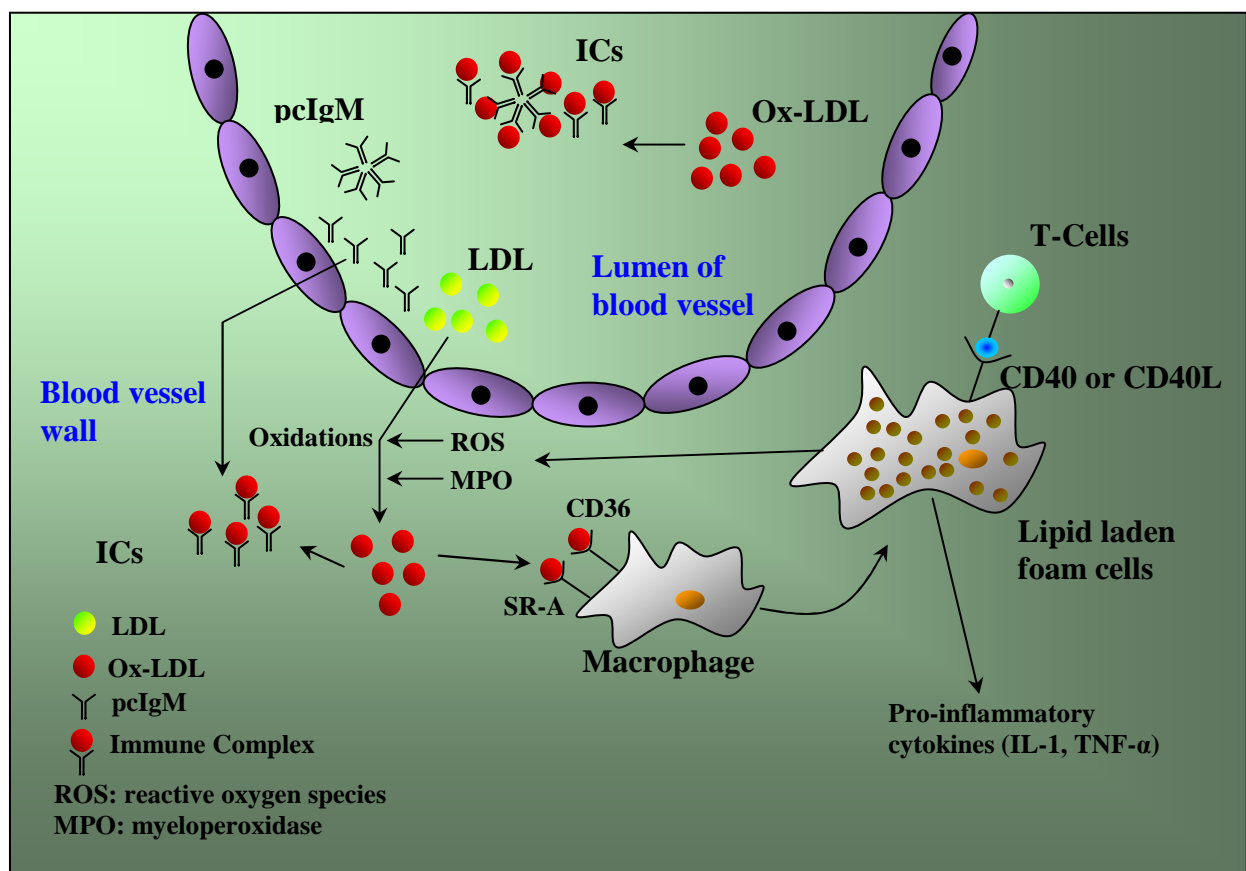


Figure 3.6 Oxidized LDL formation in the endothelial cells and arterial intima. Ox-LDL was taken up by macrophages through scavenger receptor CD36. Lipid laden foam cell formation was depicted in the figure which showed high adhesion properties for binding to the arterial wall.

3.6. Targeted proteomics with LC-MS/MS based methodologies

Evolution of the versatile mass spectrometric instrumentation in post-genomic era revealed new horizons in protein mass spectrometry. Marc Wilkins coined the term “proteomics” in 1994 while working with a 2D-Gel based protein samples following a notion that all proteins were expressed from the genome.¹⁵⁷ In biological studies, however, the concept of proteomics was expanded to a new level in order to answer more critical and demanding questions. Proteomics is no longer confined in protein sequencing and profiling but also used to study

protein post translational modifications and protein-protein interactions in cells and tissues.¹⁵⁸⁻¹⁶⁰ Discovery and development in the field of protein and peptide ionization techniques in mass spectrometry helped the biologists and chemists to perform complex protein/peptide mixture analysis.

Two “soft” ionization techniques were extensively used in protein mass spectrometry. Matrix assisted laser desorption ionization (MALDI)¹⁶¹ was extensively used in identifying proteins. In this method the samples are mixed with a specific matrix (organic compounds such as sinapinic acid or dihydroxybenzoic acid). A laser beam transfers energy to the matrix which in turn ionizes the peptides or the proteins.

Electrospray ionization^{161, 162} is also a soft ionization technique where a high electrical potential is applied to the analyte solution such that the analyte molecule(s) can form charged droplets (Figure 3.7). Driven by a potential gradient as well as a pressure gradient along the ion passage, the droplets follow a desolvation process leading to downsizing of the droplets. Constant evaporation continues until fully desolvated ions are formed.

In one instrument design, the ions then enter into the linear ion trap (Figure 3.7) (in our case, a Thermo Finnigan LTQ-ion trap instrument coupled with HPLC). By a suitable combination of constant direct current (DC), radio frequency (RF) and oscillating alternative current (AC) in the ion traps, ions can be stabilized (precursor ion isolation), fragmented (collision induced dissociation) and detected. Helium is used as a collision gas in the traps for damping and cooling of ions (detailed discussion in chapter 4).

Liquid chromatography adds physical separation capabilities in LC-MS/MS based approach. In a complex mixture of proteins and peptides the separation before the mass

spectrometric detection increases the sensitivity and specificity of the detection. Overall this methodology provides a robust and a powerful detection in protein sequencing.

3.6.1. Database search

Database search is by far the most crucial parameter in identification of post translational modifications in proteins. Mass spectrometric data for biomacromolecules are complex. Various algorithms exist to identify the peptides from complex biological mixtures. A full length cDNA sequence of apo B-100 (accession # P04114) was employed in our database search using SEQUEST algorithm.

When a sequence is added to a database, the software theoretically cleaves the sequence to peptides (precursor ions). The precursor ions are fragmented to its corresponding B-ions (charge stays with amine terminal) and Y-ions (charge stays with carboxylic acid terminal). In mass spectrometric data both precursor ion and corresponding B- & Y-ions are observed. The observed tandem mass spectra were matched with the theoretical B- and Y- ions.

$$\Delta C_n = \frac{\text{Primary Xcorr} - \text{Secondary Xcorr}}{\text{Primary Xcorr}} \quad \text{Equation 3.8}$$

Where, Primary Xcorr is the best match and secondary Xcorr is the second best match.

The match between the theoretical and experimental spectra generates a score known as X-correlation score which defines goodness of fit. ΔC_n score on the other hand indicates the specificity of the match and mathematically defined as Equation 3.8. Although there is a certain degree of uncertainty involved in the database search method, improvements are on their way.¹⁶³ Based on the Xcorr, ΔC_n value, identification of peptide was initially filtered. The identified peptides that passed the filter were further verified manually by matching the spectra and theoretically calculating the masses.

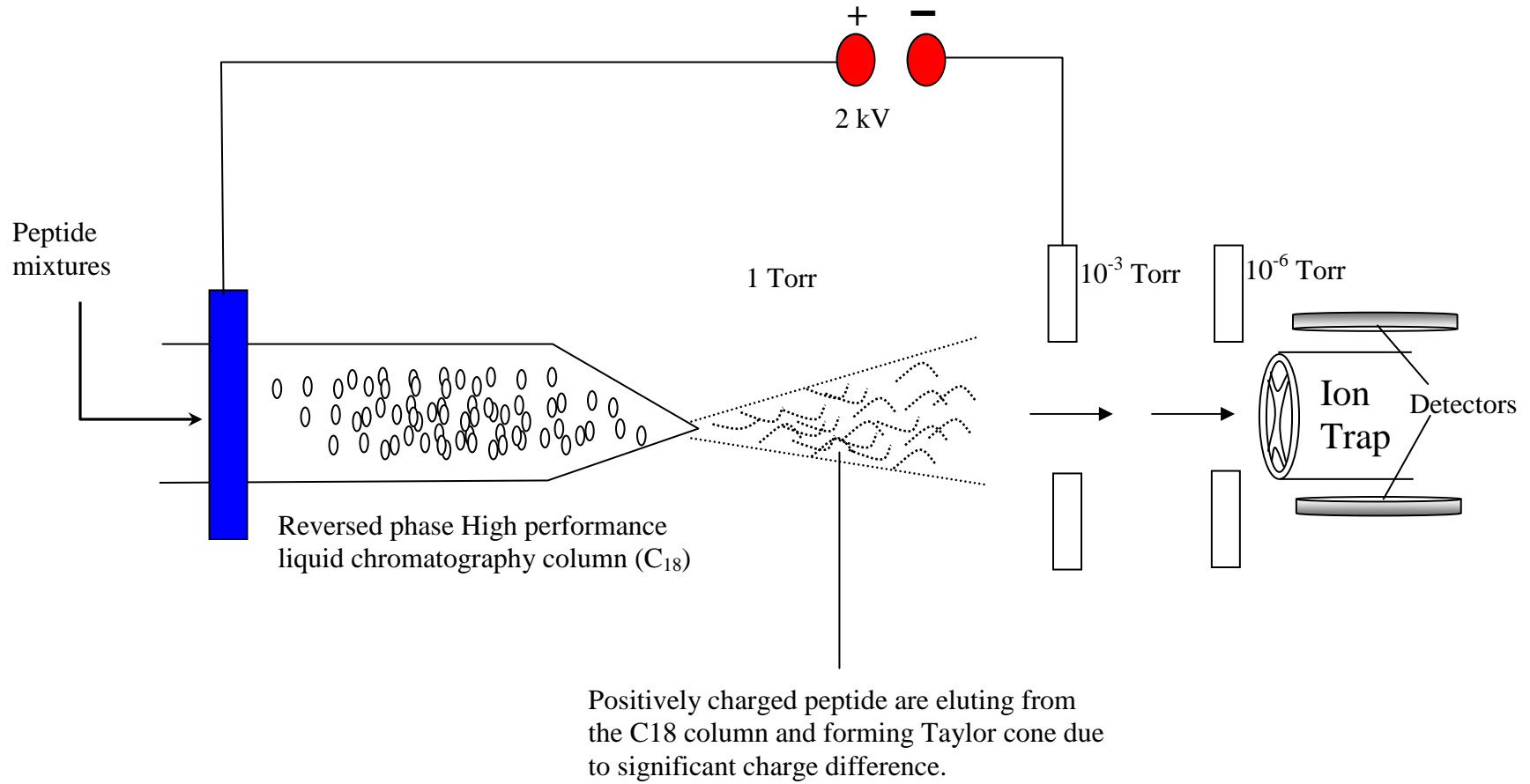


Figure 3.7 Schematic representation of liquid chromatography (LC) – mass spectrometry (MS) setup for analysis of peptide mixture in positive ion mode.

3.6.2. Protein surface mapping by LC-MS/MS

Protein footprinting¹⁶⁴⁻¹⁶⁸ has become popular in the last decade. By using laser flash photolysis method hydroxyl radical can be generated. Generated hydroxyl radical can react with amino acid residues in protein and leave a stable footprint on them. Kinetics of amino acid side chain reactions were well documented.¹⁰⁶ These stable footprints could be determined by LC-MS based methodologies. Aye *et. al.* tuned the laser flash photolysis method and identified several surface accessible oxidation sites in model protein ubiquitin and apomyoglobin.¹⁶⁴ Hambly and co-workers worked on laser flash photochemical oxidation of proteins (FPOP) and identified important structural characteristics of apo and holo myoglobin.^{165, 166} Sharp *et. al.* reported solvent accessible surface mapping on apomyoglobin, lysozyme and β -lactoglobulin A.^{169, 170} Hydroxyl radical reactivity towards the side chain of the amino acids depends on the accessibility of the side chain and reactivity of the side chain residue.¹⁷¹ Bridgewater and co-workers studied amino acid-metal binding interactions through metal catalyzed oxidation and mass spectrometry.^{154, 172, 173} Shcherbakova *et. al.* proved the usefulness of using inexpensive Fenton method for generating hydroxyl radical during the footprinting of protein, DNA and RNA.¹⁶⁷

3.6.3. LC-MS/MS studies on LDL

Considerable attention was focused on HDL cholesterol and apo A-I oxidation^{8, 9, 81, 130}, while studies on apo B-100 in LDL was fairly limited.^{115, 121} Ample evidence indicated strong positive correlation between oxidation of apo B-100 and atherosclerosis.^{31, 33, 36, 68, 150, 174, 175} Free radical mediated oxidations in apo B-100 brings about non-specific heterogeneous changes in the protein. Heinecke and co-workers have studied both HDL and LDL proteins for specific

footprints by employing various analytical techniques.^{31-33, 35, 77, 130, 176} Leeuwenburgh *et. al.* used advanced GC/MS methodologies to identify oxidation products of apo B-100.⁶⁸

A great deal of emphasis was exhausted to understand Cu (II) mediated oxidations of LDL. Moreover, as discussed earlier Cu (II) can bind to amino acid residues such as His and Trp. Therefore, it will initiate oxidation in the protein by lipid mediated pathways. Previously, Yang *et. al.* identified Cu (II) mediated oxidation of Trp in apo B-100 with HPLC followed by a MS methodology which was robust but required a tedious complicated follow-up work.¹⁷⁷ Obama *et. al.* identified Cu (II) mediated oxidation sites in apo B-100. In *in vitro* experiments with apo B-100, HNE and acrolein, several different binding sites were identified by LC-MS/MS based methodologies.¹¹⁵ Oxidation product of Trp (NFK) was quantitatively measured by HPLC method after apo B-100 was oxidized in LDL.¹⁷⁸

Butt *et. al.* detected nitrated proteins by 2D-gel and mass spectrometric approach.¹⁷⁹ Recently, Hamilton and co-workers reported some oxidized residues in apo B-100 during LDL incubation with peroxynitrite. Extensive circular dichorism and LC-MS/MS methodology were used to identify the oxidized residues¹²¹ on apo B-100.

Effect of hypochlorous acid on LDL was studied extensively by Mike Davies's group.^{75, 79, 82, 131, 180, 181} Several interesting mechanistic and kinetic work on hypochlorous acid and amino acids, peptides and proteins were published over last fifteen years.^{75, 78, 82, 180} However, free radical based footprinting combined with proteomics would render new insights in apo B-100 oxidation.

3.7. Total hydroxyl radical quantitation

The flux of hydroxyl radical and hydrogen peroxide formed in a cellular system varies significantly depending on the stress condition. Also the flux of H₂O₂ can be generated

periodically in the cells. Chance and co-workers quantitated concentration of hydrogen peroxide *in vivo* which is on the order of 10^{-7} to 10^{-8} M.¹⁸² In H_2O_2 induced cell apoptosis studies, Antunes *et. al.* used 10^{-3} to 10^{-5} M H_2O_2 which is two to five orders of magnitude higher than the cellular concentration.¹⁸³ Complex mechanisms drive hydroxyl radical mediated oxidation of proteins in cells. Kinetic studies involving hydroxyl radical in cells and tissues yield complicated and interfering results. However, total hydroxyl radical formed by Fe (II) and H_2O_2 (Fenton method) can be quantitated by a probe based approach. Benzoic acid can efficiently trap hydroxyl radical formed to yield hydroxybenzoic acids (o-, m-, p-hydroxybenzoic acid).

High performance liquid chromatography (HPLC) (Figure 3.8) was extensively used to identify and quantitate hydroxybenzoic acids.^{184, 185} If benzoic acid concentration is considerably high compared to the hydroxyl radical formed, it would be possible to trap all of the hydroxyl radical formed by the Fenton system. This hypothesis however is good only if other scavengers were present in low quantities and/or show lower radical trapping efficiency. Zhou *et. al.* showed an effective trapping efficiency of benzoic acid to hydroxyl radical in aqueous samples.¹⁸⁵ Lindsey *et. al.* provided an improved analytical methodology to detect low concentration of p-HBA in hydroxyl radical trapping experiment with benzoic acid.¹⁸⁴ Therefore effective hydroxyl radical concentrations for LDL exposure were calculated in this study by benzoic acid experiments. Hydroxyl radical formed from low micromolar to low millimolar H_2O_2 was quantitated by benzoic acid method (for details please see chapter 4).

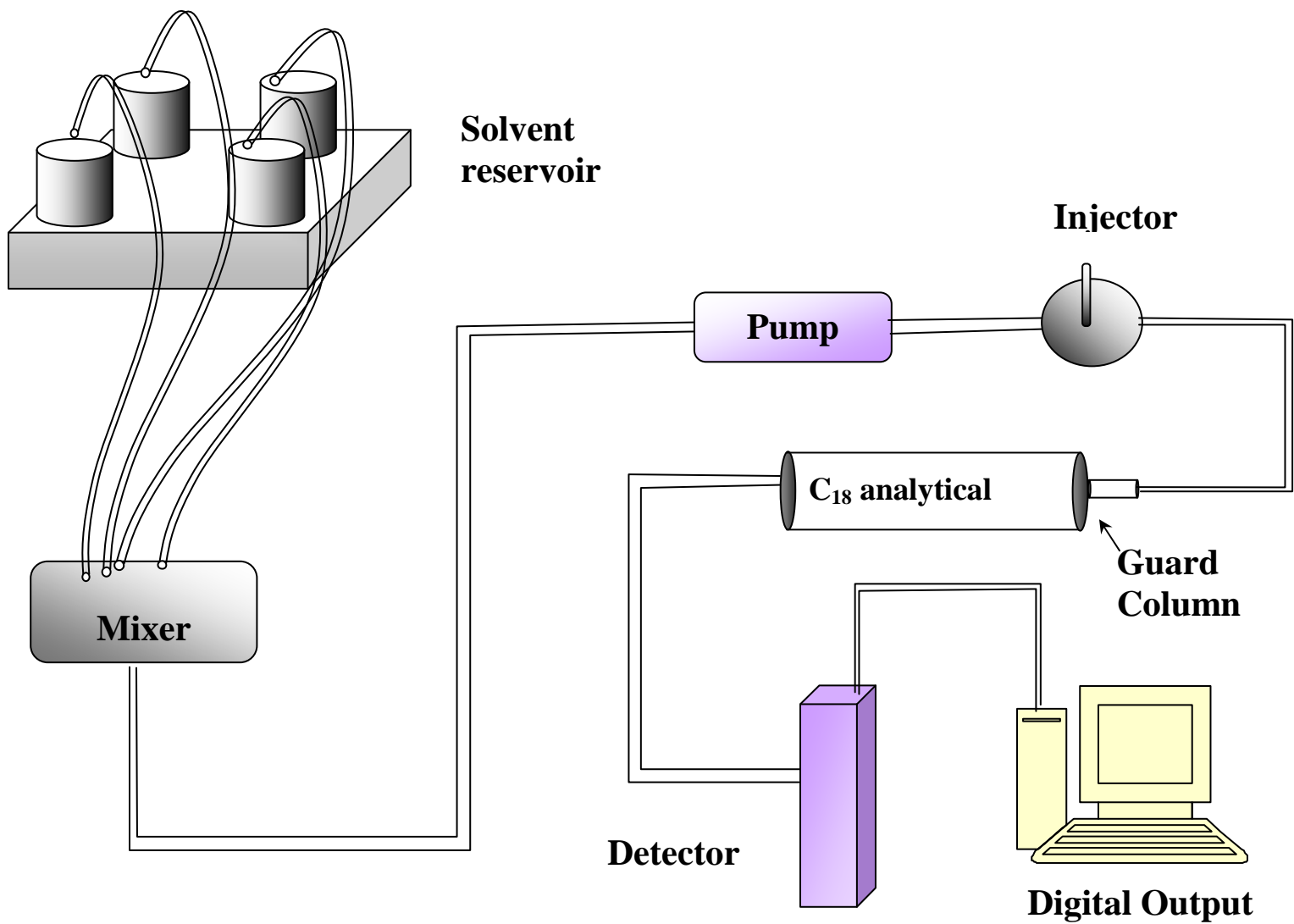


Figure 3.8 A typical High performance liquid chromatography set up for analyte detection.

3.8. Analysis of natural variants

3.8.1. What are natural variants?

Natural variants are defined as variations in the amino acids in a reported sequence (canonical sequence) in the sequence database. Canonical sequence contains most common polymorphs or the most conserved orthologous genes. Various types of natural variants exist in proteins. Single amino acid polymorphisms appear due to a single nucleotide change at the codon level. Disease associated mutations in amino acids are also possible in the sequence. During RNA editing events the protein primary structure changes (changes in the amino acid sequence).

3.8.2. Mass spectrometry database search on natural variants

Mass spectrometry was not fully exploited in determining natural variants in low density lipoproteins. However, various efforts were made to identify natural variants in hemoglobin by using mass spectrometric method.¹⁸⁶⁻¹⁸⁹

Fifty one natural variants are reported for apo B-100 on Swissprot over last twenty four years.¹⁶ A novel database search method was employed in this study to identify some of the reported natural variants in apo B-100. By slightly tweaking the database search, rapid identification of reported natural variants was possible. By simply analyzing tandem mass spectra it is not possible to differentiate the natural variants leucine (Leu) and isoleucine (Ile) (m/z: 113). However, these amino acids are translated from different cDNA codons. In database search, our endeavor was to include those natural variants which were reported by other methods to develop a rapid screening technique for clinical samples. This unique methodology could

expand the horizon of mass spectrometry to determine amino acid variants in protein clinical samples.

3.9. References

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Chapter 4.

Mapping oxidations of apo B-100 in human low density lipoprotein

by LC-MS/MS

4.1. Abstract

Human low density lipoprotein (LDL) is a major cholesterol carrier in blood. Elevated concentration of low density lipoprotein, especially when oxidized, is a risk factor for atherosclerosis and other cardiac inflammatory diseases. Past research has connected free radical initiated oxidations of LDL with the formation of atherosclerotic lesions and plaque in the arterial wall. The role of the LDL protein in the associated diseases is still poorly understood, partially due to lack of structural information. In this study, LDL was oxidized by hydroxyl radical. The oxidized protein was then delipidated and subjected to trypsin digestion. Peptides derived from trypsin digestion were analyzed by LC-MS/MS. Identification of modified peptide sequences was achieved by database search against apo B-100 protein sequences using the SEQUEST algorithm. At different hydroxyl radical concentrations, oxidation products of tyrosine, tryptophan, phenylalanine, proline and lysine were identified. Oxidized amino acid residues are likely located on the exterior of the LDL particle in contact with the aqueous environment or directly bound to the free radical permeable lipid layer. These modifications provided insight for understanding the native conformation of apo B-100 in LDL particles. The presence of some natural variants at the protein level was also confirmed in our study.

Keywords: LDL, hydroxyl radical, LC-MS/MS, oxidative mapping, natural variants.

4.2. Introduction

Low density lipoprotein (LDL) acts as a major cholesterol carrier in the human body. LDL consists of a lipid core of triacyl glycerol and cholesteryl ester and an outer layer of phospholipids with free cholesterol and apo B-100 (Figure 3.3). Apo B-100 was reported as a single monomeric protein consisting of 4563 amino acid residues¹ encircling the LDL molecule. This protein plays an intricate role in LDL receptor binding.² Oxidative modification of LDL (ox-LDL) is considered as a major risk factor since it plays a critical role in the pathogenesis of atherosclerosis.³⁻⁷ During oxidative stress various radicals are generated in the human body which can induce the oxidation of LDL. Thereafter ox-LDL can be taken up by macrophages through scavenger receptors in smooth muscle cells, which lead to foam cell formation.^{7, 8} Foam cells interact with pro-inflammatory cytokines and enhance the adhesion of ox-LDL to the arterial wall, a major early event of atherosclerotic plaque formation (Figure 3.4). In addition, it was also reported that interaction of aberrant apo B-100 with proteoglycans induces the generation of atherosclerotic plaques.^{9, 10}

The primary source of free radical in the human body is superoxide.^{11, 12} By various physiological mechanisms, less reactive superoxide is converted to reactive peroxy, alkoxy and hydroxyl radicals. The last of this list is known to be highly reactive and cause non-specific, extensive damage to lipids and proteins.¹³ *In vitro* studies have shown the generation of hydroxyl radical by the well established mechanism of Fenton chemistry.^{14, 15} The nature of oxidation of apo B-100 by hydroxyl radical and its implications in atherosclerosis^{4, 7} is still poorly understood.¹⁶ Finding the oxidation sites for apo B-100 is relevant and important for its structure-function relationship. Several attempts were made to elucidate the structure of apo B-100 by 3-D modeling and a pentapartite domain arrangement was proposed.^{17, 18} However, these

studies have some uncertainties involved, making the protein structure predictions speculative. Oxidation of LDL can occur by various pathways. One group of researchers strongly believes that lipid peroxides (oxidation products of LDL lipids) are involved in the atherosclerotic plaque formation^{8, 19}, while other researchers reported oxidation of apo B-100 (LDL protein) as the important step in atherogenesis.^{7, 20-22} There are two reported pathways (Table 4.2) for hydroxyl radical mediated apo B-100 oxidation in LDL. The direct oxidation pathway of apo B-100 involves the oxidation of amino acid residue by extensive cross-linking, peptide bond cleavage and also oxidation of side chains.^{4, 13, 15, 21, 23, 24} The hydrophobic amino acid residues such as Phe, Tyr, and Trp undergo simple hydroxylation reactions. This pathway prevails when the radical generation site is in close proximity to the apo B-100. The indirect oxidation pathway of apo B-100 involves indirect oxidation through an intermediate lipid reactive species. Several lipid peroxidation products such as 4-hydroxynonenal (HNE) and acrolein can form. These molecules contain unsaturated aldehydes and are highly reactive electrophiles which can react with histidine, lysine and cysteine residues to form Michael and Schiff base adducts. The ϵ -amino group of lysine specifically reacts with acrolein to form N^ϵ -(3-formyl-3,4-dehydropiperidino)lysine (FDP-Lys) and N^ϵ -(3-methylpyridinium)lysine (MP-Lys).^{25, 26} The ϵ -amino group of lysine also reacts with HNE to yield a HNE-Lys Schiff base adduct which readily loses water to yield 2-pentylpyrrole-Lys adduct.²⁷ These two processes result in mass increases of 138 Da (Lys+138) and 120 Da (Lys+120) in lysine residues. Histidine also forms adducts such as HNE-His.^{6, 25, 26, 28} All of these modified amino acid residues are listed in (Table 4.1). As mentioned earlier, hydroxyl radical mediated oxidation of apo B-100 in LDL can be of two distinct types, namely direct oxidation (hydroxyl radical must have direct access to the amino acid residue of apo B-100) and oxidation via lipid intermediates (hydroxyl radical

oxidizes the lipid and the reactive lipid species subsequently reacts with the protein). During oxidative modification of LDL, the variety and magnitude of modifications can be substantially diverse for each LDL particle. Uchida, *et al.* explored protein modifications in ox-LDL by apo B-100 hydrolysis and used antibodies to verify formation of HNE and acrolein adducts. Those studies, however, did not elucidate the oxidation sites of apo B-100.^{25, 26}

Table 4.1 List of oxidatively modified amino acids (hydroxyl radical mediated) previously reported in literature.

Amino Acid	Major Amino acid Oxidation Products Reported in Literature	
	Direct Oxidation	Oxidation via Lipid Pathway
Tyrosine	Dihydroxyphenylalanine ^{12-14, 20} (DOPA) (Y+16), Dityrosine	N.D.
Phenylalanine	<i>o-/m</i> -tyrosine ^{12, 13, 20}	N.D.
Tryptophan	5-/7-Hydroxytryptophan, Kynurenine, N-formylkynurenine ^{12-14, 20}	N.D.
Histidine	2-Oxohistidine ^{12-14, 20}	Michael & Schiff Base adduct formation ^{14, 24-26}
Lysine	Aminoadipic semialdehyde ^{22, 23, 52}	Michael & Schiff Base adduct formation ^{14, 24-26}
Arginine	Glutamic semialdehyde ^{22, 23, 52}	N.D.
Proline	Glutamic semialdehyde ^{22, 23, 52}	N.D.

N.D. Not determined

Mass spectrometry (MS) is one of the most powerful tools to identify post translational modifications of proteins. LC-MS/MS based studies on proteins were demonstrated previously as

powerful techniques in determining the stable oxidative products in proteins.^{22, 29-32} Research has been done on oxidative modifications of apolipoprotein A-I (30 KDa protein) in high density lipoprotein (HDL) by Heinecke and coworkers.^{33, 34} Bolgar, *et al.* identified HNE adducts of histidine by MS/MS for the first time.³⁵ Several different approaches were taken by various researchers to follow the oxidation pattern of apo B-100 in LDL. Karlsson, *et al.*³⁶ used 2D-CE and MS analysis, and Heller, *et al.*³⁷ utilized SDS-PAGE followed by MS analysis. However, these studies were focused on small apolipoproteins. The determination of oxidation sites is complicated since the modifications are non-specific in nature. Recently Obama, *et al.* identified some oxidized amino acid residues using polyvinylidene (PVDF) membranes²² by incubating LDL with Cu²⁺ ion. Obama's method showed improved efficiency in removing the lipid from apo B-100 for LC-MS/MS analysis. Also, in PVDF membranes, digestion with trypsin showed greater peptide recovery. The current study focused on oxidative mapping of amino acid residues including those with hydrophobic side chains such as Phe, Pro, and Trp. Several other amino acid residues were found to be oxidized with variable concentrations of hydrogen peroxide mixed with Fe (II).

Amino acid residues in the human apo B-100 sequence sometimes differ from the reported sequence (P04114).¹ These changes in the amino acids are known as natural variants. Apo B-100 has been shown to contain several natural variants in its sequence.³⁸⁻⁴⁷ Often times these variants were attributed to polymorphism and/or disease.⁴¹ Fifty-one natural variants were reported so far in the cDNA or expressed sequence tag (EST) of apo B-100.¹ In addition to oxidative mapping, the presence of some reported natural variants in the LDL was revealed in this study from direct protein analysis via LC-MS/MS experiments.

4.3. Materials and methods

Human low density lipoprotein (LDL) was purchased from VWR chemicals. Each batch of LDL was acquired from a single, healthy human subject. LDL was stored with 150 mM ethylenediaminetetraacetic acid (EDTA) and was kept refrigerated (4 °C). Iron sulfate ($\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$), disodium ethylenediaminetetraacetic acid (Na_2EDTA), and all solvents were purchased from VWR. Micro-dialysis tubes and floats were also purchased from VWR. High purity (99%) 4-hydroxybenzoic acid (4-HBA), ammonium bicarbonate (ABC), dithiothritol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). In all experiments, purified water was obtained by deionization and UV irradiation.

4.3.1. Quantitation of hydroxyl radical

High performance liquid chromatography (HPLC) was used to quantify the total amount of hydroxyl radical formed. In this approach, a high loading of benzoic acid (10 mM) was added in order to scavenge all radical that was formed (detailed method described elsewhere^{48, 49}). The reaction between benzoic acid and hydroxyl radical has a known second order rate constant and forms a characteristic product, 4-HBA. The amount of 4-HBA formed during the reaction between hydrogen peroxide and Fe^{2+} was determined. In short, 400 μL of 50 mM FeSO_4 (final concentration 1 mM), 400 μL of 50 mM Na_2EDTA (final concentration 1 mM), and different concentrations of hydrogen peroxide (final concentrations 9 μM , 90 μM , 900 μM , 4500 μM and 9000 μM) were added to 20 mL of 10 mM benzoic acid (aq). In these experiments hydrogen peroxide was added as a bolus addition. The reaction was carried out for 25 minutes and was quenched by addition of 200 μL of methanol and acidified with 2 drops of concentrated HCl. The

sample was loaded onto a 1.5 mL loop. An Alltech 15 cm C18 (4.6 mm I.D., and ODS 5 μm) column was used for separation of 4-HBA from the reaction mixture with detection by UV absorbance at 234 nm. The elution gradient was water acidified to pH \sim 2.5 with trifluoroacetic acid (A) and acetonitrile (B): 0-3 min 10% B; 3-12 min linearly to 44% B; 12-13 min linear to 100% B. The analytes were pre-concentrated on column in the first 3 minutes, and then were eluted by increasing the solvent strength. A calibration curve was formulated from peak area under the HPLC trace daily ($R^2 > 0.99$) with 4-HBA standards (at least 2 replicates) at concentrations of 0.5 μM , 10 μM and 25 μM . Oxidized benzoic acid samples were injected using at least 3 replicates for each concentration of hydrogen peroxide exposure. Peak area corresponding to 4-HBA in the reaction mixture was compared to that of the standards used in calibration (Figure 4.1).

4.3.2. Oxidative modification of LDL

Ten microliters of LDL (5.3 mg/ml) was diluted to 100 μL with purified water to yield a concentration of 0.53 $\mu\text{g}/\mu\text{L}$ of apo B-100. Eight samples were placed in separate micro-dialysis tubes (MW cut off 3500 Da) and were then dialyzed against 500 mL ABC buffer (pH = 7.4, 25 mM) for 24 hrs at 4 $^{\circ}\text{C}$. The dialyzed LDL samples were collected and placed in small micro-centrifuge tubes. Each exposure was carried out in duplicate. Hydroxyl radical was generated by adding 2 μL of 50 mM Na_2EDTA , 2 μL of 50 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (aq) and 6 μL of aqueous hydrogen peroxide (0.15 mM, 1.5 mM or 15 mM). Immediately after addition and mixing, the concentrations of the added hydrogen peroxide were 9 μM , 90 μM or 900 μM . The reaction was carried out for 25 minutes at room temp in an Eppendorf Thermomixer R in the dark with constant shaking at 800 rpm. Control experiments with no peroxide addition were carried out at about the same time when hydrogen peroxide exposures were performed. Immediately after the

reaction period the treated samples were placed in micro-dialysis tubes for dialysis against acetone (24 hrs at 4 °C) to remove lipids and other hydrophobic molecules. Then the treated samples were further dialyzed against methanol:aqueous 50 mM ammonium bicarbonate (3:2) for 24 hrs at 4 °C. Samples were collected in plastic tubes and were placed in boiling water for 5-10 minutes for partial denaturation of the protein. Thirty molar excess dithiothritol (DTT) was added and samples were incubated for 30 minutes at 37 °C to cleave the disulfide linkages. Next, 30 molar excess iodoacetamide was added and let react for 90 minutes at 37 °C for alkylation of the cysteinyl residues. Tryptic digestion was carried out overnight following previous methods.⁵⁰
⁵¹ The tryptic peptides were quenched with liquid nitrogen, vacuum dried, and reconstituted in 5% aqueous formic acid with 5% acetonitrile for LC-MS/MS analysis. Samples were stored at -80 °C until analysis.

4.3.3. LC-MS/MS analysis of apo B-100

The peptide mixture obtained from the procedure noted above (maximum amount equivalent to 3 µg protein) was first loaded onto a reversed-phase trap column, and then washed with 3% acetonitrile and 0.1% formic acid (aq) to desalt the sample. After the wash/desalt step, the trap column was switched into a gradient flow (60 minutes, water with 3% acetonitrile and 0.1% formic acid at the beginning and 65% acetonitrile with 0.1% formic acid at the end) to elute peptides to a C₁₈ analytical column before introduction into a linear trapping quadrupole (LTQ) mass spectrometer.

4.3.4. MS setup

Mass spectrometry analyses were performed on a LTQ mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nano-flow (200-500 nL/min) electrospray source. The

peptides were ionized via electrospray and recorded in a full scan mass spectrum in a positive ion mode. For each full MS scan, the top 3 most intense peptide precursor ions were selected and fragmented via collision induced dissociation (CID). Low pressure helium was employed as damping (for ion storage and isolation) and collision (fragmentation of activated ions) gas. The instrument was operated in a highly automated data dependent acquisition mode.

4.3.5. Database search

The database search was performed on an in-house search engine (Bioworks 3.3, Thermo Electron) using the SEQUEST algorithm. Apo-B sequences (P04114) and seven other sequences that include reported natural variants were compiled in a small database for searching (Table 4.2). Peptides with MW of 600-3500 Da were chosen for identification. All modifications were defined as differential (unmodified peptides were also searched) and are listed in (Table 4.2). Trypsin was selected as the proteolytic enzyme, and the database search included identification of one missed cleavage of Lys or Arg. An Xcorr score was generated from the matching of experimental spectra to the theoretical spectra of apo B-100 peptides. A charge state-Xcorr score filter was applied to all MS/MS spectra. Singly charged peptides with Xcorr score less than 2, doubly charged peptides with Xcorr score less than 2.5, and triply charged peptides with Xcorr score less than 3.0 were considered as false positives and removed from the final report.

4.4. Results

Reaction of hydroxyl radical in cells follows complicated pathways making it difficult to understand the underlying mechanisms and kinetics. The total amount of hydroxyl radical reacted can however be calculated. This information was helpful to determine the total hydroxyl radical generated during hydrogen peroxide incubation with LDL. We have quantified the total

amount of hydroxyl radical^{48, 49} that was formed after a bolus addition of hydrogen peroxide to aqueous iron in the presence of EDTA.

4.4.1. Total hydroxyl radical quantitation using benzoic acid with HPLC

LDL was exposed to hydroxyl radical at three different doses obtained by varying hydrogen peroxide concentration in the oxidation step. Quantitation of the total amount of hydroxyl radical formed at each of these peroxide/hydroxyl radical doses was carried out by reaction with excess benzoic acid. Hydroxyl radical reaction with benzoic acid yields several products, including 2-hydroxybenzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid. 4-Hydroxybenzoic acid makes up approximately 1/6 of the total products formed.^{48, 49} Consequently, the total amount of benzoic acid that reacted with hydroxyl radical can be calculated by measuring only the amount of 4-HBA formed and then multiplying this amount by a factor of 6.⁴⁸ The total amounts of hydroxyl radical formed after 25 minutes of reaction at different peroxide concentrations were calculated to be 0.78 ± 0.03 micromoles, 1.7 ± 0.3 micromoles and 3.1 ± 0.04 micromoles for 9 μM , 90 μM , and 900 μM H_2O_2 doses, respectively (95% confidence limits). These data are presented in Figure 4.1. The total radical formed was also quantitated for 4.5 mM and 9 mM hydrogen peroxide concentrations, but the total amount of hydroxyl radical produced did not increase beyond that observed for 900 μM peroxide (Figure 4.1).

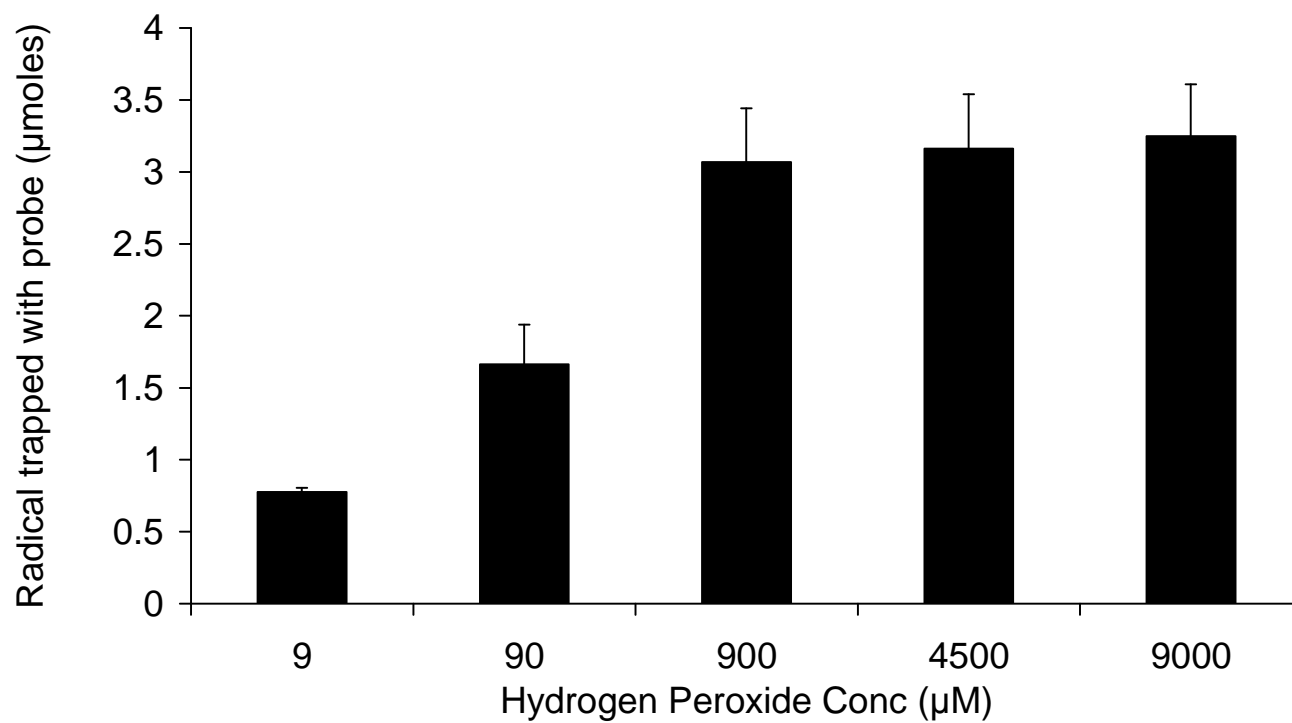


Figure 4.1 Total hydroxyl radical produced by adding different hydrogen peroxide concentrations to 10 mM benzoic acid in the presence of 1 mM FeSO₄·7H₂O and 1 mM Na₂EDTA in water.

4.4.2. Sequence coverage

As mentioned earlier tryptic peptides were searched against a full length cDNA sequence of apo B-100 protein (P04114) listed in the IPI-human database¹ using the SEQUEST algorithm. For large proteins such as apo B-100 it is difficult to get high sequence coverage via LC-MS/MS analyses of the peptides derived from trypsin digestion.^{19, 23} We achieved $(48 \pm 1) \%$ (2 samples) sequence coverage from untreated LDL samples (Figure 4.2). For the sample with no peroxide added and for the LDL treated with $9 \mu\text{M H}_2\text{O}_2$ (both with 1 mM Fe^{2+} and $1 \text{ mM Na}_2\text{EDTA}$) the recovery was $(45 \pm 2) \%$. These results are comparable to earlier work.²² With high concentrations of H_2O_2 ($90 \mu\text{M}$ and $900 \mu\text{M}$), however, our recovery decreased to $(33 \pm 2) \%$ and $(26 \pm 3) \%$, respectively. The decrease in the sequence coverage is plausible since high hydroxyl radical concentrations would result in substantial protein degradation including lysine and arginine modifications and additional unknown modifications of amino acid residues. Such highly degraded peptides would be unlikely to match a database search based on the unmodified protein sequence. A figure (Figure 4.2) indicating the sequence coverage for all samples is presented to indicate the regions of matching observed peptides to those in the cDNA sequence.

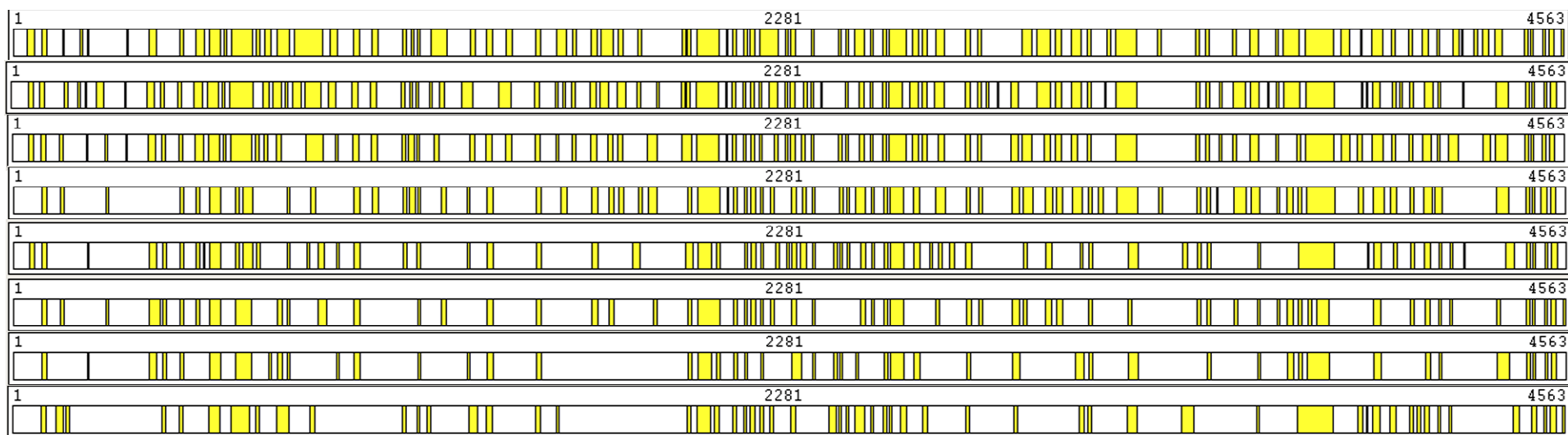


Figure 4.2 Sequence coverage was represented by yellow region in apo B-100 sequence (P04114). Amino acid 1 through 4563 was shown in the above figure. “A” represents sample without H₂O₂ treatment (Blank). This sample was run twice and the sequence coverage data for both run was furnished. “B” represents low peroxide concentration exposure to LDL (9 μM) (duplicate experiment). “C” represents intermediate peroxide concentration (90 μM) (duplicate experiments). “D” represents exposure to high peroxide concentration (900 μM) (duplicate experiments). Sequence coverage decreases with increase in peroxide concentration.

4.4.3. Oxidation of amino acid residues

As mentioned above, tryptic peptides were analyzed by LC-MS/MS. The peptides were first eluted according to their hydrophobicity immediately followed by introduction to the mass spectrometer. Individual ions were then selected for collision induced dissociation (CID). Resulting fragment ions (b and y ions) were used to determine the peptide sequence and possible modifications to the original peptide sequence[^]. Amino acids with aromatic side chains such as Phe, Trp, and Tyr were previously observed to follow +16 Da mass changes.^{15, 52} All amino acid modifications observed in this study are listed in Table 4.3 from duplicate analyses. Oxidations that were observed in both unoxidized controls and in oxidized samples were omitted from the table, so all entries in this table indicated peptides that were oxidized by the hydroxyl radical treatment. In the exposure with the lowest peroxide concentration (9 μ M), six different phenylalanine residues (Figure 4.3) were oxidized by addition of one oxygen atom. Under these conditions, eight Tyr residues also underwent O addition (Table 4.3 and Figure 4.4).

Direct oxidation of Trp yields kynurenine and N-formylkynurenine. 3898Trp was found to be oxidized to N-formylkynurenine (+32 Da) (Table 4.3). Trp+16 oxidation product was not observed. Also, three directly oxidized His residues were observed (Table 4.3 and Figure 4.6). Under direct hydroxyl radical attack, arginine and proline both take similar pathways and form a characteristic product, glutamic semialdehyde (Figure 4.7). It should be noted that widely reported formation of 5-hydroxy-2-aminovaleric acid from arginine and proline was previously observed in other studies only if a strong reducing agent (NaBH₄) was present in the reaction

[^] All positively matched peptides were listed in Appendix A from Table A-1 through Table A-5. Corresponding Xcorr value and ΔC_n values were also listed.

medium.⁵³ In our studies, ten oxidized Pro, and no Arg residues were observed in the low peroxide experiments.

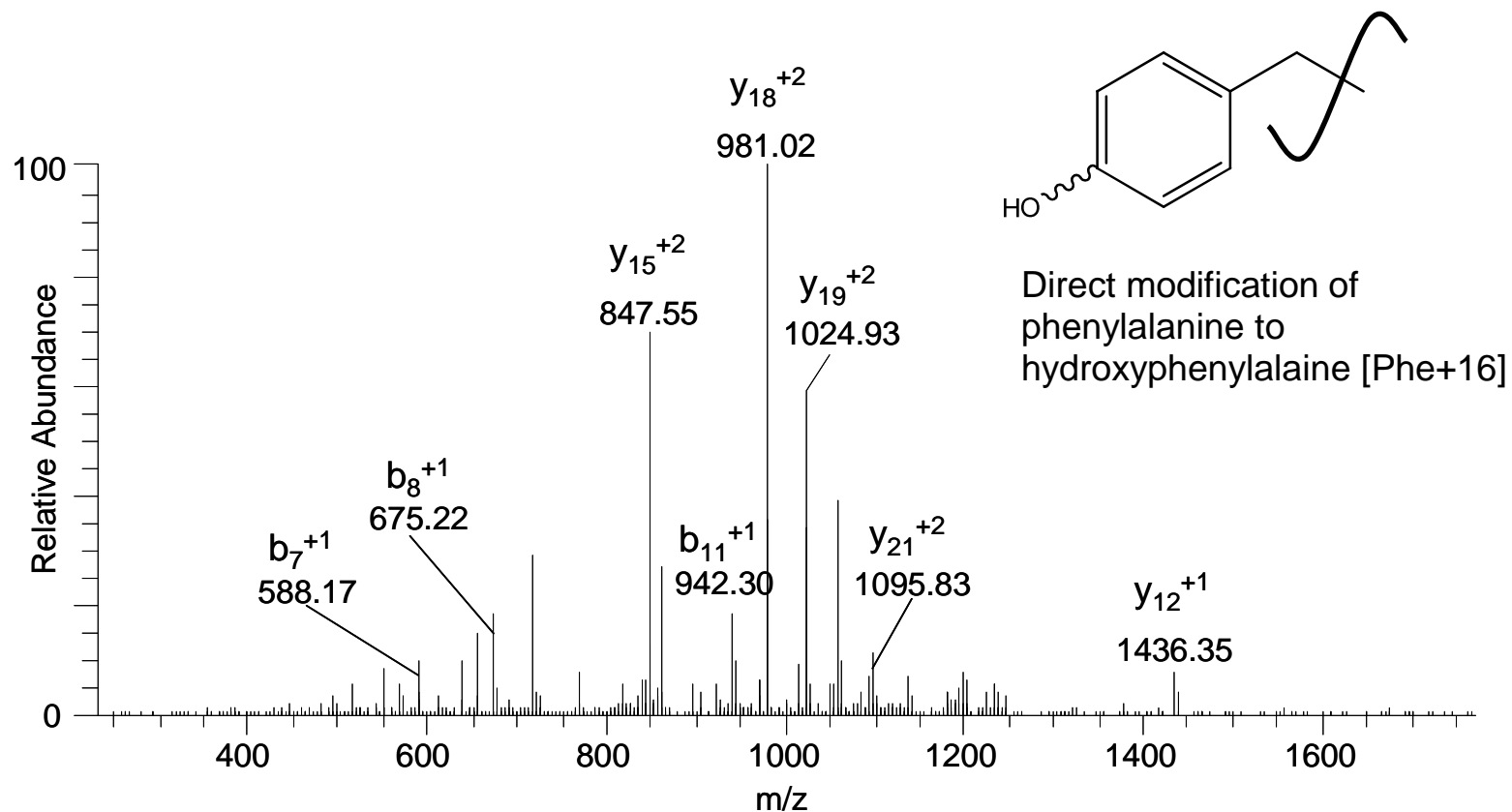
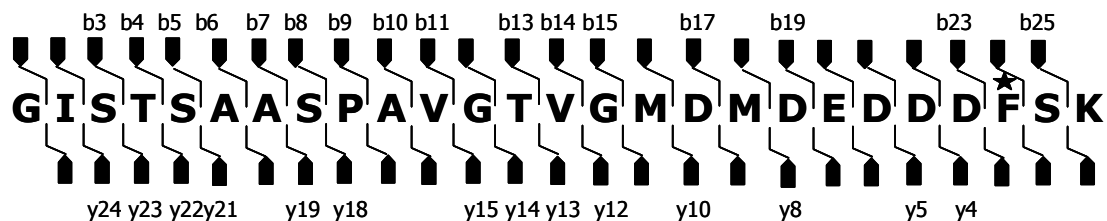


Figure 4.3 MS/MS spectra of tryptic peptide 3996-4021 from low peroxide concentration depicting modification of 4019Phe (Phe+16 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was not observed.

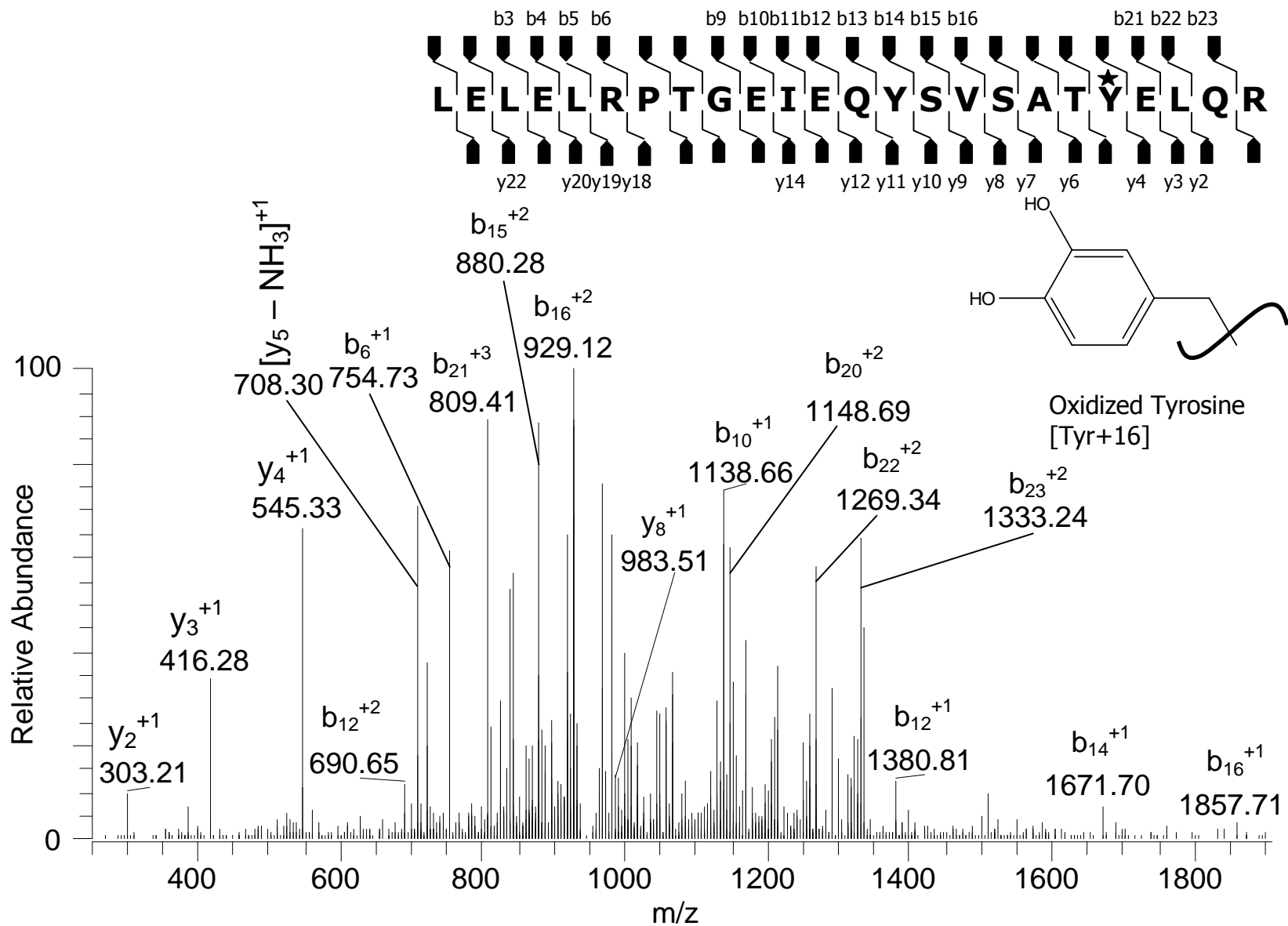


Figure 4.4 MS/MS spectra of tryptic peptide 1001-24 from low peroxide concentration depicting modification of 1020Tyr (Tyr+16 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was observed and compared with.

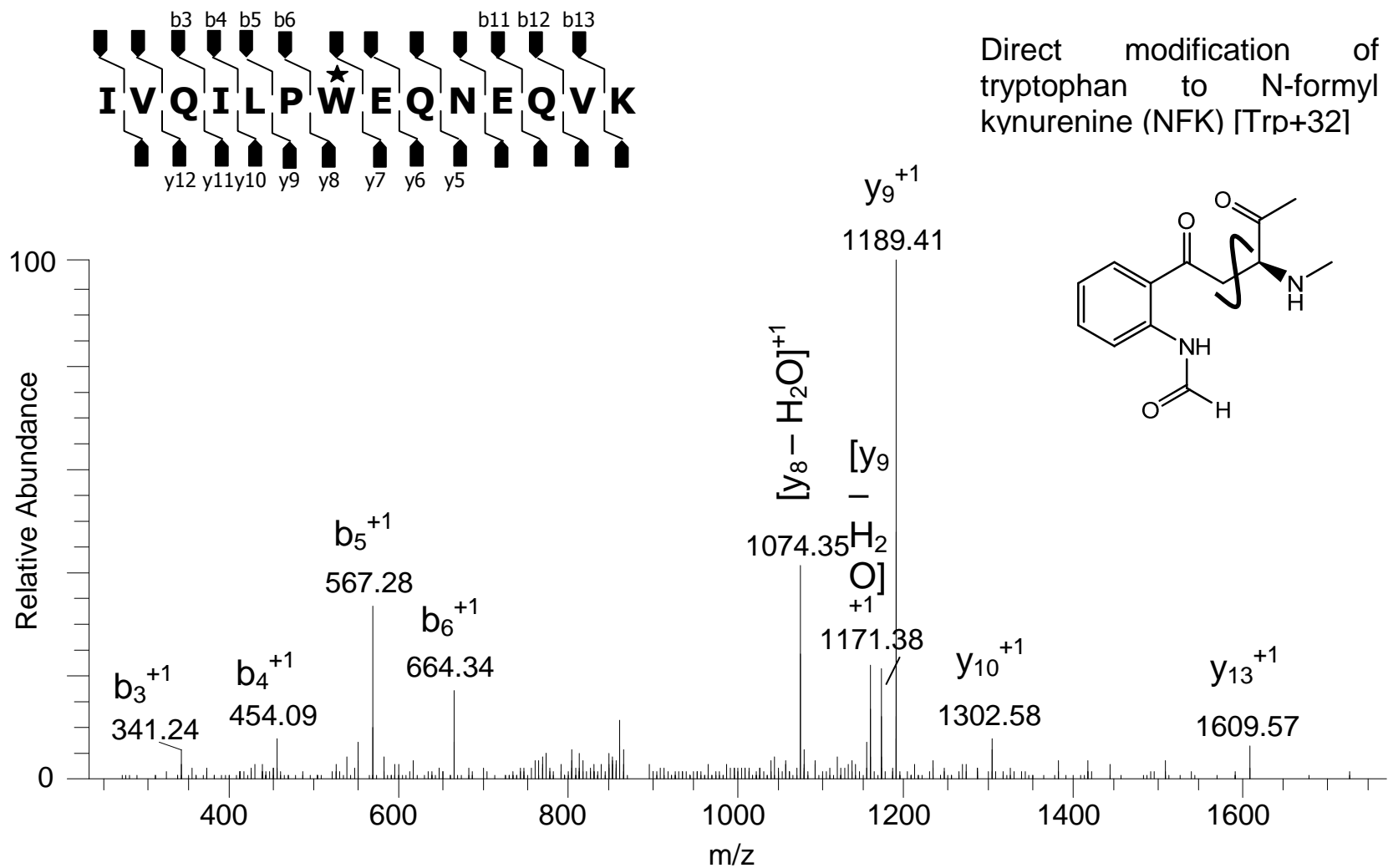
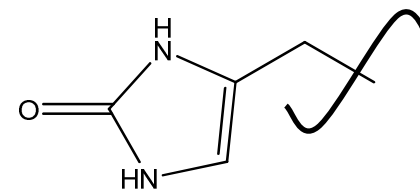
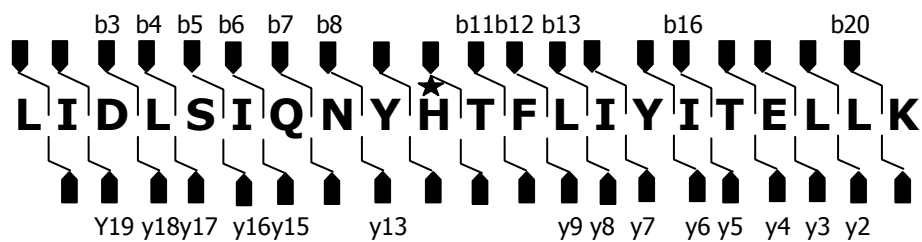


Figure 4.5 MS/MS spectra of tryptic peptide 577-590 from low peroxide concentration depicting modification of 583Trp (Trp+32 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was observed and compared with.



Direct modification of histidine to oxidized histidine [His+16]

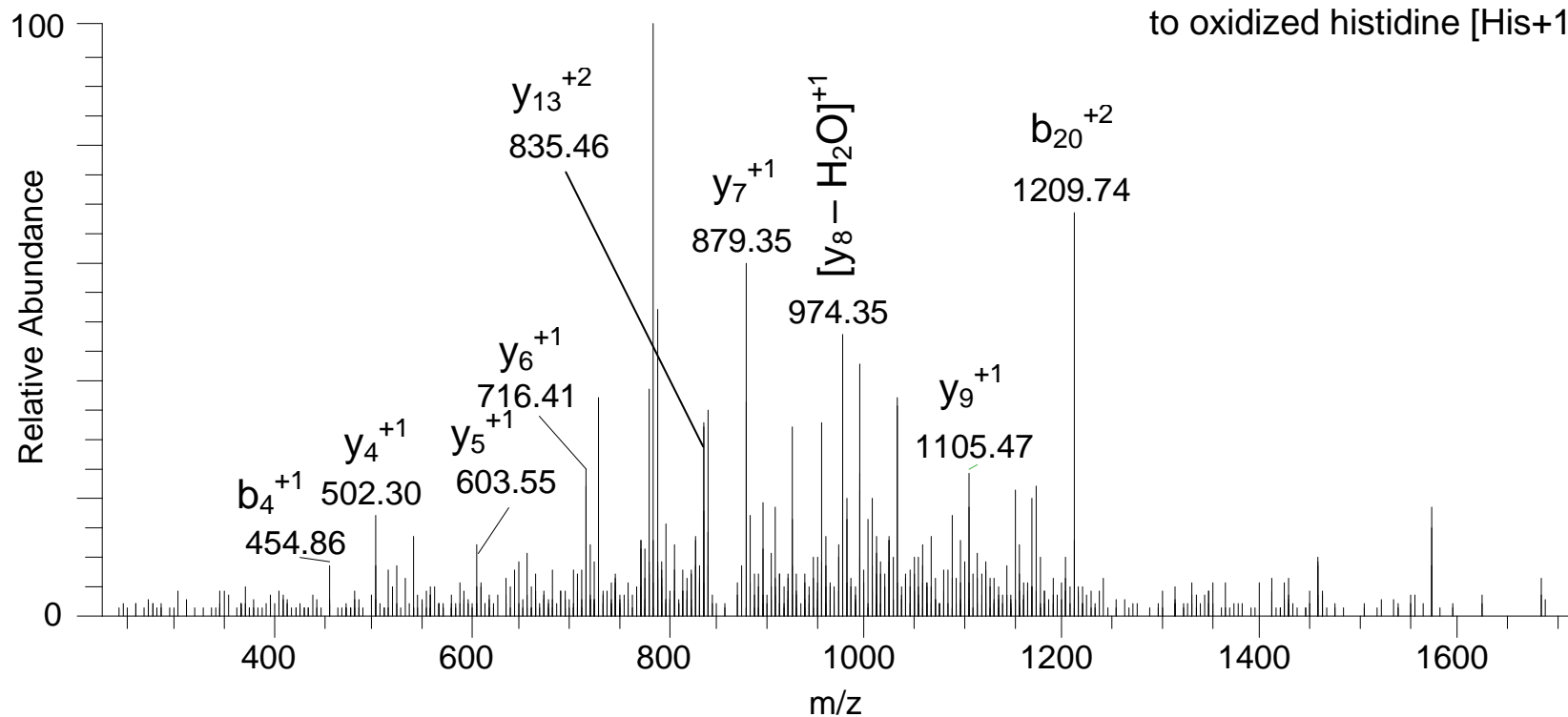


Figure 4.6 MS/MS spectra of tryptic peptide 4520–40 from low peroxide concentration depicting modification of 4529His (His+16 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was not observed.

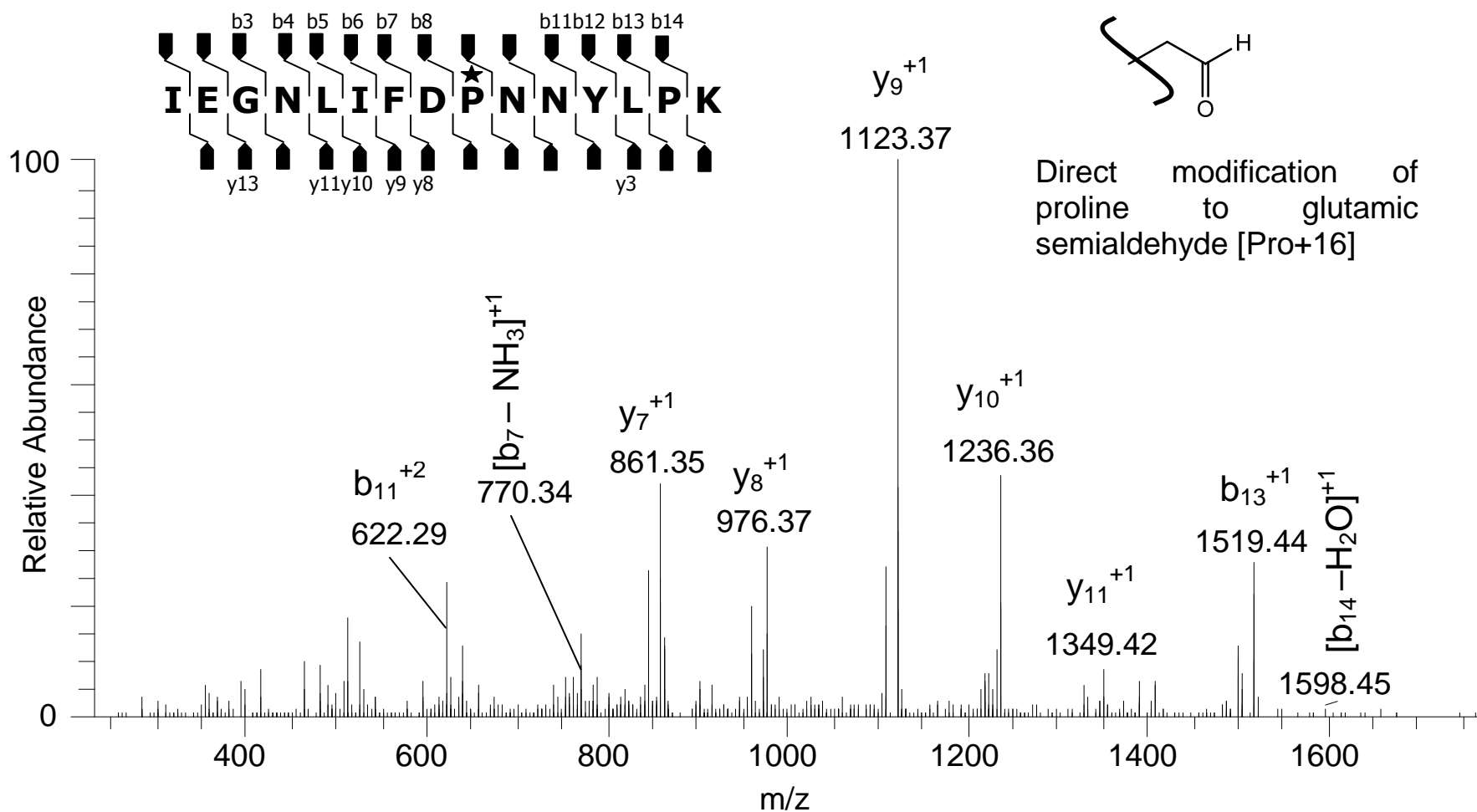


Figure 4.7 MS/MS spectra of tryptic peptide 655-69 from low peroxide concentration depicting modification of 663Pro (Pro+16 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was observed, compared and accepted as partial modification.

Figure 4.8 presents a map of all 4563 amino acids for apo B-100. The distribution of oxidized amino acids is depicted. The figure represents the presence of directly oxidized amino acids (upward line) and amino acids oxidized indirectly via a lipid radical pathway (downward line). In addition different colors indicate oxidations observed at low (red), intermediate (blue), and high (green) peroxide concentrations, respectively. Other colors (black, turquoise and yellow) represent amino acids that were oxidized in two or more different concentrations (for example black represents low and high, turquoise represents intermediate and high and yellow represents the amino acid was oxidized with low, intermediate and high peroxide concentrations). The red, upward lines indicate amino acids that are most likely solvent accessible in the intact LDL particle. Because additional oxidations only occurred at higher concentrations of peroxide, at which there was evidence for loss of protein structural integrity, it is unclear whether the blue and green lines represent solvent accessible amino acid residues. These amino acid residues could be solvent accessible but resistant to oxidation (e.g. nearby residues or lipids might scavenge radicals), or they could be isolated from the aqueous environment in the protein's native state, which may have been disrupted by excessive protein damage.

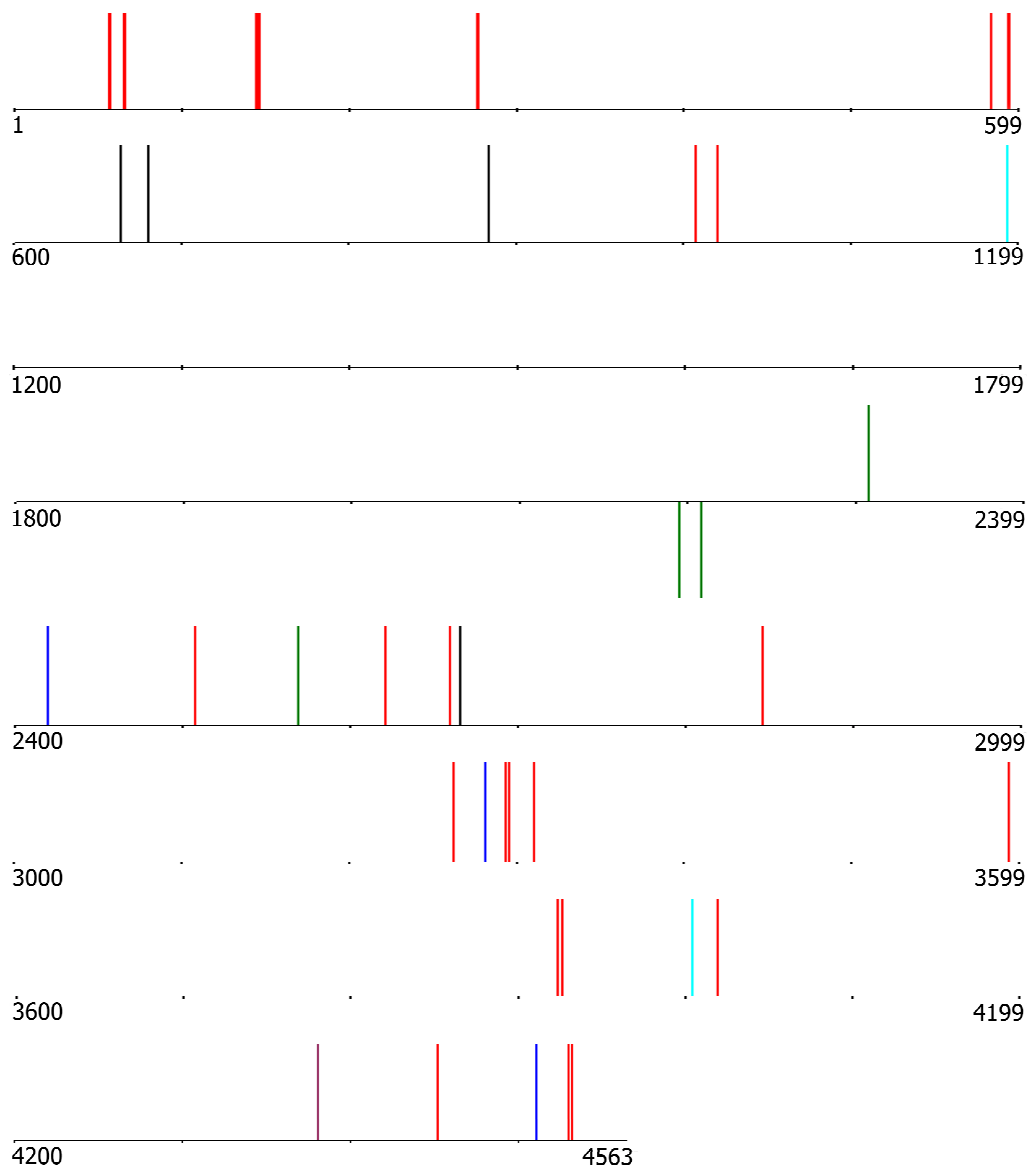


Figure 4.8 Plot of observed apo B-100 oxidized amino acids by sequence number [P04114]. Lines indicate locations of amino acid modification by direct oxidation (upward line) and indirect oxidation/lipid mediated oxidation (downward line). Red, blue and green lines indicate observed oxidations at low, moderate and high peroxide concentrations, respectively. Black lines indicate oxidations observed at (low + high), turquoise represents (intermediate + high) and yellow represents (low + intermediate + high) hydrogen peroxide concentration. Numbers indicate amino acid residue in the apo B-100 sequence.

At the intermediate peroxide concentration (90 μM), only two Phe, no His and one Tyr residue were found to be oxidized by addition of an O atom (Table 4.3). The number of observed oxidized amino acid residues was smaller at intermediate and high peroxide concentrations compared to the low peroxide concentration. At intermediate or high concentrations of hydroxyl radical, amino acid modification pathways become more complicated and result in unknown structural changes. Due to the complexity of protein damage, the method suffers from a decreased ability to identify apo B-100 peptide fragments by LC-MS/MS.

At high peroxide concentrations (900 μM), oxidations were observed at seven Phe residues, two Tyr residues, and no Trp residue (Table 4.3). Interestingly, a double modification of 2307Phe (dihydroxyphenylalanine) (Figure 4.9) was observed. Three Pro modifications and no Arg modification were observed at high peroxide concentration.

Oxidized residues were found to be widely distributed in the protein sequence as represented in Figure 4.8. In the LDL receptor domains (3130-60 and 3259-67),¹⁷ 3262Pro oxidation (Figure 4.7) was observed at low peroxide exposure. This oxidation may lead to alteration of LDL uptake by LDL receptors in the cell. The wide distributions of oxidation sites indicate that solvent accessible amino acid residues are spread throughout the protein.

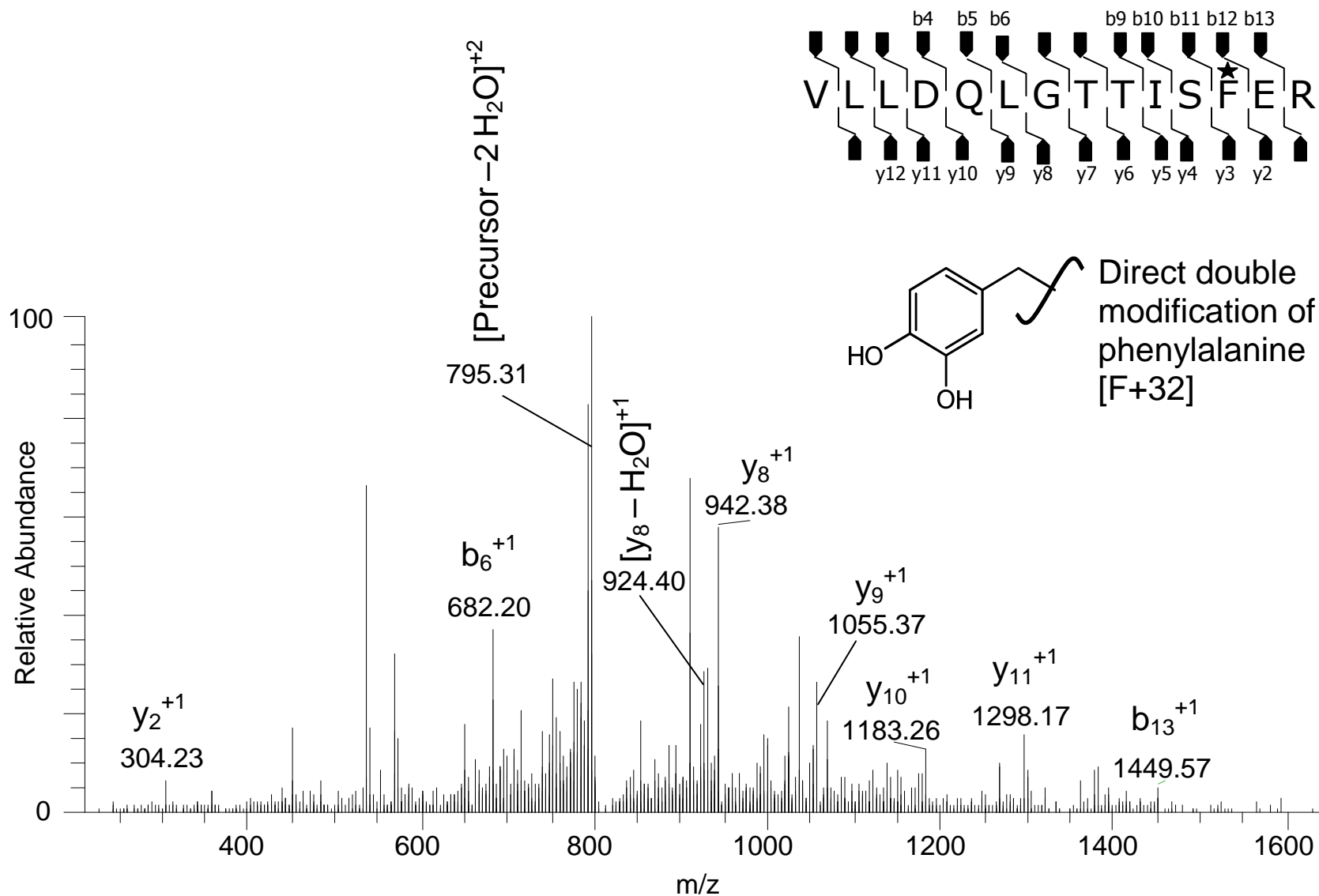


Figure 4.9 MS/MS spectra of tryptic peptide 2296-2309 from high peroxide concentration depicting modification of 2307Phe (Phe+32 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was observed and compared.

4.4.4. Amino acid residues undergoing oxidation via lipid oxidation route

Oxidation of lipids produces stable α,β -unsaturated aldehydes (acrolein or 4-hydroxynonenal). These products are hydrophilic in nature and therefore increase the polarity of some parts of the apo B-100 protein. HNE is prone to react with histidine residues to produce a mass increase of 156 Da. We did not observe this characteristic mass increase in the oxidized LDL. However, HNE also attacks the ϵ -NH₂ group of lysine to yield a Schiff base adduct (+138 Da) followed by a water loss and 2-pentylpyrrole adduct formation (+120 Da).²⁷ At low and intermediate concentrations of peroxide, no HNE-Lys adduct was observed, while at high concentrations of peroxide, 2195Lys and 2208Lys formed HNE-Lys (Figure 4.10). Previous attempts were made to identify HNE-Lys adducts in apomyoglobin and apo B-100 by tryptic digestion method without success.^{22, 27, 54}

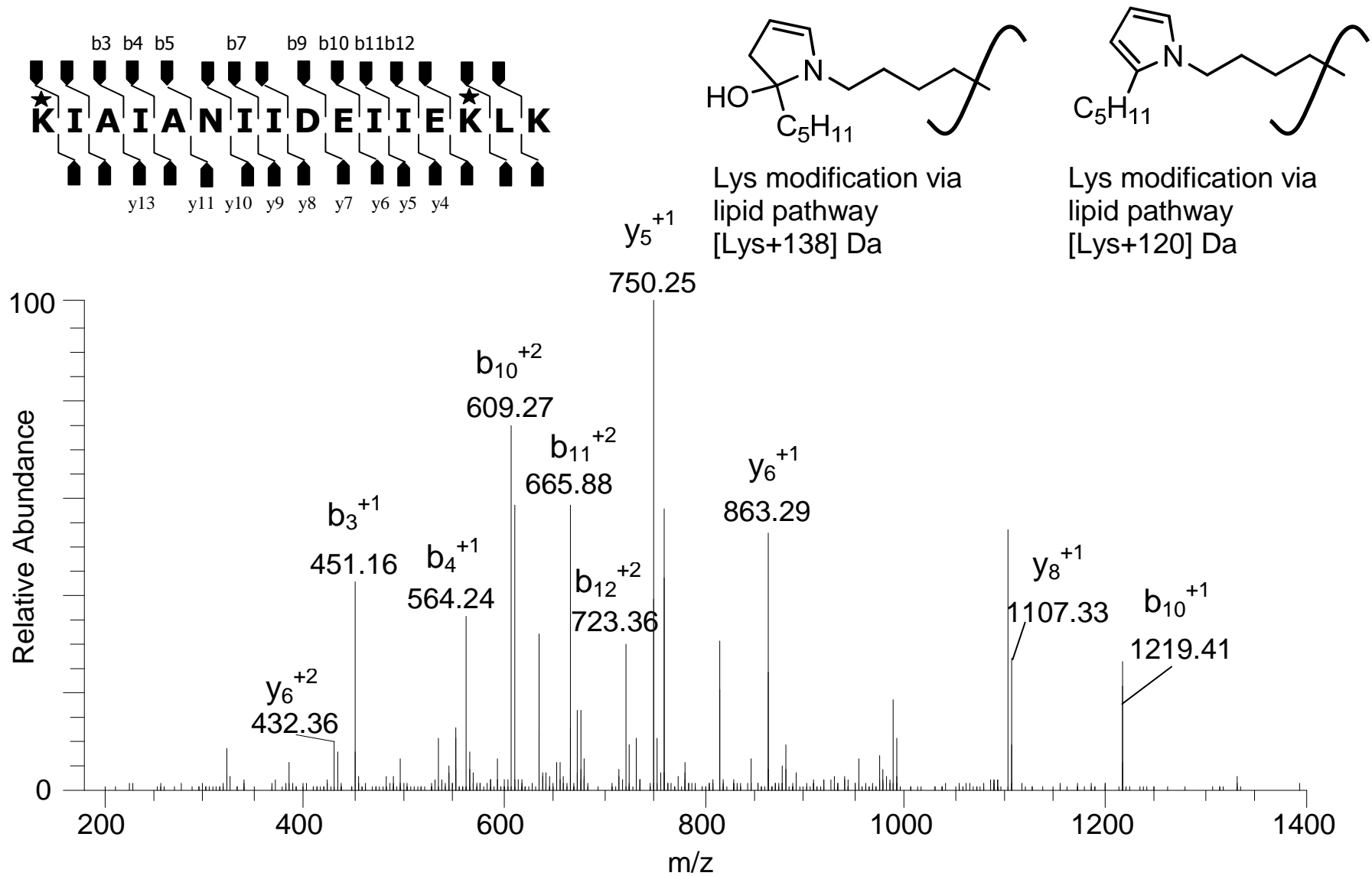


Figure 4.10 MS/MS spectra of tryptic peptide 2195-2210 from high peroxide concentration depicting modification of 2195Lys (K+138 Da) and 2208Lys (K+120 Da). Corresponding B- and Y- ions are shown. Unmodified peptide was not observed.

4.4.5. Analysis of natural variants

Seven modified apo B-100 amino acid sequences were constructed to include fifty-one natural variants and compiled in a small amino acid sequence database (Table 4.2) along with the apo B-100 sequence (P04114) obtained from the human IPI database. Using tryptic digestion followed by LC-MS/MS, unique peptides representing a total of four natural variants^{38-43, 46, 55} were observed in this study (Table 4.4). Mass spectral evidence for the presence of the natural variant with 2092Leu instead of Val is depicted in Figure 4.11. These natural variants were confirmed by manual verification of the high quality tandem mass spectra that matched the variant containing sequences. So far, different studies reported the presence of the natural variants of LDL based on nucleotide sequence. However, no previous research has directly identified the presence of these variant amino acid residues in LDL samples via mass spectrometric data analysis.

Table 4.2 Reported natural variants were compiled in seven small subsequence databases modified from published sequence (P04114) [[http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-e+\[UNIPROT:APOB_HUMAN\]+-newId](http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-e+[UNIPROT:APOB_HUMAN]+-newId)]. In order to efficiently identify the reported natural variants by MS/MS, these databases were constructed by incorporating a small group (3-10) of amino acid variants into the P04114 sequence. The sequence modification table given below demonstrates the subsequences [Seq mod 1, seq mod 2, and so on] created for database search. The natural variants observed by MS/MS in our LDL experiments are indicated by an asterisk (*).

Seq mod 1		Seq mod 2		Seq mod 3		Seq mod 4		Seq mod 5		Seq mod 6		Seq mod 7	
103	Tyr>His	741	Thr>Asn	618	Ala>Val	273	Asn>Lys	145	Pro>Ser	733	Val>Ile	98	Thr>Ile
490	Arg>Trp	3527	Arg>Gln	1128	Arg>His	730	Val>Ile	554	Pro>Leu	3182	His>Asn	408	Ile->Thr
1086	Gly>Ser	4181	Lys>Glu	2299	Asp>His	1218	Glu>Gln	1113	Asp>His	3558	Arg>Cys	877	Pro>Leu
1437	Phe>Leu			2739	Pro>Leu	2092*	Val>Leu	1914	Asn>Ser	3964*	Phe>Tyr	1388	Arg>His
1923	His>Arg			3427	Lys>Thr	2680*	Gln>Leu	2365	Thr>Ala			2566	Glu>Lys
2456	Ala>Asp			3949	Leu>Phe	3294	Ser>Pro	2785	Asn>His			3319	His>Asp
3121	Ala>Thr			4481	Ala>Thr	3732*	Thr>Ile	3279	Ser>Gly			3921	Val>Ile
3432	Glu>Gln					3945	Thr>Ala	3638	Arg>Gln			4394	Val>Ala
3801	Ser>Thr					4338	Asn>Ser	4128	Val>Met				
4270	Arg>Thr							4484	Thr>Met				

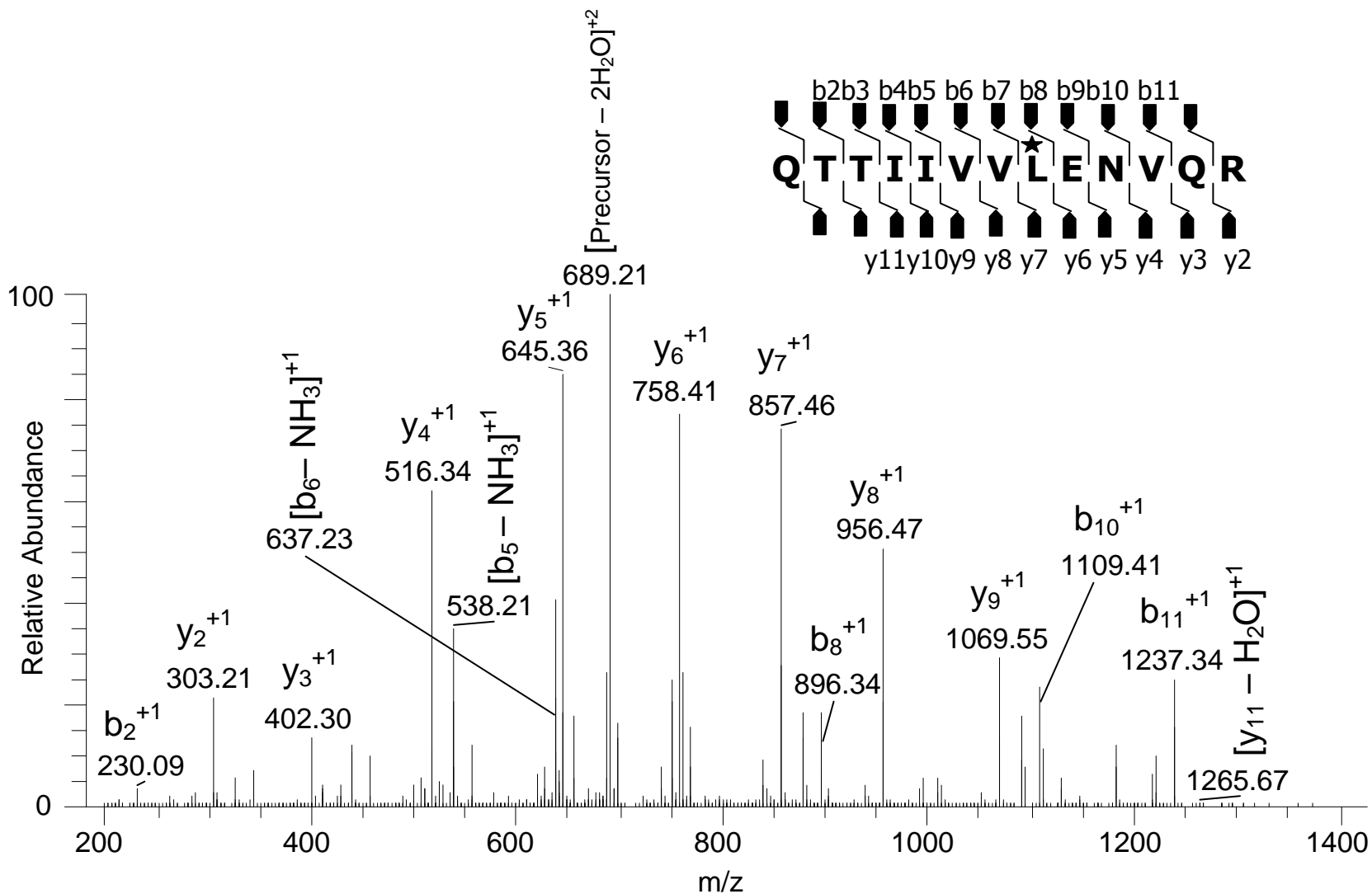


Figure 4.11 MS/MS spectra of tryptic peptide 2086–97 from untreated sample depicting presence of 2092Leu as a natural variant. Corresponding B- and Y- ions are shown. Ammonia loss from b5, b6, b7, b8, b9 was observed in the MS/MS spectra.

4.5. Discussion

4.5.1. Methionine modification

Methionine can be easily oxidized to methionine sulfoxide even by simple exposure to ambient oxygen (Scheme 3.15). We frequently observed methionine modifications. These oxidations were not reported because they were prevalent in control experiments which made it difficult to verify that the oxidation was the result of treatment with hydroxyl radical.

Some observed oxidized peptides contained both methionine and another oxidizable residue (e.g. phenylalanine, tryptophan and tyrosine). In these cases, the mass spectral data was manually checked to determine which of these residues was actually oxidized. These peptides are identified in Table 4.3 with an asterisk. None of these peptides showed methionine oxidation in control experiments (no peroxide added), suggesting that these methionine residues were resistant to oxidation. Methionine modification is characterized by a loss of CH_3SOH (-64 Da) from the precursor ion.⁵⁶ In Fe^{2+} /peroxide treated LDL, no major peaks in product ion spectra confirmed this loss. Hence there is no convincing evidence that treated apo B-100 had methionine oxidations. The presence of inaccessible methionine residues indicates that some parts of the protein retained their structure during hydroxyl radical exposure. Also, in peptide 2523-34 (observed in high peroxide dose and blank), 2526Met was oxidized and 2523Met remained intact. Hence it was perceived that some methionines such as 2523Met were in the hydrophobic pockets of the proteins which are inaccessible to both ambient oxygen and hydroxyl radical.

4.5.2. LDL oxidation

It is well recognized that ox-LDL is one of the major factors in promoting atherogenesis. *In vivo* formation of ox-LDL and atheroma was extensively demonstrated by several different studies.^{4, 5, 7, 8, 57} Our focus was to study apo B-100 oxidation in LDL in the presence of hydroxyl radical in order to identify the positions of amino acid residues that undergo oxidations under these conditions. Upon exposure to hydroxyl radical, the most commonly observed modification was addition of an oxygen atom (e.g. hydroxylation), which resulted in a mass change of +16 Da. It is expected that the location of these oxidation sites reveals solvent accessible portions of apo B-100 in LDL. Sharp *et al.* and other researchers^{30, 31, 58, 59} demonstrated the usefulness of hydroxyl radical mapping techniques in model proteins such as lysozyme and β -lactoglobulin A. In short, the solvent accessible amino acid residues of the protein, particularly those containing aromatic side chains, react readily with the hydroxyl radical and form an aromatic alcohol. These are stable markers that can be determined by mass spectrometric studies.

Addition of two oxygens to 2307Phe from low peroxide dose and addition of two oxygens to 3923Phe from high peroxide dose (Figure 4.9) depicts extensive reactions in some protein regions. Most observed oxidations were the result of direct hydroxyl radical attack on the amino acid residues, and only a small portion of the observed oxidations were representative of lipid mediated oxidation (i.e. involving a lipid radical). The overwhelming majority of direct oxidation products suggests that at the lowest radical concentration used in this study, little indirect oxidation occurred in the time frame of these experiments, which was likely too short to exceed the lag period.^{60, 61} Furthermore, peptide sequence coverage by LC-MS/MS was unchanged between LDL not exposed to oxidants and LDL exposed to low concentrations of hydroxyl radical. This result indicates that low concentrations did not cause enough structural

damage to interfere with the analysis. However, for higher radical concentrations, dramatic decreases in sequence coverage were observed indicating that the higher radical concentrations resulted in extensive protein damage.

Table 4.3 Modifications of amino acids observed in LDL exposed to three different hydroxyl radical concentrations. Numbers represent the position of the amino acid in the apoB sequence (P04114). All experiments were done in duplicate. The positions of the oxidized amino acids were compared with the control. The table includes only oxidations not also appearing in control experiments (no peroxide added). All modifications from each replicate were listed.

Modifications Observed	Low Peroxide concentration	Intermediate peroxide concentration	High Peroxide concentration
F+16	680 [*] , 883 [*] , 2665 [*] , 2845 [*] , 4019 [*] , 4531	1193 [*] , 2419 [*]	680 [*] , 883 [*] , 1193 [*] , 2307, 2419 [*] , 2665 [*]
F+32	3923	N.D.	2307
Y+16	56, 144, 276 [*] , 1020, 3295 3926, 4380 [*] , 4451	4510	2568, 4380 [*]
W+32	583	N.D.	N.D.
H+16	596, 2507 [*] , 4529	N.D.	N.D.
P+16	65, 145, 663, 1007, 2620, 2659 [*] , 3262 [*] , 3293, 3310, 3593	4004 [*]	663, 3281, 4004 [*]
K+120	N.D.	N.D.	2208
K+138	N.D.	N.D.	2195

*These peptides contained a methionine residue. Differentiation between methionine oxidation and oxidation of another amino acid in the same peptide is difficult. However, characteristic loss of 64 Dalton from oxidized methionine was not observed, so methionine oxidation was ruled out.

Previously, FDP-Lys (Lys+94)⁶² and MP-Lys (Lys + 96)⁶³ oxidation products were observed in samples exposed to hydroxyl radical. Also acrolein forms as a lipid oxidation product from hydroxyl radical mediated oxidation that can react with amino acid residues such as Lys and His. FDP-Lys is the result of these oxidations.^{22, 26} Uchida's pioneering work in this area already revealed that by MS/MS methods, these products were extremely difficult to identify due to extensive cross-linking. In previous work, only 4 molecules of FDP-Lys/molecule of apo B-100 were formed during hydroxyl radical mediated oxidations.^{25, 26} FDP-Lys was not observed in our studies. It is apparent that in our experiments, the lipid mediated pathway is not predominant. In previous researches the abundance of MP-Lys, FDP-Lys and HNE-His formation from Cu (II) mediated oxidations were substantial,²² indicating that Cu (II) mediated pathways oxidize the protein in a substantially different way compared to direct hydroxyl radical attack.

Exposing LDL to three different hydroxyl radical concentrations showed concentration-dependent oxidation. At low hydroxyl radical concentrations (low peroxide doses), amino acid oxidations were observed, but no extensive protein structural damage was observed. At higher hydroxyl radical concentrations, apo B-100 was modified extensively and loss of its native structure was observed. During *in vivo* oxidative stress, a lag phase is generally observed in which the cell or the tissue can recover from the damage by means of its defense mechanisms^{60, 61} such as antioxidants and repair processes. Nevertheless, oxidative stress in the lag phase can still induce some damage to apo B-100. In our low hydroxyl radical concentration experiments, apo B-100 was partially oxidized, but based on the fact that sequence coverage remained high, it is not likely that extensive structural damage occurred. The low hydroxyl radical dose in this study is likely representative of the lag phase, and is therefore more relevant to *in vivo* systems. The *in vitro* studies reported here with different peroxide concentrations are significant since

physiological concentrations of hydroxyl radical are low, but during oxidative burst, neutrophils generate micromolar amounts of peroxide⁶⁴ in the cells. The choice of low, intermediate and high concentrations of hydrogen peroxide elucidated varying degrees of protein damage as a function of hydroxyl radical concentration.

Ox-LDL uptake by CD-36 into macrophages is a complicated process and not yet completely understood⁶⁵. Recent research has correlated the uptake of oxidized phosphatidyl choline by the scavenger receptor CD-36⁶⁶. However, dose dependent formation of oxidized epitopes in apo B-100 can play a crucial role in macrophage uptake and thereby pathogenesis of atherosclerosis.

Natural variants were previously reported for hemoglobin with the help of mass spectrometric data.^{67, 68} Natural variants in apo B-100 were previously observed by other researchers based on cDNA and expressed sequence tag data. The peptide sequence for apo B-100 is extremely complex bearing two alleles in the gene, both of which can be expressed. Gene expression from two alleles can lead to more than one form of protein or even a mixture of proteins. The presence of a large number of natural variants could be a result of this complex gene expression. For our work, all previously reported natural variants were incorporated in seven small sequences and added to the main database for apo B-100 for peptide searching in mass spectral data. The search results against the small databases revealed unique peptides depicting the presence of natural variants in the protein itself. A total of four different amino acids were found to be present as natural variants in the LDL sample used in this study. These natural variants can possibly be attributed to polymorphism, mutations or disease state of apo B-100 genes.

Table 4.4 List of natural variants observed in native and ox-LDL from LC-MS/MS experiments, for native LDL a total of two samples was analyzed. For ox-LDL six samples were analyzed and tabulated.

Natural variants observed native LDL	Natural variants observed in ox-LDL
2092Leu	2092Leu
2680Leu	2680Leu
3964Tyr	3732Ile
	3964Tyr

4.6. Conclusions

In LDL protein several amino acid residues were found to be oxidized directly or indirectly by hydroxyl radical. The mass changes due to the oxidation of amino acid residues such as phenylalanine and tyrosine were analyzed by LC-MS/MS. Oxidized amino acid residues were distributed widely throughout the apo B-100 sequence. At the lowest radical concentration used in this study, protein modifications were minor enough to allow successful MS/MS mapping, and the results suggest that this concentration is effective for mapping solvent accessible amino acid residues. Higher radical concentrations, however, resulted in lower recovery of recognizable peptides, indicating substantial structural damage may have occurred to the protein. Consequently, these higher concentrations are not as reliable for mapping solvent accessibility. The vast majority of peptide modifications observed in this study were from direct hydroxyl radical attack, indicating that our methods represent probing of protein damage in the lag phase before substantial concentrations of secondary radicals can be formed. Because natural defenses against free radicals are present *in vivo*, the lag phase is most likely representative of *in vivo* conditions. Consequently, the method reported here avoids complications arising from oxidation under conditions that are too aggressive (e.g. Cu²⁺ mediated oxidation). Modification of 3262Pro was observed in the receptor binding regions^{17, 69} of apo B-100 (3130-60 and 3259-

67), indicating that hydroxyl radical can cause direct oxidation in this important region. The identity and location of the solvent accessible peptides, as well as their oxidation products, will be helpful in understanding the mechanisms of *in vivo* LDL oxidation. Furthermore, the protein modifications identified here may allow studies that elucidate the impact of these modifications in model systems.

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Chapter 5.

Hypochlorous acid and peroxynitrite treated apo B-100 in LDL: A study of the oxidized regions of LDL protein

5.1. Abstract

Low density lipoproteins (LDL) act as a major cholesterol carrier in the human physiological system. Oxidized LDL (ox-LDL) has been found to be pro-atherogenic by several previous studies. Free radical mediated oxidations of apo B-100 (LDL protein) plays a critical role in early stages of plaque formation in the arterial wall. The structure of apo B-100 is still poorly understood, although this structure may be crucial in identifying the role of the protein in atheroma formation. In this study we mapped the oxidized regions of apo B-100 in human LDL using peroxynitrite and hypochlorous acid as probes. In a separate experiment, tyrosine was exposed to peroxynitrite in PBS buffer spiked with bicarbonate. The resultant reaction mixture was analyzed by reversed phase HPLC and the concentration of 3-nitrotyrosine was calculated. This result was correlated with the effective concentration of peroxynitrite for protein damage. LDL was incubated with various concentrations of sodium hypochlorite in bicarbonate buffer. Previously, we treated LDL with hydroxyl radical and identified oxidized amino acids of apo B-100. We employed the same method to identify positions at which peroxynitrite and hypochlorous acid oxidizes apo B-100. After exposure to peroxynitrite and hypochlorous acid, LDL was delipidated and dialyzed against methanol:water followed by a treatment with DTT and iodoacetamide to break disulfide linkages. The resulting solution from peroxynitrite was digested with trypsin to yield peptides. Trypsin and pronase both enzymes were used separately

in hypochlorite treated samples. The peptides are then analyzed by LC-MS-MS to identify modifications in the protein. Nitrotyrosine and nitrotryptophan, along with hydroxylated amino acids, are specific markers for peroxynitrite mediated oxidations in proteins. Chlorotyrosines were specific markers for hypochlorous acid mediated damage in proteins. By employing dose dependent oxidations of peroxynitrite and hypochlorous acid we have identified site specific oxidations caused by the oxidants in apo B-100 associated with intact LDL particle. Oxidative mapping of apo B-100 will reveal solvent accessible regions of apo B-100 and provide valuable protein structural information related to oxidative protein damage.

Keywords: LDL, ox-LDL, apo B-100, peroxynitrite, hypochlorous acid, oxidative mapping, LC-MS/MS, trypsin, pronase.

5.2. Introduction

Low density lipoproteins (LDL) are cholesterol transporters in physiological systems. LDL particles are comprised of three components: the LDL core has cholesteryl esters and triacylglycerol, the outermost layer contains phospholipids and free cholesterol, and a single monomeric protein apo B-100 (4563 amino acids) encircles the lipid assembly (Figure 3.3) and is partially exposed at the surface of LDL.^{1, 2} A variety of strong oxidants can damage LDL and induce formation of oxidized LDL (ox-LDL). Pathological implication of ox-LDL in body is severe and could be attributed to atherosclerosis.³⁻⁸ Atherosclerosis is a complex disease involving plaque formation in arterial intima. Plaque renders inelasticity in arteries and causes complicated symptoms in cardiovascular diseases.

Oxidants can damage LDL in a bidirectional fashion. Oxidative stress renders formation of lipid peroxides and highly reactive aldehydes which are known to disrupt the protein structure.⁹⁻¹¹ Oxidized apo B-100 could also be a key factor in plaque formation. This hypothesis was supported by the observation that elevated concentrations of the oxidized protein were accumulated in the atherosclerotic lesions.^{4, 12, 13} Post translational modification of this protein by free radical or enzymatic processes may be a key parameter in initiating plaque formation. Current methodologies are inadequate to completely understand the tertiary structure of this protein. Molecular modeling, although very useful in some cases, becomes speculative for this protein¹⁴⁻¹⁶ and requires additional information¹⁴ to predict the structure (4563 amino acids and 550 KDa). Due to lipid association, it would be even harder to crystallize this protein. Previously we observed that liquid chromatography coupled with mass spectrometry (LC-MS/MS) could be a useful approach in understanding the structure of LDL protein.¹⁷ Oxidatively and/or enzymatically modified LDL particles are inflammatory and activate cytokines and monocyte

adhesion molecules.¹⁸⁻²⁰ Generally, LDL particles are internalized by LDL receptors which are expressed ubiquitously in cells. However, oxidative modification in apo B-100 in endothelial cells promotes binding and uptake of ox-LDL by scavenger receptor CD-36²¹⁻²⁶ in macrophages. This phenomenon becomes more relevant in endothelial dysfunction and initiation of atherosclerotic plaques.

Due to the enzymatic activity of endothelial nitric oxide synthase (eNOS), L-arginine is converted to citrulline, releasing nitric oxide in the process.^{27, 28} Nitric oxide works as a signaling molecule and helps in vasodilation.^{27, 28} In vascular endothelial cells, peroxynitrite (ONOO⁻) is formed (Scheme 3.3) due to the reaction of nitric oxide and superoxide.²⁹⁻³² Peroxynitrite is a very strong oxidant and cause nitration in LDL.^{18, 33, 34} Indeed peroxynitrite can adapt multiple pathways (Scheme 3.4) to damage the proteins.³⁵ One of the key reactions of peroxynitrite is nitration of tyrosine residues to form 3-nitrotyrosine (3-NT)³⁶ (Table 5.1) in LDL protein.³⁷ Myeloperoxidase (MPO) can catalyze this reaction by forming intermediate complexes. Bicarbonate or dissolved carbon dioxide play an important role in forming tyrosyl radical and enhance reaction between tyrosyl radical and nitrite radical to form 3-NT.³⁸ Tryptophan oxidation products were also reported in proteins including changes in the phenyl ring and nitration in the phenyl ring (Table 5.1). Other amino acids can undergo hydroxylation reaction by this pathway since hydroxyl radical is also involved as a dissociation product of peroxynitrite.³⁹

During respiratory burst, phagocytes release superoxide and hydrogen peroxide. In the presence of the heme enzyme myeloperoxidase, hydrogen peroxide reacts with physiological concentrations of chloride to form reactive hypochlorous acid (HOCl).⁴⁰ Also, HOCl can be generated in an extracellular matrix which can cause damage to circulatory LDL in blood plasma. The damage arising out of hypochlorous acid could be substantially diverse.^{41, 42} It can

cause side chain oxidation, peptide bond fragmentation and aggregation of proteins.^{42, 43} A range of amino acid oxidation products were previously published in literature and some of them are listed in Table 5.2. Controversial results existed on the more susceptible oxidizable regions of LDL; however, Hazell and co-workers proved that amino acids in apo B-100 are more oxidizable^{44, 45} by HOCl compare to its lipid counterpart. It was shown that initial oxidation in protein apo B-100 can oxidize the lipids and lead to the formation of cholesteryl ester hydroperoxide. Hazen *et. al.* depicted tyrosine chlorination in LDL to form chlorotyrosine in atherosclerotic lesions.⁴⁶ An increase in oxidation of LDL particles increased the uptake of ox-LDL by scavenger receptor CD36, which in turn induced foam cell formation.^{21, 22} These steps are considered to be major early events in plaque formation in the arterial wall.

Bergt *et. al.* presented valuable information on chloramine transfer reactions between HOCl in apo A-I and high density lipoprotein (HDL).⁴⁷ This reaction was coined as one of the key reactions in hypochlorite mediated damage to proteins. Interestingly, Bergt's quantitative work showed that the MPO/H₂O₂/Cl⁻ system chlorinated Tyr192 to a greater extent than that observed for the other six Tyr residues in the protein. These results indicate that the protein structure influences the chlorination reactions.⁴⁷ Cystein, methionine and lysine react directly to hypochlorous acid. Cys yields corresponding sulfonic acid (Cysteic acid, +48 Da) while methionine forms methionine sulfoxide (+16 Da).⁴⁸ Chloramine transfer reactions at the ε-NH₂ group of Lys was important since this chloramine can transfer chloride to a nearby Tyr residue (+34 Da) and go back to its native state.⁴⁹ Also, aromatic amino acid such as histidine can participate in transferring the chloramine by initial side chain oxidation. Tryptophan can give oxidation products of the indole residue (+16 Da).⁴² The extent of chlorination with MPO/H₂O₂/Cl⁻ and reagent hypochlorous acid sometimes appeared to be controversial.

Although it was proposed that under physiological conditions MPO binds directly to the amino acids in the proteins and induces reactions, reagent hypochlorous acid had produced similar extent of chlorination in Tyr residues.^{47, 50}

Yang *et al.* presented pioneering work by modifying apo B-100 in LDL with HOCl. His methodology was tedious but nonetheless effective.⁵⁰ His methods involved purification and isolation of tryptic peptides from apo B-100 by liquid chromatography after HOCl or MPO treatment followed by analysis with mass spectrometric method (ESI-MS).⁵⁰ Recent developments in LC-MS/MS based methodologies are of paramount importance since it attenuated analysis time and prodigious sample handling skills.^{51, 52}

Free radical⁵³⁻⁵⁶ and non-free radical^{36, 46} mediated oxidations leave permanent footprints in the side chains of amino acids of proteins (Table 5.1 and Table 5.2). By identifying these footprints, the solvent accessible parts⁵⁷⁻⁵⁹ (rather aqueous accessible regions) could be identified. Previously we have shown usefulness of LC-MS/MS based methodologies in determining oxidative post translational modifications (with hydroxyl radical) in apo B-100.¹⁷ In this article we have assessed the site specific oxidations of apo B-100 in LDL with oxidant peroxynitrite and hypochlorous acid. We hope this result will help us to understand the solvent accessible residues in apo B-100, the structure of the protein in LDL and important oxidation products.

Table 5.1 Reported amino acid oxidations from literature and associated structural changes after oxidation by peroxynitrite. ^{33, 35, 60-62}

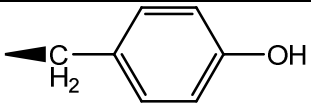
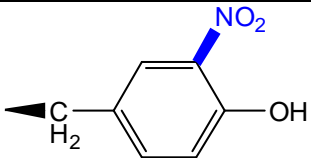
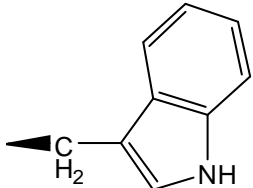
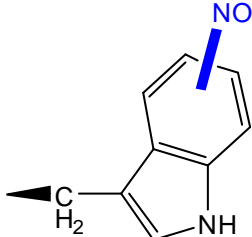
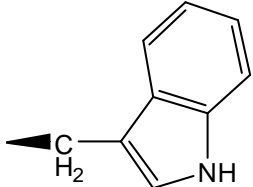
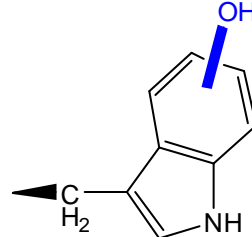
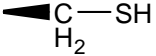
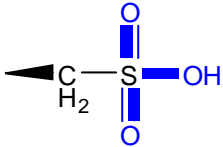
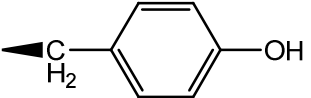
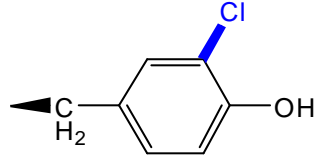
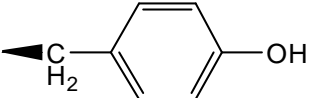
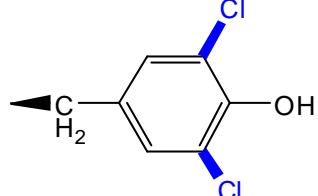
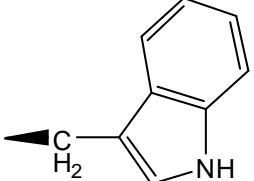
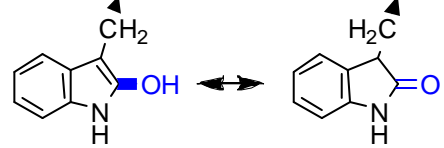
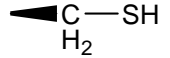
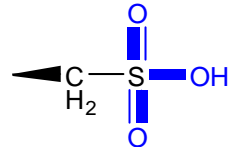
Amino acid	Amino acid	Modified amino acid	Modified amino acid	Associated mass change
Tyrosine		3-Nitrotyrosine		+45 Da
Tryptophan		Nitro-tryptophan		+45 Da
Tryptophan		Hydroxylated tryptophan		+16 Da
Cystein		Cysteic acid		+48 Da

Table 5.2 List of amino acid oxidations by hypochlorous acid. ^{36, 46, 48, 50, 63-66}

Amino acid		Modified amino acid		Associated mass change
Tyrosine		3-Chlorotyrosine		+34 Da
Tyrosine		3,5-Dichlorotyrosine		+68 Da
Tryptophan		2-oxindole		+16 Da
Cystein		Cysteic acid		+48 Da

5.3. Materials and methods

Human low density lipoprotein (LDL) was purchased from VWR chemicals. Each batch of LDL was acquired from a single, healthy human subject. LDL was stored with 150 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) and was kept refrigerated (4 °C). Pronase was purchased from VWR chemicals. Micro-dialysis tubes and floats were also purchased from VWR. Sodium nitrite (NaNO_2), hydrogen peroxide (30%) and all solvents were purchased from Sigma-Aldrich (St. Louis, MO). High purity (98%) DL-tyrosine, sodium hypochlorite (NaOCl) (15% chlorine content), ammonium bicarbonate (ABC), formic acid, dithiothritol (DTT) and iodoacetamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). In all experiments, purified water was obtained by distillation, deionization, carbon filtration and UV irradiation.

5.3.1. Synthesis of peroxyxynitrite

Peroxyxynitrite was synthesized by using two peristaltic pumps. This is a slight improvement to the conventional method.^{67, 68} In short 0.60 (M) NaNO_2 solution was mixed with 0.65 (M) H_2O_2 (in HCl) at 29ml/min. This mixture was added drop wise to a constantly stirred 3 (M) NaOH solution for quenching. Since, peroxyxynitrite is extremely unstable in acidic medium (half life <1 ms) it is necessary to quench the reaction without any delay. The yellow colored solution indicates the existence of peroxyxynitrite in solution. This reaction is exothermic in nature therefore NaOH solution was placed over ice. Excess H_2O_2 was degraded by transferring the mixture to a beaker containing MnO_2 . Bubbles issuing from the solution indicated degradation of peroxide. Solutions were transferred to 2 mL centrifuge tubes and stored at -80 °C until further use.

5.3.2. Characterization of peroxynitrite and quantification of 3-nitrotyrosine

Each day one centrifuge tube of peroxynitrite was taken out from the freezer and thawed over ice. A pinch of MnO_2 was added to ensure residual H_2O_2 removal from the solution. 25 μl of this solution was diluted to 3 ml in 0.1 M NaOH and absorbance measurement was taken. Concentration of the said peroxynitrite solution was calculated from its absorbance at 302 nm ($\epsilon = 1670 \text{ M}^{-1} \text{ cm}^{-1}$).⁶⁹

High performance liquid chromatography (HPLC) was used to measure the amount of 3-nitrotyrosine (3-NT) formed after reacting peroxynitrite with DL-tyrosine (0.5 mM) in phosphate buffer saline (PBS at pH 7.4) and 25 mM ammonium bicarbonate. Initially peroxynitrite concentration was estimated by previously described absorbance method.⁶⁹ Three different peroxynitrite concentrations (10 μM , 100 μM and 1000 μM) (duplicate experiments) were used for exposure. Reactions were stirred for 30 minutes, acidified and injected into the HPLC to measure the amount of 3-NT formed. A total of six samples were analyzed.

An Alltech 150 mm C_{18} (5 μ particle size, I.D. 4.6 mm) column equipped with a 100 μl loop was used in the HPLC. An isocratic solvent system, 95% water (0.1% Trifluoroacetic acid) and 5% acetonitrile was used as eluent with a ten minute run time. 3-Nitrotyrosine was injected as an external standard and a calibration curve was formulated from peak area under HPLC trace everyday ($R^2 > 0.99$) with three 3-nitrotyrosine standards analyzed in duplicate.

5.3.3. Reagent hypochlorite characterization

Sodium hypochlorite was stored at 4°C until use. Absorbance of NaOCl was measured in 0.01 M NaOH. Concentration was calculated from molar extinction coefficient ($\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$

¹) at 292 nm.⁷⁰ Hypochlorite solution was diluted to desired concentration with 0.01 M NaOH and stored over ice until addition to LDL.

5.3.4. LDL oxidation

Stock protein concentration was 4.10 mg/mL. For each sample, 12.19 μ L LDL was diluted to 100 μ L with ammonium bicarbonate buffer (pH adjusted to 7.4) and placed in micro centrifuge tubes. A total of eight samples were used in each set of experiments. Each exposure was carried out in duplicate. Three microliters of peroxyxynitrite (stock concentration 0.273 mM, 2.733 mM or 27.33 mM) was added to exposed samples maintaining the final concentration of peroxyxynitrite in LDL sample as 10 μ M, 100 μ M and 1000 μ M respectively. Three microliters of 0.1 M NaOH was added as blank.

Sodium hypochlorite exposure to LDL was carried out similarly. 12.19 μ L NaOCl stock was diluted to 100 μ L by ammonium bicarbonate (pH 7.4). Three microliters of NaOCl (concentration 0.343 mM, 3.43 mM and 34.3 mM) was added to each LDL sample to start with the concentration 10 μ M, 100 μ M and 1000 μ M respectively. 3 μ L 0.01 M NaOH served as blank. Again each exposure was carried out in duplicate.

The micro centrifuge tubes are transferred to a temperature controlled shaker (Eppendorf Thermomixer R). At 37 °C and 900 rpm the reaction was shaken for 10 minutes. An additional 3 μ L of peroxyxynitrite/NaOCl solution was added and shaken for another 10 minutes. Finally, 3 μ L of peroxyxynitrite/NaOCl was added again and shaken. After 10 minutes these reaction mixtures were transferred to previously labeled microdialysis tubes.

The samples were then dialyzed against 500 mL methanol:toluene:methylene chloride (3:1:1) (v/v) for two hours at 4 °C for delipidation. The dialysis float was transferred to another

500 mL solution containing 300 mL methanol with 200 mL water for another two hours of dialysis. The cloudy protein solutions were collected and transferred to micro-centrifuge tubes. BCA assay method was used to estimate the protein concentration at this stage. The protein solution was boiled in water for 10 minutes to initiate denaturing. Thirty molar excess dithiothritol was added, and samples were incubated for 30 minutes at 37 °C to cleave the disulfide linkages. Next, 30 molar excess iodoacetamide was added and let react for 90 minutes at 37 °C for alkylation of the cysteinyl residues. Tryptic digestion (one blank and three exposed samples) was carried out overnight following previous methods.^{17, 71, 72} The tryptic peptides were quenched with liquid nitrogen, vacuum dried and stored until further use.

Pronase digestion^{73, 74} was carried out on one batch of NaOCl (exposed to 10 µM, 100 µM and 1000 µM) treated samples. The enzyme was dissolved in water immediately before addition to protein samples that were exposed to NaOCl (one blank and three exposed samples). The protein to pronase ratio was calculated by using the enzymatic digestion of bovine serum albumin (BSA) and optimized as 50:1 (protein:enzyme) (data not shown). Based on the estimated LDL protein concentration, required pronase was added and samples were incubated at 37 °C for 24 hours. 5 µl formic acid (99%) was added to the samples to quench the reaction followed by boiling for five minutes. The reaction mixture was then evaporated and dried peptides were stored at -80 °C. Dried peptides were reconstituted in 5% aqueous formic acid with 5% acetonitrile for LC-MS/MS analysis. Samples were stored at -20 °C until analysis.

5.3.5. LC-MS/MS of apo B-100 peptides

The peptide mixture obtained from the procedure noted above (maximum amount equivalent to 3 µg protein) was first loaded onto a reversed-phase trap column, and then washed with 3% acetonitrile and 0.1% formic acid (aq) to desalt the sample. After the wash/desalt step,

the trap column was switched into a gradient flow (60 minutes, water with 3% acetonitrile and 0.1% formic acid at the beginning and 65% acetonitrile with 0.1% formic acid at the end) to elute peptides to a C₁₈ analytical column before introduction into a linear trapping quadrupole (LTQ) mass spectrometer.

5.3.6. MS setup

Mass spectrometry analyses were performed on an LTQ mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nano-flow (200-500 nL/min) electrospray source. The peptides were ionized via electrospray and recorded in a full scan mass spectrum. For each full MS scan, the top 3 most intense peptide precursor ions were selected and fragmented via collision induced dissociation (CID). Low pressure helium was employed as damping (for ion storage and isolation) and collision (fragmentation of activated ions) gas. The instrument was operated in a highly automated data dependent acquisition mode.

5.3.7. Database search

The database search was performed on an in-house search engine (Bioworks 3.3, Thermo Electron) using the “SEQUEST” algorithm against human IPI database for apo B-100 (accession # P04114).⁷⁵ Peptides with MW of 600-3500 Da were chosen for identification. All modifications were defined as differential (i.e. the amino acid residue may or may not be modified) and are listed in Table 5.1. An Xcorr score was generated from the matching of experimental spectra to the theoretical spectra of apo B-100 peptides. A charge state-Xcorr score filter was applied to all MS/MS spectra. Singly charged peptides with Xcorr score less than 2,

doubly charged peptides with Xcorr score less than 2.5, and triply charged peptides with Xcorr score less than 3.0 were considered as false positives and removed from the final report*.

5.4. Results and discussion

5.4.1. Quantification of 3-nitrotyrosine with HPLC

In vitro nitration of tyrosine using reagent peroxyntirite followed a complicated mechanism. In physiological systems, the heme enzyme myeloperoxidase and other artifacts may facilitate 3-nitrotyrosine formation in proteins. It was important to know the concentration of peroxyntirite added to the sample. Therefore conventional absorbance method (for peroxyntirite) and HPLC method (quantitation of 3-NT) were used to verify the consistency between the results obtained from both of these methods. We have measured amounts of 3-nitrotyrosine (duplicates) in three different reactions containing three different concentrations of peroxyntirite. For estimated 10 μM , 50 μM and 100 μM peroxyntirite exposures, we calculated the total amounts of 3-NT as $(1.1 \pm 0.1) \times 10^{-10}$ moles, $(5 \pm 1) \times 10^{-10}$ moles and $(11 \pm 2) \times 10^{-10}$ moles. This data is presented in Figure 5.1. 3-NT formation was a measure of nitrite radical formation through nitrosoperoxy-carboxylate pathway after reaction of peroxyntirite and carbon dioxide (bicarbonate) (detailed discussion in chapter 3). It should be noted that the actual yield of 3-NT in this reaction is only ~10% compare to the total peroxyntirite added to the reaction mixture.

* All positively matched peptides were listed in Appendix B from Table B-1 through Table B-6. Corresponding Xcorr and ΔC_n values were also included.

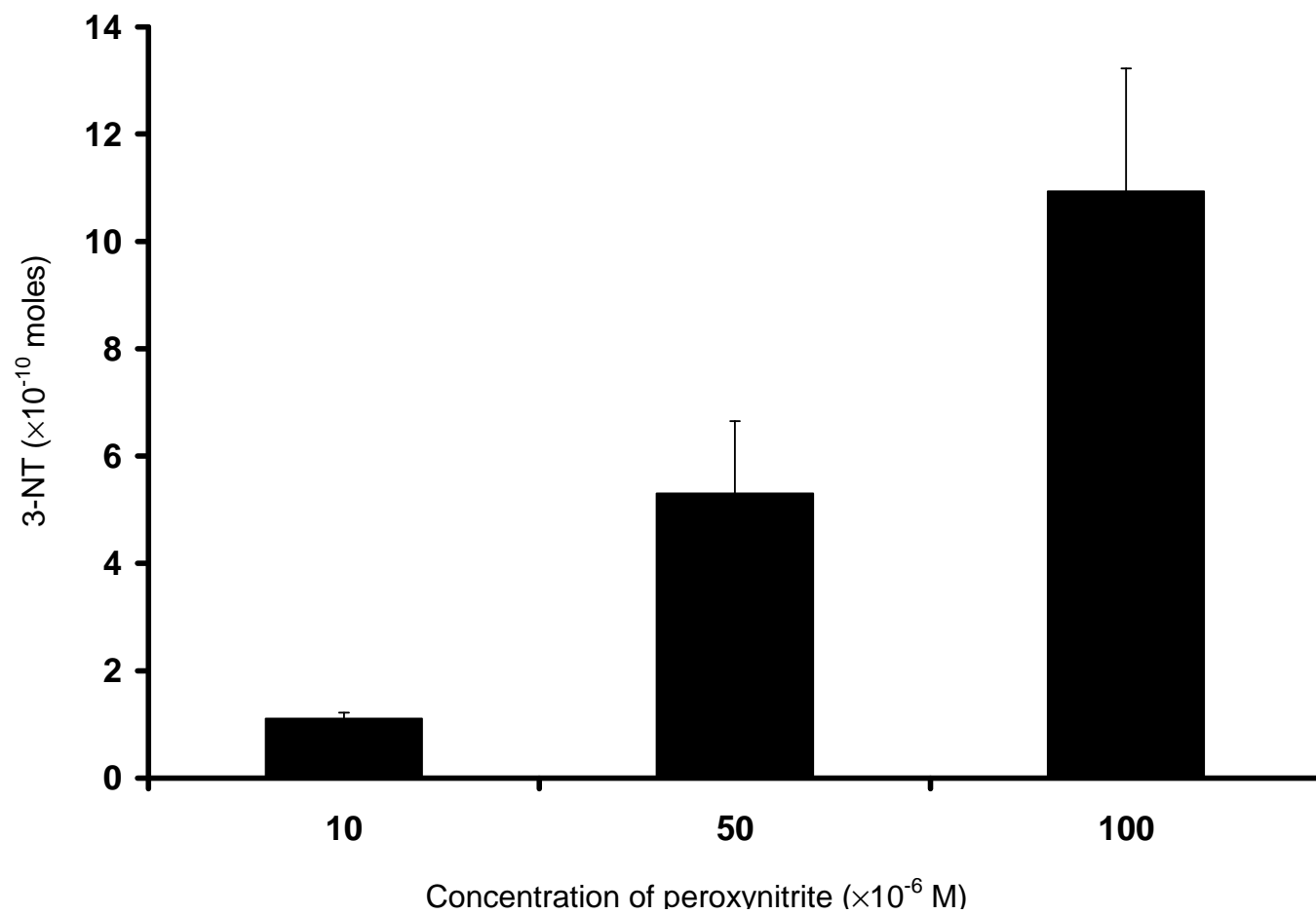


Figure 5.1 3-Nitrotyrosine formation from three different concentrations of peroxyntirite. An increase in 3-NT formation with increasing peroxyntirite concentration is apparent. Errors are represented as one standard deviation.

5.4.2. Modification of apo B-100 by peroxynitrite

Identification of the presence of 3-NT in atherosclerotic lesions indicated involvement of peroxynitrite in the oxidation of proteins.⁶² Previous research connected nitration of amino acid residues in LDL to peroxynitrite activity. Eiserich *et. al.*⁷⁶ and Kettle *et. al.*³⁶ suggested involvement of MPO in protein tyrosine nitration. We however worked in an enzyme free environment and explored nitration through nitrosoperoxy-carboxylate³¹ pathway. Previously Alvarez *et. al.* depicted nitrotryptophan formation by reacting peroxynitrite directly with tryptophan.⁶¹ Yamakura *et. al.* emphasized on formation of 6-nitrotryptophan formation in Cu, Zn-superoxide dismutase with peroxynitrite⁷⁷. From our experiments with reagent peroxynitrite, we have rarely observed nitration products on Tyr residues. Tryptophan nitration was not observed at all in the three different peroxynitrite doses.

In low peroxynitrite dose (10 μ M), we have observed peptide 1830-52 which represented both 1840Tyr nitration and 1831Tyr hydroxylation (Figure 5.2) from low peroxynitrite exposure. Three hydroxylated tryptophans were observed. Oxidation of Cys1505 to cysteic acid (Figure 5.3) was also observed (Table 5.3).

With intermediate peroxynitrite concentration, no nitrotyrosine was observed. Hydroxylation of the phenyl ring in tryptophan was obviously easier compared to nitration, although it was mediated through the formation of hydroxyl radical³⁹ by homolytic fission of peroxynitrite[§]. It seems plausible that some of the peroxynitrite molecules escape the nitrosoperoxy-carboxylate pathway and simply follow homolytic fission even though the reaction

[§] Due to the enormous volume of data tyrosine and phenylalanine hydroxylation was not exclusively considered at this stage. Hydroxylation of both the amino acids occurs if hydroxyl radical pathway is prevalent. We have mapped hydroxyl radical mediated oxidations in apo B-100 previously. Due to similar reason this database search was focused on hydroxylation of the phenyl ring in tryptophan and not on the ring dissociation products kynurenine and NFK.

is slower. Trp2248 (Figure 5.4) and Trp3970 oxidation were observed, which is consistent with the samples treated with the low peroxynitrite dose. Cystein oxidation at intermediate peroxynitrite concentration revealed interesting events. Hamilton *et. al.* reported modification of Cys1112 to its corresponding sulfonic acid by peroxynitrite treatment.³⁷ We have observed modification of the same amino acid without peroxynitrite treatment. It could be possible that Cys1112 is highly susceptible to oxidation by ambient oxidants. Three different peptides 1100-18, 1101-18 and 1001-15 confirmed this modification revealing both +2 and +3 charge state of the peptides. We have not observed the unmodified peptide in the untreated sample. It will therefore difficult to be claimed as a modification originating from peroxynitrite in our case.

Due to paucity of the nitrated peptides in our high peroxynitrite experiments, another set of 1000 μ M peroxynitrite exposure experiments were carried out keeping all parameters the same (Table 5.3). Four tyrosine residues appeared to be nitrated, of which Tyr1602 was not reported previously. Two products for modification of Tyr2524 were observed. One modified peptide was nitrated at this position (+45 Da) (Figure 5.5) and the other was hydroxylated (+16 Da) (Figure 5.6). It should be noted here that previous experiments with hydroxyl radical always showed oxidation of Tyr2524.¹⁷

Although Protparameter analysis⁷⁸ of apo B-100 sequence showed the presence of 151 Tyr residues in the apo B-100, very few of them were nitrated. Hamilton and coworkers³⁷ reported nitration of tyrosine residues in LDL with both reagent peroxynitrite and SIN-1 (nitric oxide and superoxide donor). Their observations included nitration of 10 Tyr residues in the presence of 1 mM peroxynitrite. However, it must be noted that physiological peroxynitrite concentration even in severe conditions could hardly mount to such a high level. Thus our initial intention was to look at dose dependent nitration of apo B-100 which might reveal important

aspects of peroxynitrite mediated nitration in LDL. From the results it was apparent that at low peroxynitrite local concentrations, tyrosine nitrations are not highly favorable. Also peroxynitrite stays in equilibrium with peroxynitrous acid (ONOOH). If the tyrosine residues are not completely exposed to the solvent, peroxynitrite/peroxynitrous acid will be unable to access them. In addition, site specific generation (closer to certain amino acids) of nitrosoperoxy-carboxylate could be harder even in *in vitro* system. If the Tyr residues are close to lipid associated domains lipid nitrite formation will be facilitated which will minimize the possibility of formation of 3-NT in LDL.

Furthermore, in all most all of our experiments sequence coverage had decreased dramatically (data not shown) which could be attributed to severe aggregation and cross-linking in the protein following peroxynitrite mediated oxidations followed by digestion with trypsin.

5.4.3. Hypochlorous acid mediated oxidations of apo B-100

In vivo flux of hypochlorous acid was attributed to phagocytic activity in and out of the cells. LDL in circulation can be oxidized⁷⁹ by HOCl. In vascular endothelial cells, HOCl induces damages to various reactive amino acid residues by side chain oxidations, fragmentation and cross-linking.^{65, 80} The actual species which induces protein damage by hypochlorite are controversial. The physiologically relevant model system is related to the MPO catalyzed reaction between peroxide and chloride.^{36, 66} The amount of hypochlorous acid generation can vary significantly depending on different artifacts.

In this study we have explored dose dependent site specific oxidation of apo B-100 in LDL by reagent hypochlorite. Two different enzymes were used to cleave apo B-100 in LDL. Trypsin digest only reveal ~50% of the sequence in the peptide matching. Therefore pronase was used as an alternative proteolytic enzyme. Pronase worked well on bovine serum albumin (data

not shown). However, it was not substantially effective in the case of apo B-100. Pronase could cleave the protein into smaller fragments due to non-specificity making it difficult to be detected by MS/MS methods (Table 5.4).

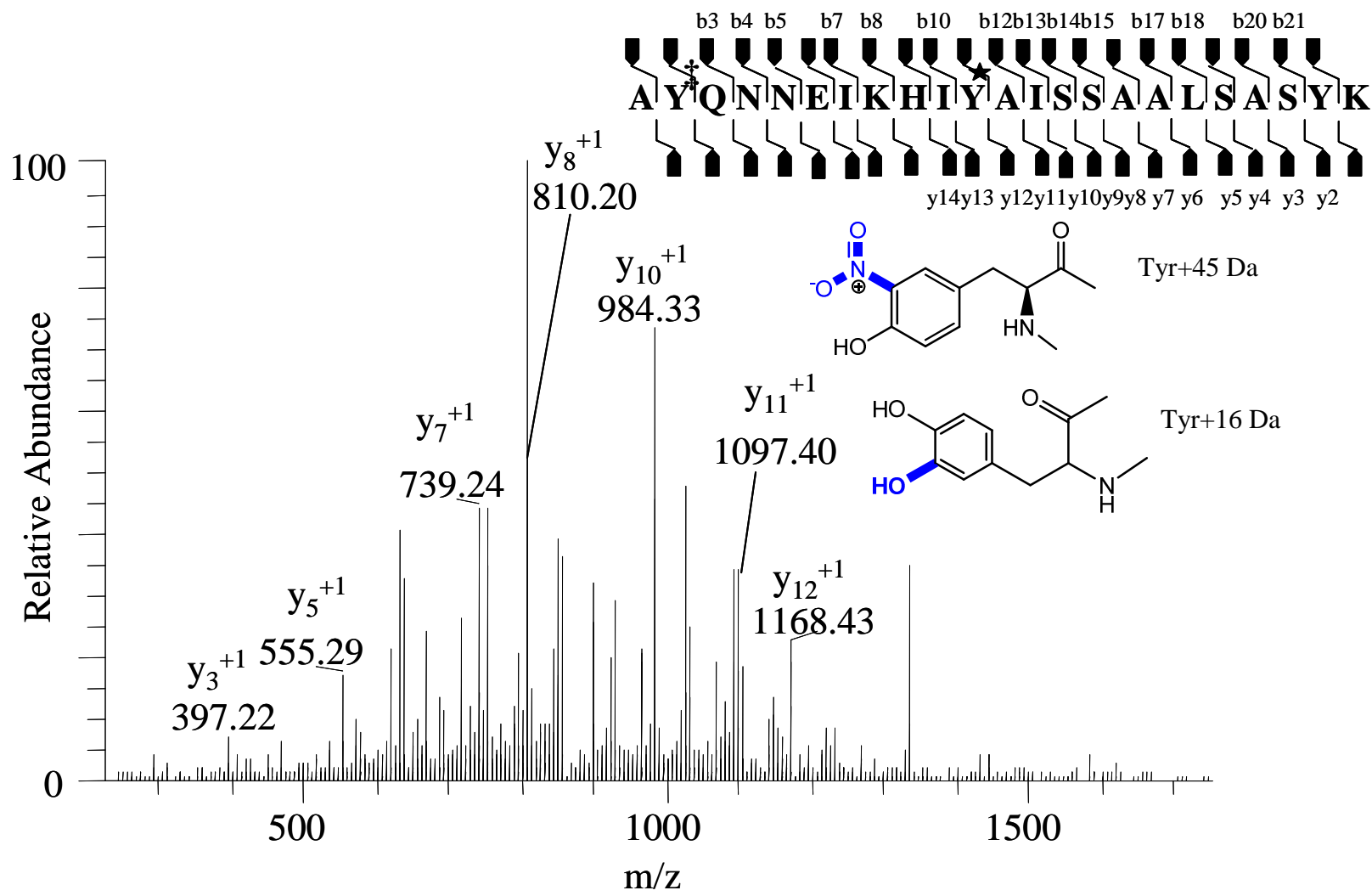


Figure 5.2 MS/MS spectra from tryptic peptide 1830-52. Tyr1840 was nitrated while Tyr1831 was hydroxylated.

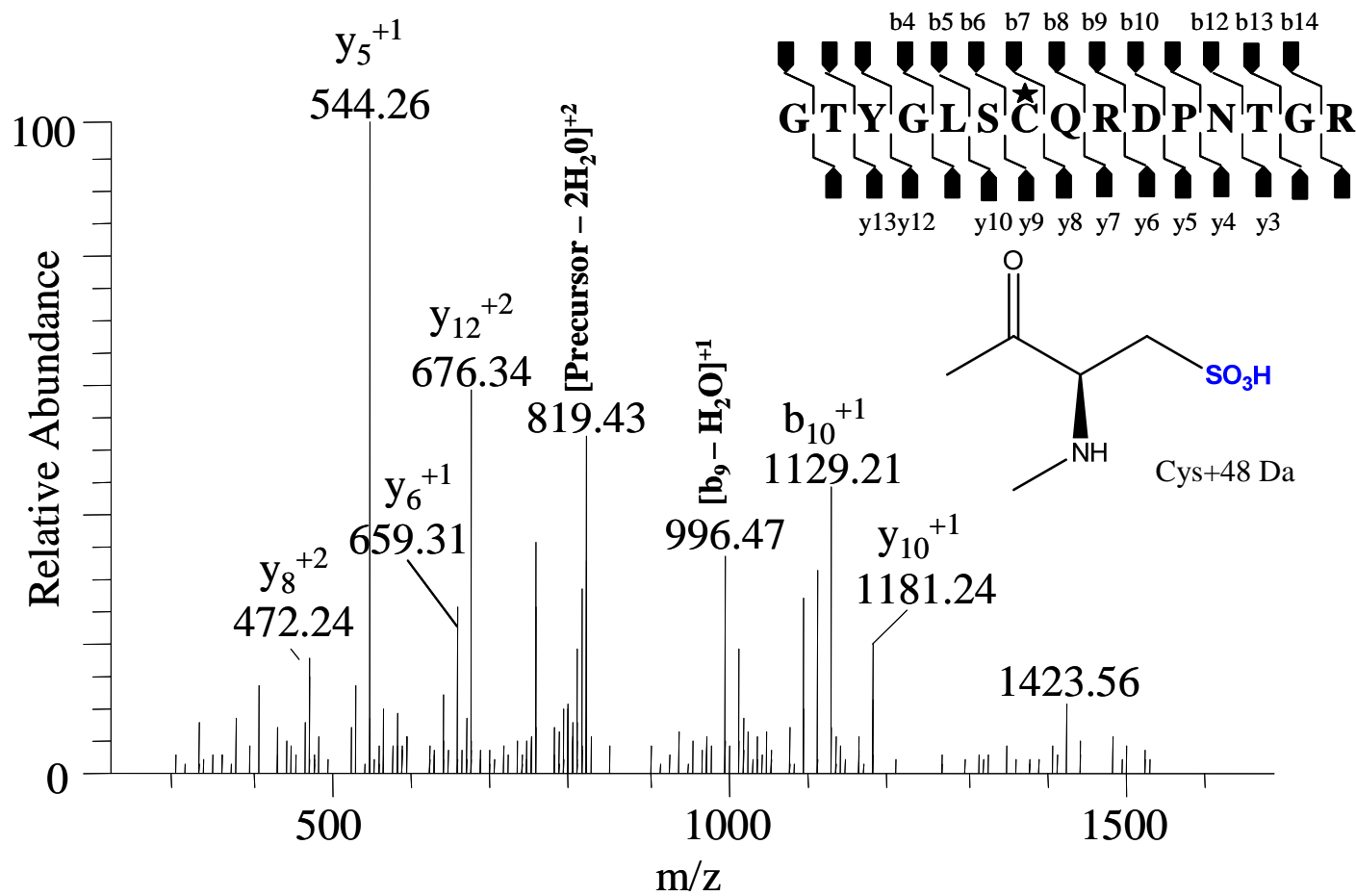


Figure 5.3 MS/MS spectra from tryptic peptide 1499-1513 from low peroxynitrite treated samples. The only one cystein oxidation observed in peroxynitrite treated sample. Cys1505 (+48 Da) was oxidized in this peptide by peroxynitrite.

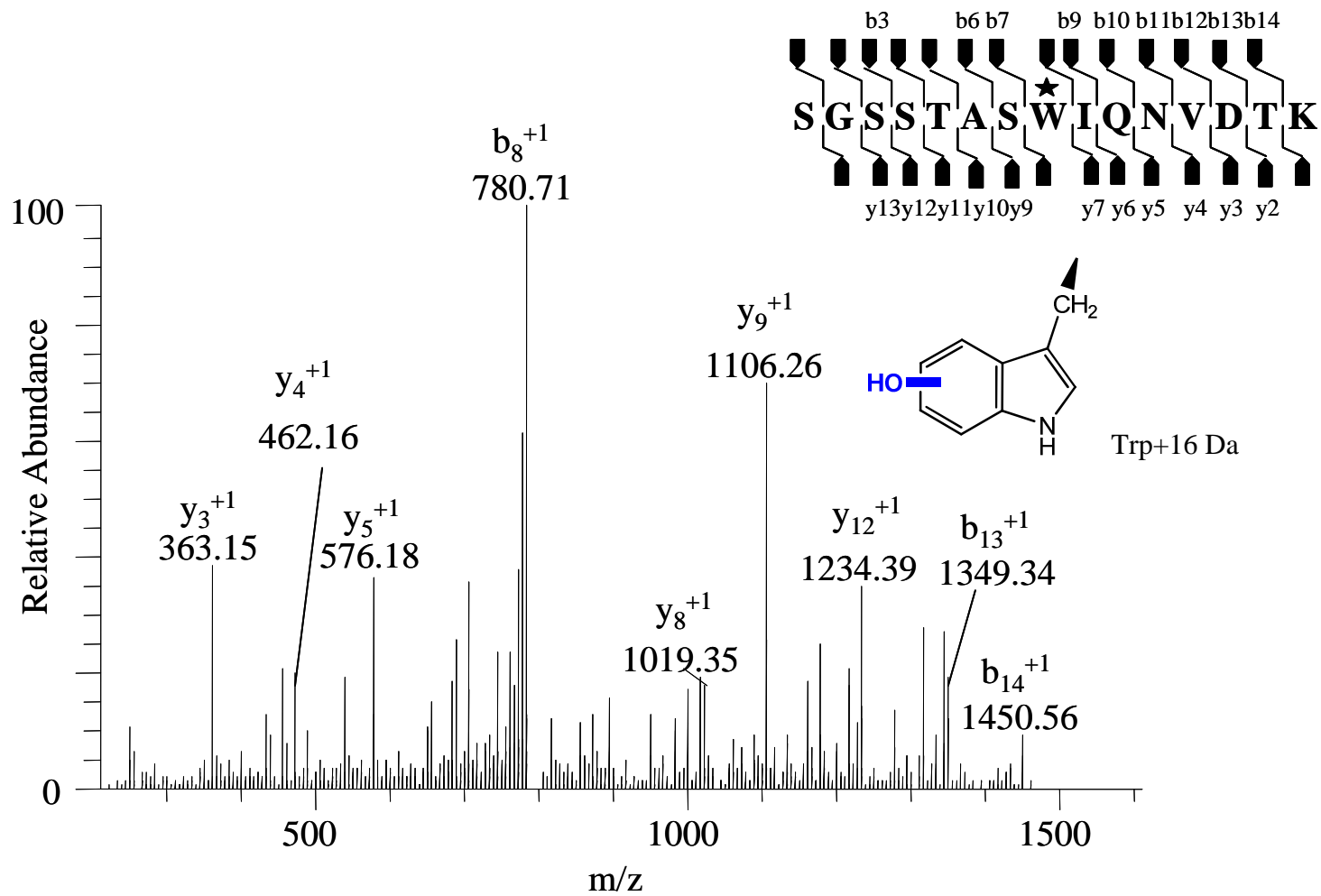


Figure 5.4 MS/MS spectra from tryptic peptide 2241-55 from intermediate peroxynitrite exposure. Trp2248 was oxidized to corresponding hydroxylated tryptophan.

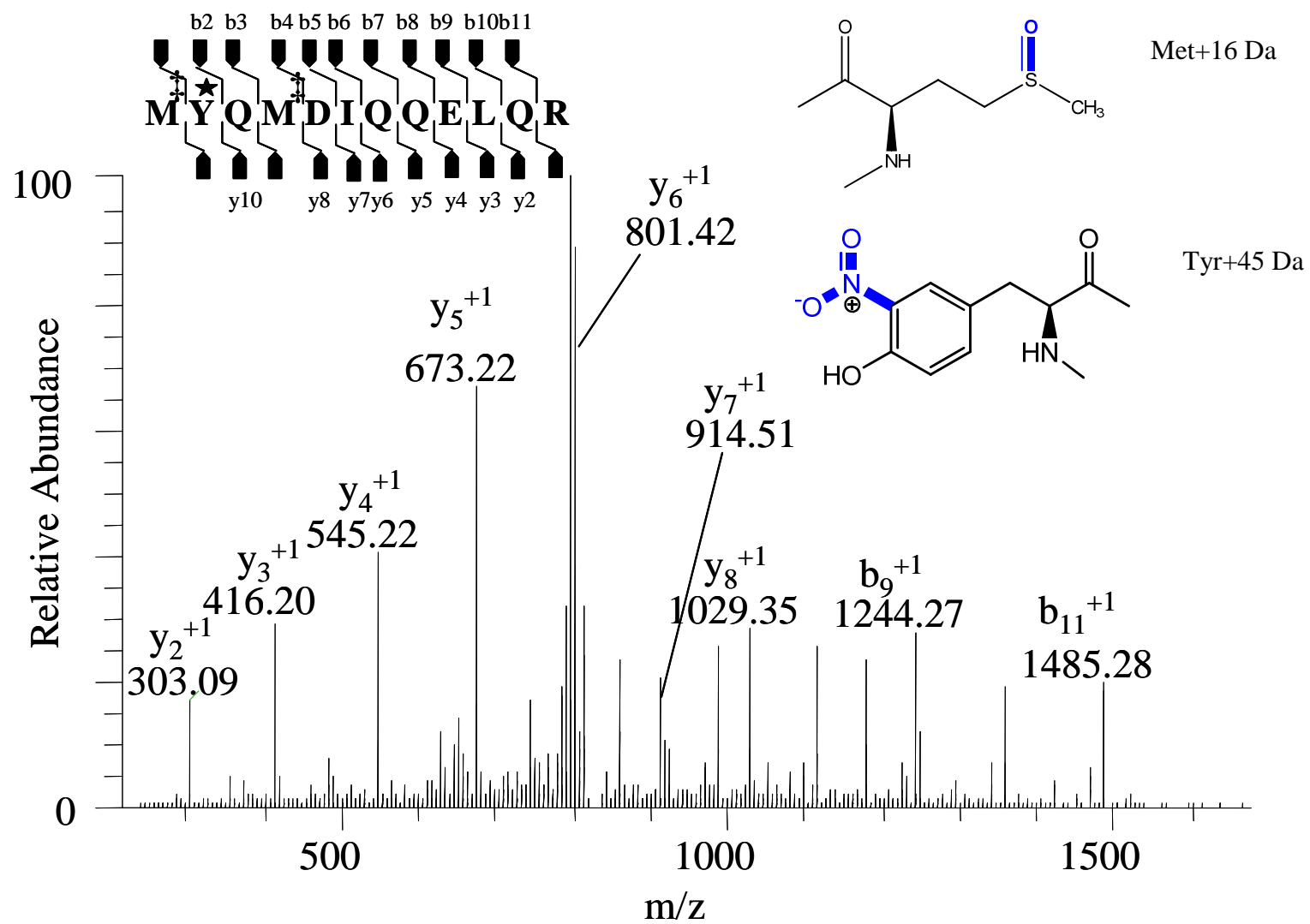


Figure 5.5 MS/MS spectra from tryptic peptide 2523-34 from high peroxynitrite exposure. Tyr2524 was chlorinated. Met2523 and Met2526 were oxidized in this peptide.

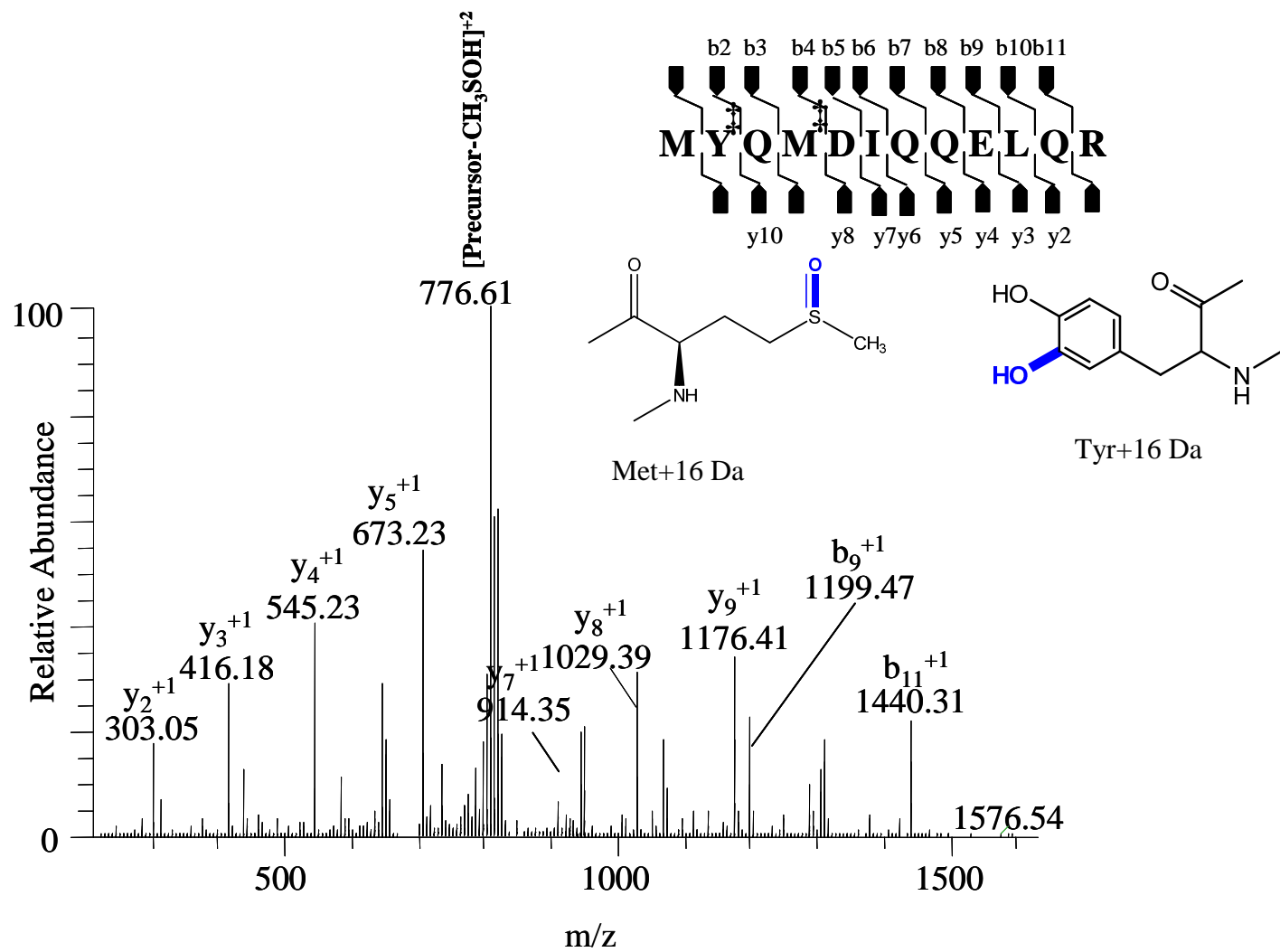


Figure 5.6 MS/MS spectra from tryptic peptide 2523-34 from high peroxynitrite exposure (same run as the previous). Tyr2524 was now oxidized (+16 Da). Met2526 were oxidized in the same peptide. Since Met2523 is adjacent it will be difficult to coin the actual oxidation site.

All observed oxidations in peptides of apo B-100 obtained after pronase digest were listed in Table 5.4. At low and intermediate hypochlorite concentrations no chlorotyrosines were observed. At the higher concentration of hypochlorite, Tyr1053 and Tyr1200 (Figure 5.7) were chlorinated. These chlorinations could be facilitated by chloramine transfers from Lys1251 and Lys1202 respectively. Interestingly Trp4022 and Trp4396 (Figure 5.8) residues were oxidized to 2-oxindolic form at the intermediate hypochlorite dose. Although these modifications were observed after digestion with pronase, they were not observed in the tryptic digests. With the moderate hypochlorite dose, peptides 4010-22 and 4385-98 showed tryptophan hydroxylation. However, due to batch to batch variability with pronase digestion, these peptides were not observed in the low or high hypochlorite doses. From pronase digested peptides, cysteic acid formation could not be confirmed although literature study stated a considerably high rate constant with hypochlorous acid.⁶⁵

Pronase is a useful enzyme in protein digestion. However, the non-specific nature of the enzyme might be detrimental for its usage in digesting large proteins such as apo B-100 which can be cleaved to very small fragments. Nevertheless, we could be able to identify some of the regions containing the modified tyrosine and tryptophan residues which did not appear in tryptic peptides. Particularly indolic ring oxidation products (Figure 5.8 and Table 5.4) were not identified before in apo B-100 peptides.

Tryptic peptides were compared between treated and untreated samples. Only those peptides are listed which did not appear in the untreated sample (Table 5.5). In low hypochlorite exposure (10 μ M) Tyr3295 was chlorinated (Table 5.5). At high hypochlorite concentration, this residue was chlorinated twice, indicating that it is readily susceptible to chlorination in the presence of hypochlorous acid. However, dichlorination of the tyrosine residues were not

observed at low hypochlorite concentration. Trp3563 was also observed to yield the corresponding 2-oxindolic residue in the side chain. No Cys oxidation was observed.

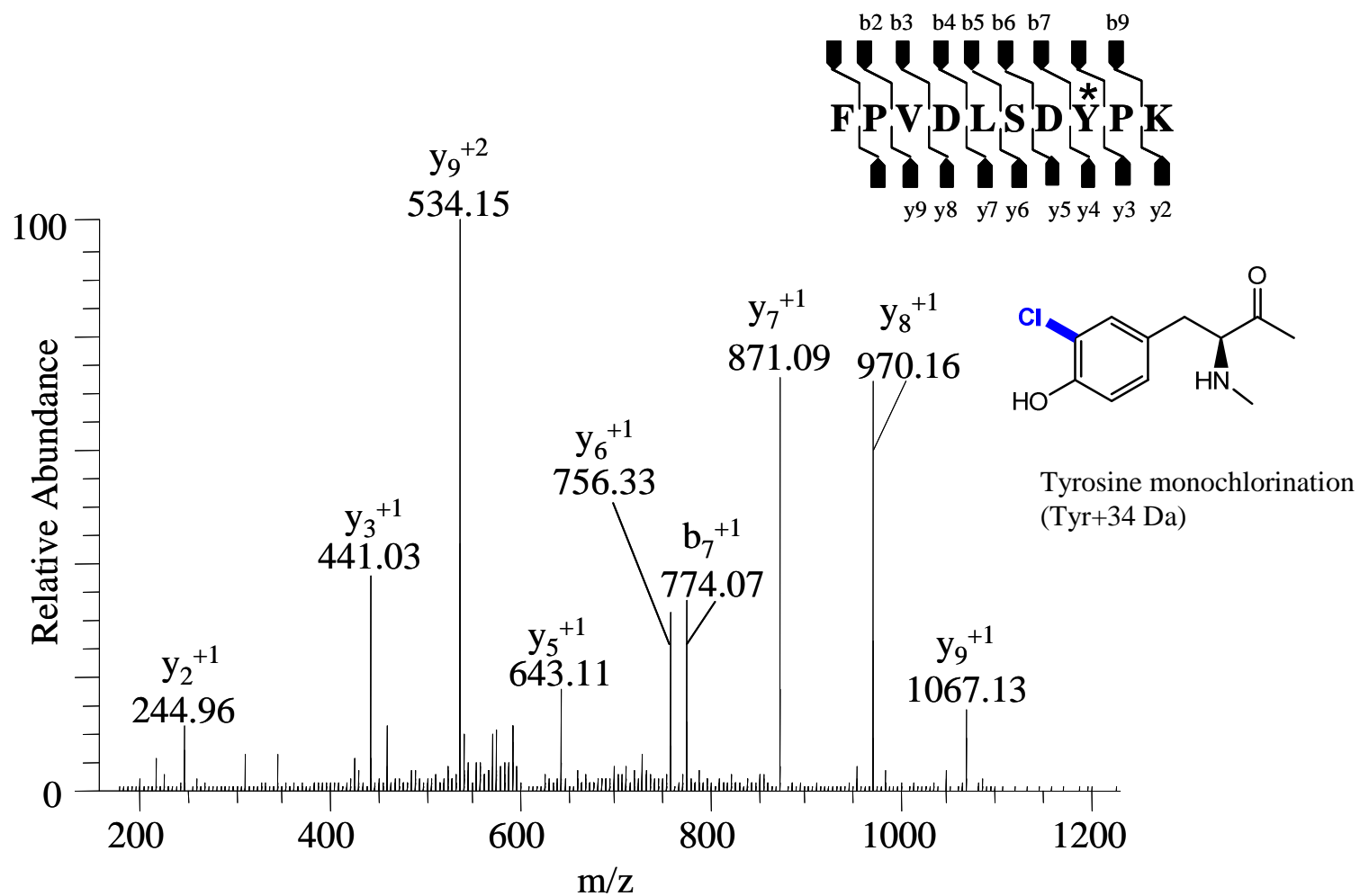


Figure 5.7 MS/MS spectra from pronase peptide 1193-1202 from high hypochlorite exposure. Tyr1200 was chlorinated was (+34 Da). Notable factor in this peptide is presence of Lys1202 which can facilitate chloramines transfers.

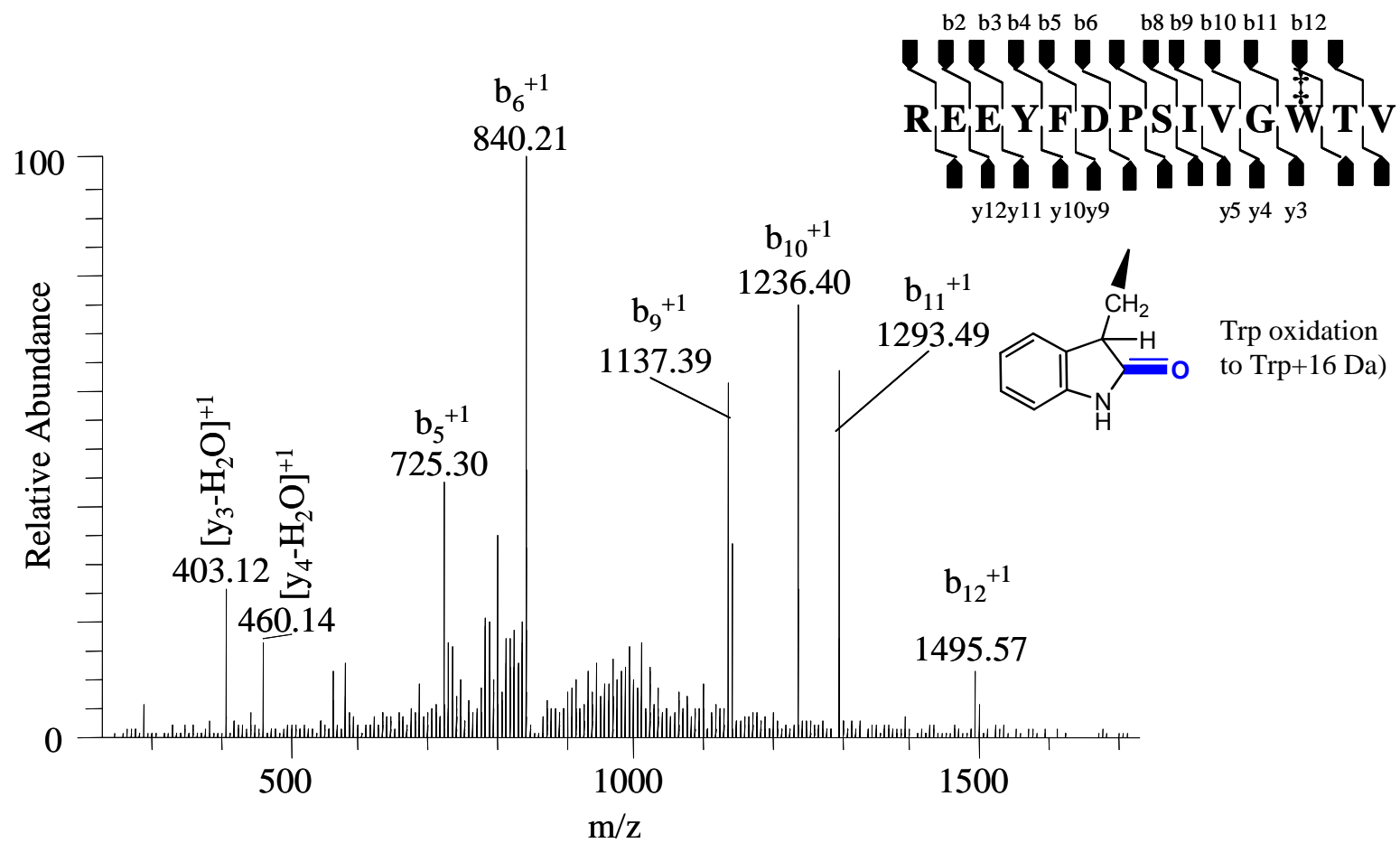


Figure 5.8 MS/MS spectra from pronase peptide 4385-98 from high hypochlorite exposure. Trp4396 was oxidized to corresponding 2-oxindole (+16 Da) derivative by intermediate hypochlorous acid dose.

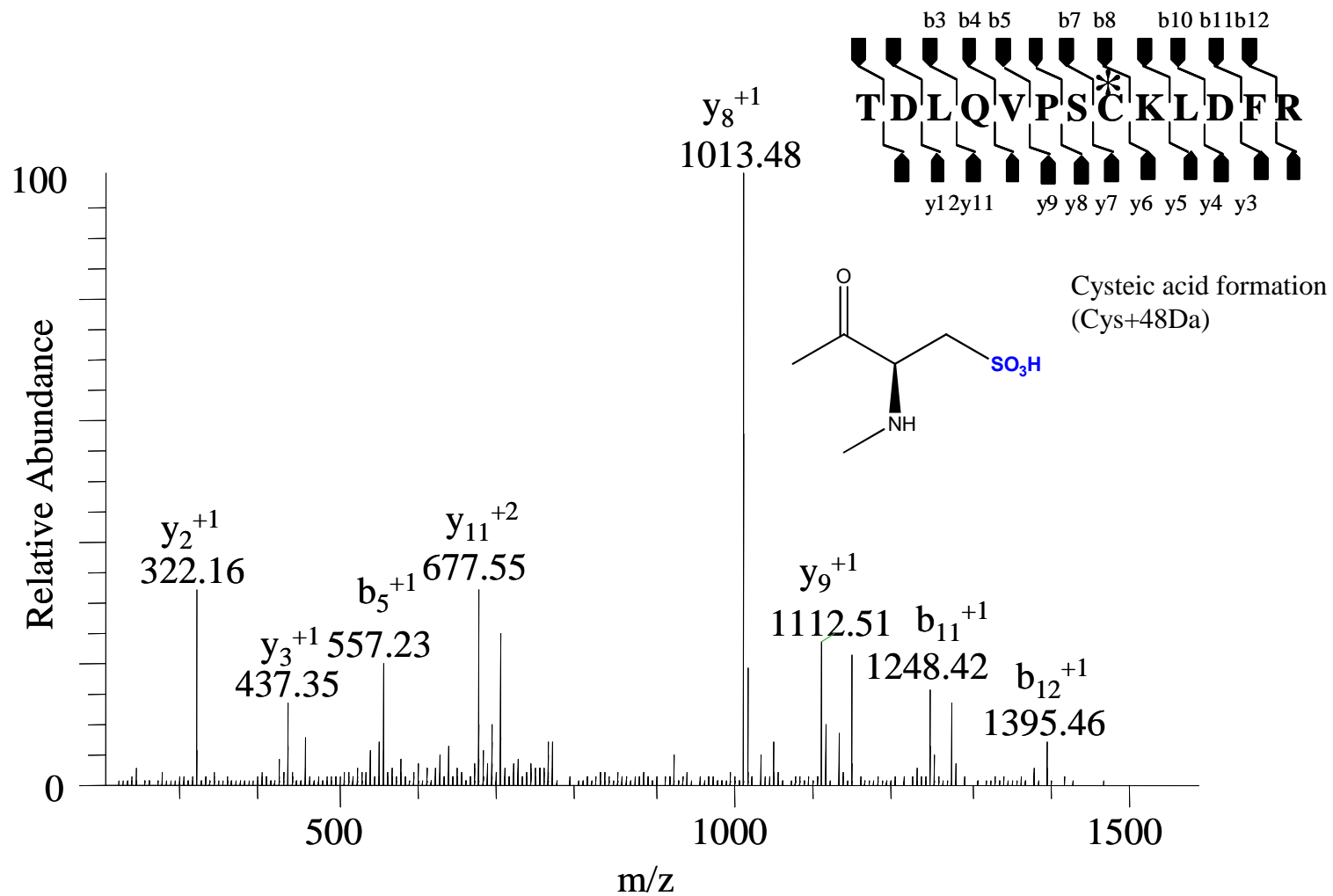


Figure 5.9 MS/MS spectra from tryptic peptide 3754-62 from intermediate hypochlorite exposure. Cys3761 was converted to cysteic acid (+48 Da).

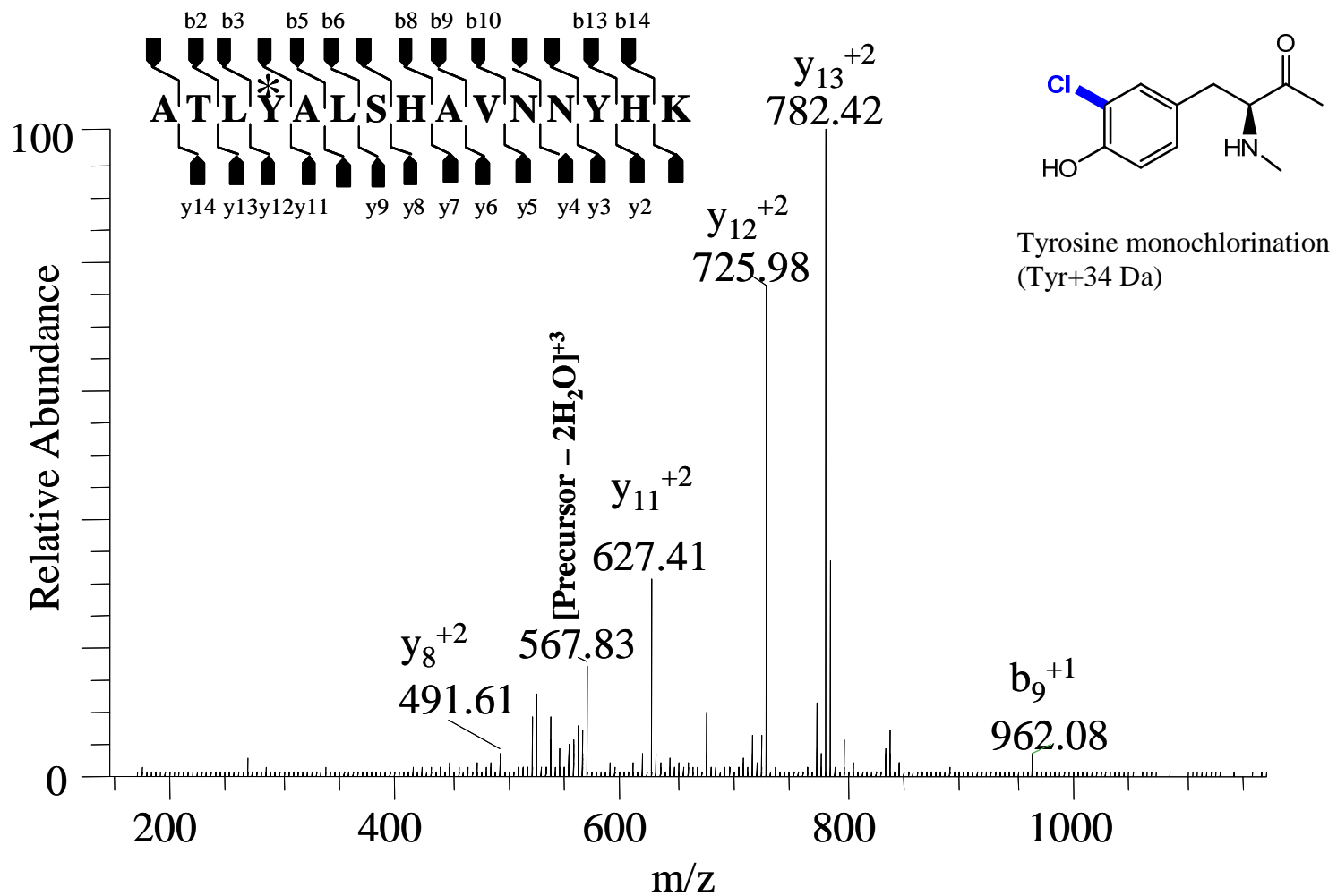


Figure 5.10 MS/MS spectra from tryptic peptide 440-54 depicting chlorination of Tyr443 from high hypochlorite exposure. Tyr452 was not modified even though His453 and Lys454 in the vicinity.

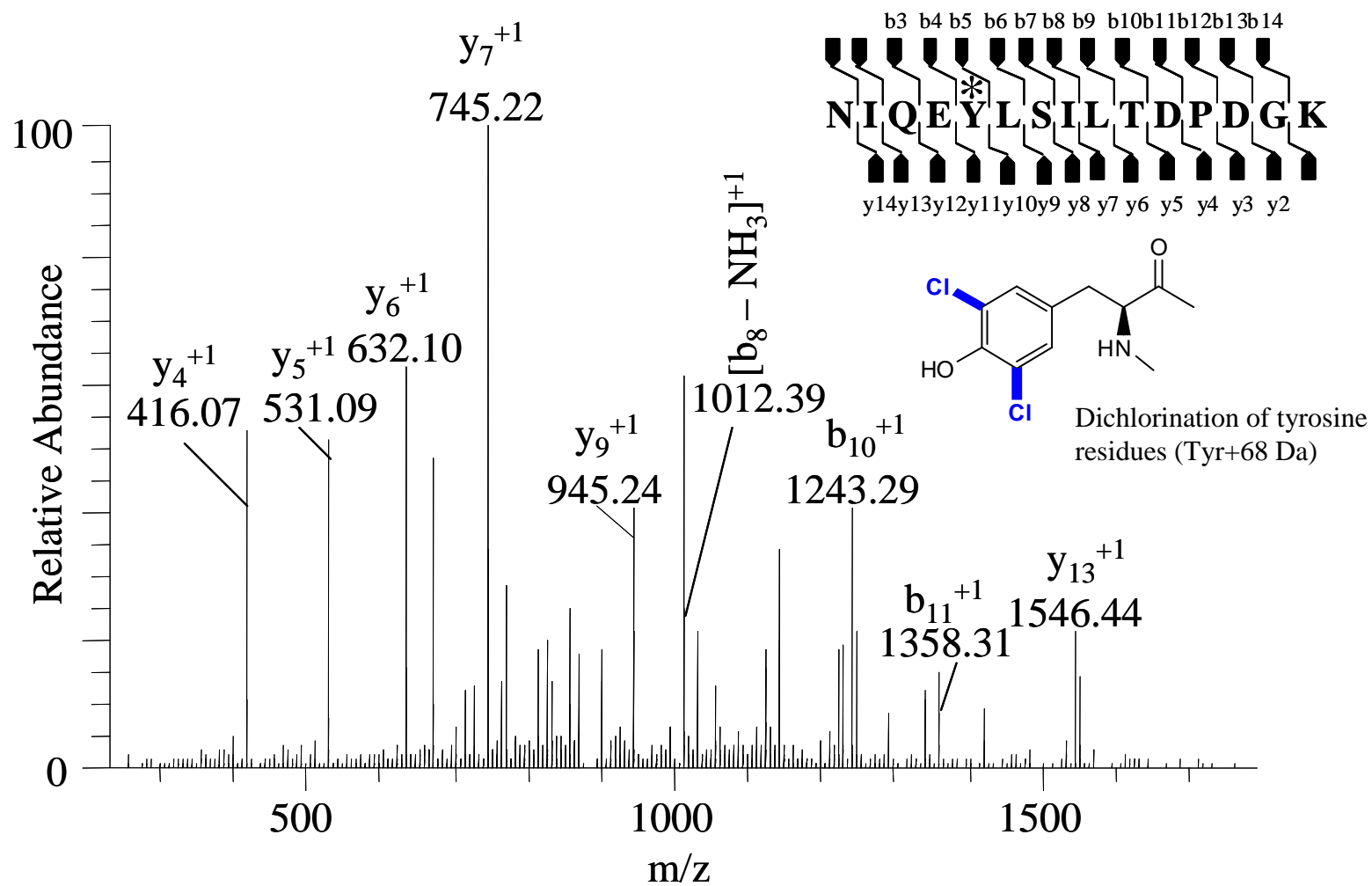


Figure 5.11 MS/MS spectra obtained from tryptic peptide 4447-61 depicting dichlorination of Tyr4451. This peptide was observed in high hypochlorite exposure.

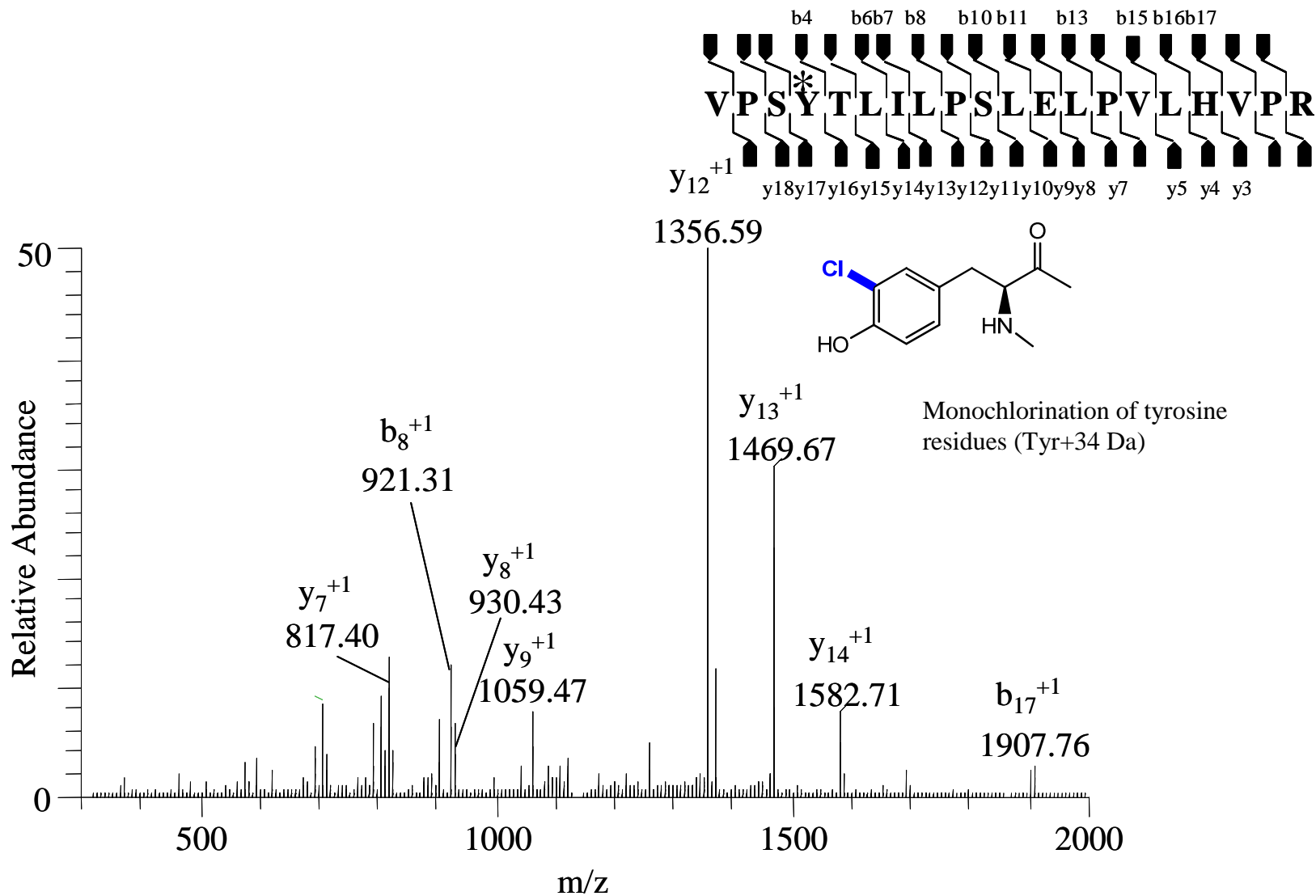


Figure 5.12 MS/MS spectra of tryptic peptide 3293-3311 indicating monochlorination of Tyr3295 (+34 Da addition).

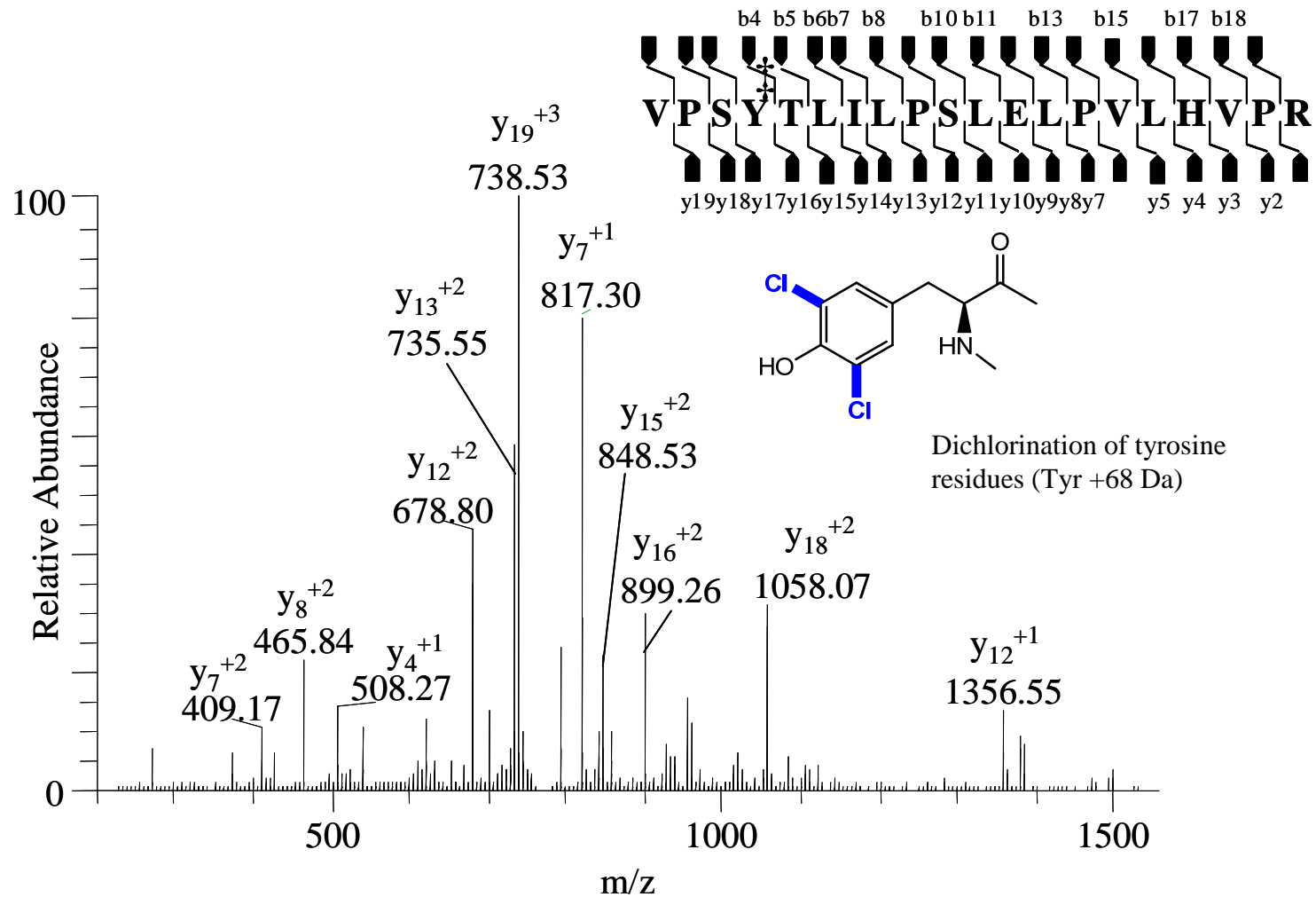


Figure 5.13 MS/MS spectra of tryptic peptide 3292-3311 depicting dichlorination of Tyr3295 (+68 Da). This peptide has even better match compare to the monochlorinated peptide.

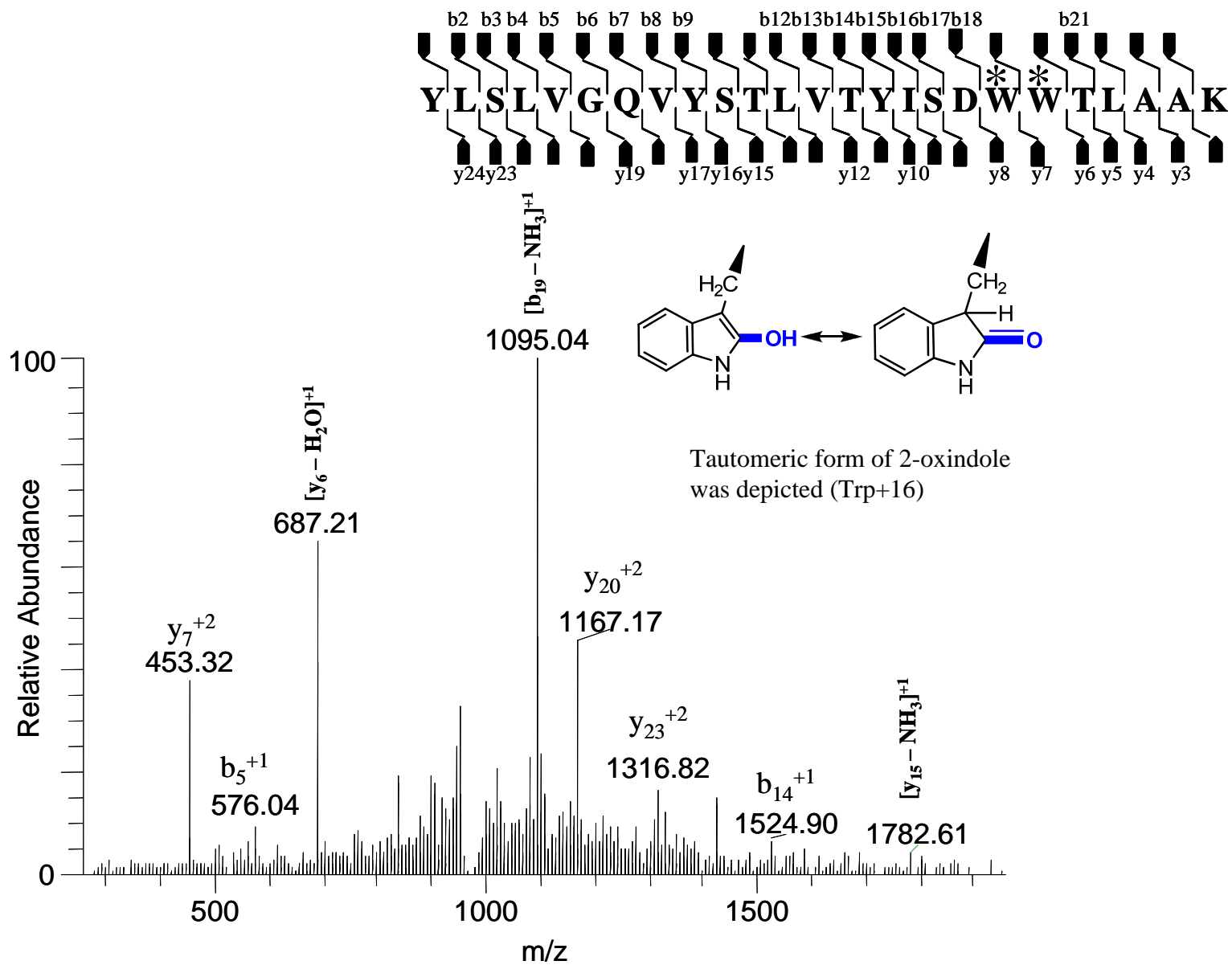


Figure 5.14 MS/MS spectra from tryptic peptide 2535-59 depicting oxidation of Trp2553 and 2554 from high hypochlorite exposure. Corresponding B-/Y- ions are observed.

Table 5.3 Observed amino acid oxidations and associated mass changes from three different peroxynitrite exposures on LDL were tabulated. Trypsin was used as the proteolytic enzyme. All these modification are unique in nature. Sequence coverage was low in all experiments (data not shown).

Modified amino acids	Low peroxynitrite (10 μM)	Intermediate peroxynitrite (100 μM)	High peroxynitrite concentration (1000 μM)	High peroxynitrite concentration (1000 μM)
Y+45	1840	–	–	413, 1602, 2524, 3295
W+45	–	–	–	N.D.
W+16	583, 3970	1461, 2248, 3970	3970	N.D.
C+48	1505	–	–	N.D.

N.D. Not determined. [Data was not searched for W+45, W+16 and C+48].

Table 5.4 Oxidation of amino acids with three different hypochlorous acid concentrations. Enzymatic digestion of apo B-100 was carried out by pronase. Results in the treated samples were compared with an untreated sample (blank containing no hypochlorous acid) were listed here.

Modified amino acids	Low hypochlorous acid (10 μ M)	Intermediate hypochlorous acid (100 μ M)	High hypochlorous acid (1000 μ M)
Y+34	–	–	1053, 1200
W+16	–	4022, 4396	–
C+48	–	–	–

Table 5.5 Oxidations of amino acids in presence of three different concentrations of hypochlorous acid. All modifications from duplicate experiments were included. Trypsin was used as a proteolytic enzyme in these experiments. Amino acids that appeared only in treater sample were listed here (compared with a blank containing no hypochlorite).

Modified amino acids	Low hypochlorous acid (10 μ M)	Intermediate hypochlorous acid (100 μ M)	High hypochlorous acid (1000 μ M)
Y+34	3295	–	144, 276, 443, 666, 1200, 1999, 3139, 3295, 3771, 4509/10 [#] , 4534
Y+68	–	–	148, 3295, 4451, 4509/10 [#]
W+16	3563	721, 1461, 2131, 2495, 2553/54, 2686, 3594, 4058	2553 [†] , 2554 [†] , 3153, 4058
C+48	–	1112, 3761, 4217	3761

#: MS/MS is not conclusive enough to pinpoint one single amino acid oxidation in this case. Tyrosine was partially modified to monochloro and dichlorotyrosine

†: Two modified tryptophan residues appeared in one single peptide.

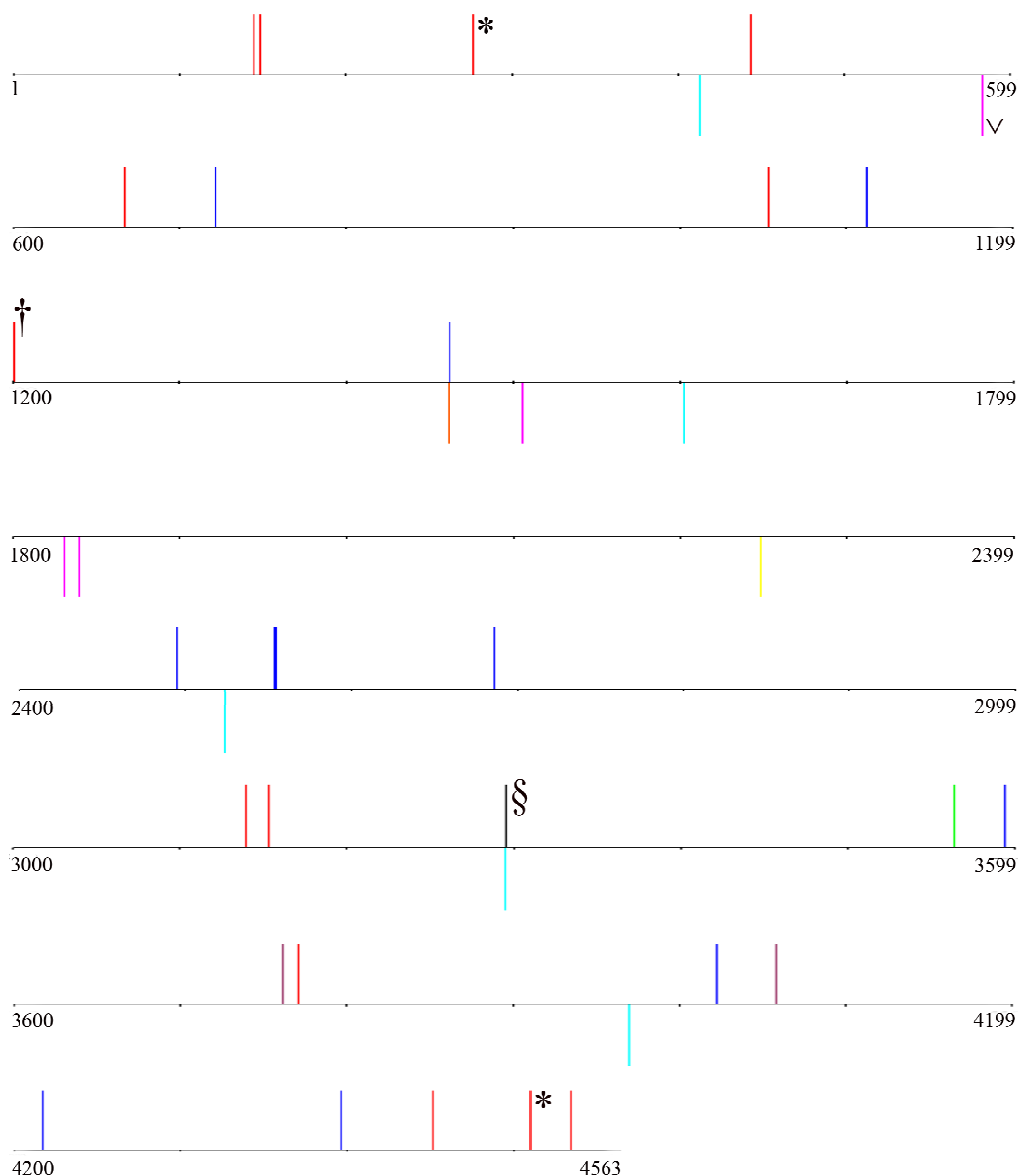


Figure 5.15 Mapping oxidized amino acids of apo B-100 in LDL with peroxynitrite (downward line) and hypochlorite (upward line). Pink represents oxidation from low peroxynitrite where as yellow and turquoise represents oxidation from intermediate and high peroxynitrite. Orange represents oxidized amino acids appearing both in low and intermediate peroxynitrite. Green, blue and red represents oxidized amino acids from low, intermediate and high hypochlorite exposure. Black represents oxidized amino acids appearing in both low and high hypochlorite. Plum represents oxidized amino acids appeared both in intermediate and high hypochlorite exposure. Special characters * represents oxidized amino acids observed both in hypochlorite and hydroxyl radical mediated oxidations. Special character ^ represents oxidized amino acid was observed both in peroxynitrite and hydroxyl radical treated samples. Special character † coins oxidized amino acid appearing in peroxynitrite and hypochlorite treated samples. Special character § coins oxidized amino acid was observed in all three treatments.

For experiments at intermediate hypochlorite concentration (100 μ M), Tyr-chlorination was confirmed. Eight tryptophan and three cystein residues were oxidized. No dichlorination products were observed (Table 5.5).

In high hypochlorite dose experiments, eleven Tyr residues were monochlorinated and four Tyr residues were dichlorinated. Tyr443 monochlorination was depicted in Figure 5.10. Tyr4451 dichlorination (mass increase of +68 Da) on the other hand was depicted in Figure 5.11. Interestingly Tyr3295 appeared in both monochlorinated (Figure 5.12) and dichlorinated (Figure 5.13) form indicating partial modification of the said residues. Cys3761 oxidation was observed (Figure 5.9) by hypochlorous acid in our samples (Table 5.5).

Positions of the all modified amino acids in apo B-100 was presented in Figure 5.15. The said figure (Figure 5.15) depicted modification from both peroxynitrite and hypochlorite including three different oxidant doses. It was evident that the number of chlorination sites increased with high concentration of hypochlorite. Lys and histidine in the nearby sites can facilitate the chloramine transfer⁴² to Tyr residues. For example peptide 3292-3311 has a histidine residue at 3308. This peptide belongs to the amphipathic β_2 strand which has a well established barrel structure.¹⁴ Therefore, His3308 could undergo initial chlorination and take part in chloramine transfer to chlorinate Tyr3295 (Figure 5.12 and Figure 5.13). Chlorination of Tyr276 occurred during high hypochlorite exposure. The N-terminal amino acid for this peptide was 275Lys, which can easily participate in chloramine transfer reactions. Tyr443 (Figure 5.10) had been one of the most interesting chlorinated tyrosines, which was part of peptide 440-54 and in close proximity to His447. No evidence was observed in support of 452Tyr chlorination which was right beside 453His and 454Lys. Probably when hypochlorite was added; it was placed right beside the His residue which took part in chloramine transfer to Tyr443. Peptide 440-54 belongs

to $\beta\alpha_1$ domain of apo B-100, which assumes an intermediate structure between helix and barrel.¹⁴ Tyr1999 is in close vicinity of C-term Lys2002 which can help chlorinating the Tyr residue. 4509/10 is an interesting special case where the match is extremely comparable in MS/MS between the Tyr residues placed side by side. However, drawing exclusive conclusion about dichlorination of one of Tyr4509 or Tyr4510 residue is difficult just with MS/MS since they are adjacent to each other. A possibility of monochlorination on both Tyr4509 and Tyr4510 cannot be overruled. Also model peptidic study involving peptide LQDFSDQLSDYYEK could reveal mechanistic details about the tyrosine chlorination(s) in the protein. C-term Lys4512 could be involved in chloramine transfer reactions. One of these residues was also found to be dichlorinated with a very high score, indicating that chlorination reaction is facile in this region. Again in peptide 140-57 two Tyr144 and Tyr153 (closer to Lys157) were present, although only Tyr144 was found to be chlorinated. It was apparent that Lys148 is closer to Tyr144 and facilitating the chloramine transfer reactions with hypochlorite. MS/MS spectra obtained from peptide 4447-61 contained Tyr4451 which was dichlorinated in the presence of high hypochlorite concentration. An excellent match in the product ion spectra was observed (Figure 5.11).

Previously, we have identified 5 and 7-hydroxytryptophan residues which were associated with hydroxyl radical addition to the phenyl ring. However hypochlorite induced oxidation of tryptophan renders favorable attack on the indole ring to yields a 2-hydroxyindole residue which stays in its resonance form 2-oxindole.⁴⁸ Oxidation of tryptophan increased as the concentration of hypochlorite increased. Also, peptide 2535-59 in intermediate hypochlorite mediated oxidations have some interesting features. From the MS/MS data it was difficult to conclude whether Trp2553 or Trp2554 was modified (Figure 5.14). In high hypochlorite

exposures, we have again observed peptide 2535-59 bearing both Trp2553 and Trp2554 oxidations. Intermediate hypochlorite contained Trp1461 oxidation, which was part of peptide 1451-74. The same peptide contained Met1465. However, CH₃SOH loss, which is common for methionine oxidations, was not observed in the MS/MS spectra. MS/MS matching for Met1465+16 is comparable to Trp1461+16. Hence methionine modification can not be completely ruled out in this case.

Cystein oxidation was observed for intermediate and high hypochlorite concentration. Cys3761 was oxidized to the corresponding sulfonic acid in presence of intermediate and high concentrations of hypochlorite (Figure 5.9). Observed oxidations at high hypochlorite dose were lower than that of the intermediate dose, which could be attributed to several reasons including the lack of accessibility of the same region by trypsin.

It was perceived that high concentration of hypochlorite can bring about various changes to a protein including peptide bond fragmentation. At this point it would be difficult to exclusively depict whether apo B-100 is fragmented by hypochlorite. Rigorous data mining are under current investigation to identify peptides which shows fragmentation by hypochlorous acid. Conceivably, hypochlorite could induce peptide bond fragmentation in apo B-100. 1-D SDS-Gel electrophoresis data might be useful, although previous attempts by other researchers were not quite successful. Several issues were raised indicating difficulties associated with loading the protein in the gel.⁷⁹

Hydroxyl radical mediated oxidative mapping and oxidative mapping from peroxynitrite and hypochlorite showed some similarities (Figure 5.15). Tyr2524, Tyr3295 and Trp583 were oxidized in hydroxyl radical mediated oxidations as well as peroxynitrite. Where as Tyr144, Tyr276, Tyr3295, Tyr4451 and Tyr4509/10 were observed both in hypochlorite mediated

oxidations and hydroxyl radical mediated oxidations. Oxidation of same amino acid residues using different oxidants emphasizes the fact that these residues are solvent accessible and would get damaged readily during oxidative stress. Also, some of these amino acids such as Tyr413, Tyr2524 and Tyr3295 were previously observed to be nitrated with peroxynitrite.³⁷ Dose dependent oxidations with peroxynitrite and hypochlorous acid to identify the site specific oxidation emerge as a novel technique which could be useful in identifying the solvent accessible residues in apo B-100. Although observed oxidations with peroxynitrite were not overwhelming, we were able to identify Tyr1602 nitration which was not reported before. Yang et. al. produced interesting work in identifying oxidation sites in apo B-100,⁸¹ however, extensive concentration dependent mapping experiments with hypochlorous acid was not carried out by any research group.⁸¹ It also revealed several different oxidation sites in apo B-100 which was not previously identified. We hope site specific oxidations data will help us comprehend the structure of the protein in intact LDL.

5.5. References

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Chapter 6.

Conclusions and future directions

6.1. Accomplishments

Thiourea based fluoride sensing using luminescence methods were investigated in the initial part of my research. In this context, 1,8-bis(phenylthioureido)naphthalene (**compound 1**) was synthesized and characterized (Chapter 2), and its fluoride sensing behavior in acetonitrile was investigated. This compound was highly selective towards fluoride compared to other halides, acetate and hydrogen phosphate. A remarkable 40-fold enhancement in fluorescence was observed in the presence of only five equivalents of tetrabutylammonium fluoride (fluoride donor). Mechanistic studies indicated strong hydrogen bonding formation between thioureido protons and the fluoride ion. The compound presented itself as a chromogenic sensor in the same solvent. Fluoride bound species showed 20 fold higher quantum yield than the unbound species at the same emission wavelength indicating a superb sensitivity. Theoretical detection limit was in the range of submicromolar level, which provided low ppm detection of fluoride.

LDL protein oxidations and their implications in atherosclerotic plaque formation is an important aspect to understand cardiovascular disease risk factors. In this research we have produced ample data to support the hypothesis that free radical mediated oxidations of apo B-100 in LDL is site specific. Extensive oxidations however might induce irrevocable changes in the protein. Metal catalyzed oxidations with variable concentrations of hydrogen peroxide revealed substantial oxidations in the proteins. Several different oxidation sites in apo B-100 were identified by using LC-MS/MS based methodologies (Chapter 4). These sites were distributed all

throughout the apo B-100 sequence. We have also identified some reported natural variants by using a novel database search method (Chapter 4).

Furthermore, we have also used the oxidants peroxyxynitrite and hypochlorite to identify oxidation sites in LDL. Different side chain oxidations in amino acid residues were observed (Chapter 5). It appears that peroxyxynitrite is very selective in nitrating the amino acids while hypochlorite could chlorinate several tyrosine residues in apo B-100. The nitration and chlorination in amino acid residues in LDL induce structural changes in apo B-100 which might be critical to its function. How these structural changes are directly propagating the plaque to cause disease advancement are still to be understood.

6.2. Future directions

Over the years fluoride remained as a fascinating ion to the scientists and researchers due to its critical behavior in health and environmental subjects. Fluorescence enhancement based fluoride sensing gives better detection than that of fluorescence quenching based fluoride sensing. Urea and thiourea based naphthalene were depicted as an excellent candidate primarily due to improve planarity^{1, 2} after binding to fluoride and also due to lack of relaxation via twisted conformations^{3, 4}. Our success in detecting micromolar to submicromolar levels of fluoride by compound 1 revealed several new vistas of research. The proposed research is furnished below:

- ❖ Synthesis and characterization of similar thioureido analogues and exploitation of their binding behavior towards fluoride. Synthesis of these analogues is complex and requires exploration of improved synthetic routes. Changing the substituents in the phenyl ring as well as in the naphthalene ring might bring about important changes in the electronic configuration of the molecule, consequently changing fluoride detection and the binding properties of the molecules. Previous articles, proposed 1:1 binding between fluoride and

anthracene thioureido compounds. However, naphthalene thiourea could be a completely different case. In our studies, we observed evidence for the presence of 1,8-bis(4-methoxyphenylthioureido)naphthalene during the reaction of 1,8-diaminonaphthalene and 4-methoxyphenylisothiocyanate. However, purification did not yield sufficient quantity of product to carry on with fluoride sensing experiments.

- ❖ Immobilization of naphthalene thiourea based analogues to a nanoparticle or a fiber optic to enable detection of the fluoride in aqueous solutions. This approach could resolve the water solubility issue of naphthalene thiourea analogues. Kim et. al. were able to immobilize anthraquinone based receptors to mesoporous silica and silica particles⁵. Immobilization of these sensing molecules on solid supports will expand the use of thiourea based sensing molecules to detect fluoride in aqueous media where interfering effects could be minimized.

Low density lipoprotein is of major interest to researchers due to its implications in atherosclerosis. Protein surface mapping is a unique technique for identifying oxidatively modified surface amino acids in proteins. Identification of these sites reveals important structural information of the proteins. In our research, several oxidized amino acid residues were identified in LDL protein resulting from oxidants such as hydroxyl radical, peroxy nitrite and hypochlorous acid. Some of the new and improved approaches are listed below which could allow us to expand the boundary of the current research. Additionally, the following approaches could also help us answer critical issues associated with current LDL research and the importance of LDL in atherosclerosis. The recommendations are:

- ❖ The oxidation sites from the *in vitro* LDL experiments provided us the insight that hydroxyl radical mediated oxidations occur to those amino acids which are solvent

accessible or close to lipid permeable layer. We, therefore, would like to map the surface of this protein on LDL samples acquired from patients with different levels of atherosclerosis (assuming that they went through *in vivo* oxidative stress) and match those site specific oxidations with our *in vitro* data. Dr. Jim Oates of MUSC, South Carolina kindly has consented to provide us LDL samples for this research.

- ❖ Oxidation of peroxynitrite was done so far with reagent peroxynitrite. A second nitric oxide donor 3-morpholinosydnonimine hydrochloride (SIN-1) (releases peroxynitrite *in situ*) should be used along with peroxynitrite in a dose dependent fashion to verify the site specific oxidations of apo B-100. Peroxynitrite is a potent oxidant and can cause fragmentation of the peptide backbone. SDS gel electrophoresis data will be useful in identifying these fragments from LDL protein. Also, oxidized LDL samples obtained from patients could again be very useful in validating the data obtained from *in vitro* experiments.
- ❖ *In vitro* hypochlorite addition showed site specific chlorination and other oxidations in the LDL protein. However, peptide backbone fragmentation was not largely explored in our current experiments. Hypochlorite being an extremely strong oxidant showed protein fragmentations in other model systems^{6, 7} Again SDS gel electrophoresis data should reveal important aspects of variable hypochlorite mediated oxidation of apo B-100 in LDL.
- ❖ In a related but slightly different subject, myeloperoxidase, hydrogen peroxide and chloride should be used to initiate more physiologically relevant hypochlorous acid oxidant system to oxidize LDL. This system will exclusively provide evidence on

whether there are any differences in site specific oxidations between reagent hypochlorite and MPO/H₂O₂/Cl⁻.⁸

- ❖ All through our LDL work we have perceived the fact that enzymatic digestion is a key factor in identifying peptides by LC-MS/MS based techniques. Trypsin was an enzyme of choice owing to its broad applicability and ease of use. However, other enzyme systems could also be useful. Use of pronase was partially explored in hypochlorite mediated oxidation of LDL. With slight modifications in the digestion protocol, pronase^{9, 10} could be considerably useful in generating substantial amounts of peptides from apo B-100, particularly due to its non-specific nature. Also chymotrypsin should be explored for the same reason⁹. Combination of enzymes might also increase the number of peptides from apo B-100, which in turn could be useful for matching them with the database.
- ❖ To address the problems associated with tryptic digestion and recovery of peptides after digestion, enzyme immobilization in microfluidic chip¹¹⁻¹³ or other devices¹⁴⁻¹⁶ could be a powerful alternative approach. Microfluidics involving in-line digestion coupled with LC-separation and tandem mass spectrometry will bring about revolutionary changes in the complete aspect of the proteomics research associated with large proteins such as apo B-100.
- ❖ Natural variant identification by novel database search method could be useful as a rapid screening method in identifying modifications in proteins. We believe this methodology will provide significant support in early screening of samples for disease related mutations in proteins.

The strong practical impetus for LDL research is associated with human health and our future research will definitely provide answers to some long standing questions associated with risk factors in cardiovascular diseases.

6.3. References

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Appendix A

Table A-1 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after low peroxide exposure (sample 1).

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg State	X _c	ΔC _n
F+16	1722-28	3996-4021	GISTSAASPAVGTVGM*DMDDEDDDF ⁴⁰¹⁹ SK	+3	4.60	0.24
	2354-68	877-96	PSVSVEF ⁸⁸³ VTNMGIIIPDFAR	+2	4.42	0.27
	2674	670-95	ESMLKTTLTAF ⁶⁸⁰ GFASADLIEIGLEGK	+3	3.48	0.23
	2734-36	2645-69	FSTPEFTILNTFHIPSFTIDF ²⁶⁶⁵ VEMK	+3	3.70	0.27
	3033	4520-40	LIDLSIQNYHTF ⁴⁵³¹ LIYITELLK	+3	3.83	0.07
F+32	2624	3906-35	ADYVETVLDSTCSSTVQF ³⁹²³ LEY ³⁹²⁶ ELNVLGTHK	+3	3.54	0.47
Y+16	1750-52	52-71	YTYNY ⁵⁴ EAESSSGVPGTADSR	+2	5.66	0.78
	2624	3906-35	Same precursor ion as 2624 in F+32			
	2877-81	4359-85	FNEFIQNELQEASQELQQIHQY ⁴³⁸⁰ IMALR	+3	6.30	0.57
H+16	2498-2504	591-613	NFVASH ⁵⁹⁶ IANILNSEEELDIQDLKK	+3	3.25	0.39
	3033	4520-40	LIDLSIQNYH ⁴⁵²⁹ TFLIYITELLK	+3	3.84	0.26
P+16	1678-80	52-71	YTYNYEAESSSGVP ⁶⁵ GTADSR	+2	3.54	0.63
	2655	3292-3311	VPSYTLILPSLELP ³³⁰⁵ VLHVP ³³¹⁰ R	+2	3.48	0.24
	2772-78	3254-76	PVVNVEVSP ³²⁶² FTIEMSAFGYVFPK	+2	3.50	0.34

Table A-2 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after low peroxide exposure (sample 2).

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
F+16	1885-92	3996-4021	GISTSAASPAVGTVGM*DMDEDDDF ⁴⁰¹⁹ SK	+2	3.42	0.67
	2403-11	877-96	PSVSVEF ⁸⁸³ VTNMGIIPDFAR	+2	4.25	0.25
	2827-34	2645-69	FSTPEFTILNTFHIPSFTIDF ²⁶⁶⁵ VEMK	+3	3.61	0.25
Y+16	1940-47	276-87	Y ²⁷⁶ GMVAQVTQTLK	+2	3.09	0.54
	2211-16	1001-24	LELELRPTGEIEQYSVSATY ¹⁰²⁰ ELQR	+3	5.22	0.03
	2223	140-57	QVFLY ¹⁴⁴ PEKDEPTYILNIK	+3	3.44	0.04
	2392	4447-61	NIQEY ⁴⁴⁵¹ LSILTDPDGK	+2	3.57	0.58
	2623	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	6.08	0.50
W+32	2159	577-90	IVQILPW ⁵⁸³ EQNEQVK	+2	3.13	0.34
H+16	2889-94	2489-2509	ITLIINWLQEALSSASLAH ²⁵⁰⁷ MK	+3	3.39	0.42
P+16	2211-16	1001-24	LELELRP ¹⁰⁰⁷ TGEIEQYSVSATYELQR	+3	5.21	0.56
	2223	140-57	QVFLYP ¹⁴⁵ EKDEPTYILNIK	+3	3.47	0.78
	2231	3587-3603	ATLELSP ³⁵⁹³ WQMSALVQVH	+2	3.79	0.67
	2303-06	655-69	IEGNLIFDP ⁶⁶³ NNYLPK	+2	3.73	0.06
	2439	2610-25	ATFQTPDFIVP ²⁶²⁰ LTDLR	+2	3.34	0.03
	2623	3292-3311	VP ³²⁹³ SYTLILPSLELPVLHVPR	+3	5.56	0.09

Table A-3 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after intermediate peroxide exposure (sample 3). Duplicate run (sample 4) did not reveal any unique peptide.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
F+16	1510	1188-1202	KMTSNF ¹¹⁹³ PVDLSDYPK.S	+3	3.23	0.09
	2433-38	2411-25	TFIEDVNKF ²⁴¹⁹ LDMLIK	+2	3.33	0.59
Y+16	1577	4499-4512	LQDFSDQLSDYY ⁴⁵¹⁰ EK	+2	4.94	0.01
P+16	1331-33	3996-4021	GISTAASP ⁴⁰⁰⁴ AVGTVGM ⁴⁰¹¹ DMDEDDDFSK	+3	3.35	0.34

Table A-4 List of peptides depicting various modifications observed in tryptic peptides obtained (unique positive match) from apo B-100 after high peroxide exposure (sample 5)

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
F+16	1621-27	1189-1202	MTSNF ¹¹⁹³ PVDLSDYPK	+2	3.11	0.73
	2323-27	2645-69	FSTPEFTILNTFHIPSFTIDF ²⁶⁶⁵ VEMK	+3	3.93	0.22
Y+16	2417-23	4359-85	FNEFIQNELQEASQELQQIHQY ⁴³⁸⁰ IMALR	+2	4.94	0.01
P+16	1367-73	3996-4021	GISTSAASP ⁴⁰⁰⁴ AVGTVGM ⁴⁰¹¹ DMDEDDDFS	+3	3.35	0.34

Table A-5 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after high peroxide exposure (sample 6)

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
F+16	2113	2296-2309	VLLDQLGTTISF ²³⁰⁷ ER	+2	3.12	0.52
	2301	877-96	PSVSVEF ⁸⁸³ VTNMGIIIPDFAR	+2	3.27	0.20
	2591-99	670-95	ESMLKTTLTAF ⁶⁸⁰ GFASADLIEIGLEGK	+3	4.30	0.02
	2799-2802	2411-25	TFIEDVNKF ²⁴¹⁹ LDMLIK	+2	3.42	0.63
F+32	2107-11	2296-2309	VLLDQLGTTISF ²³⁰⁷ ER	+2	4.94	0.01
Y+16	2131-37	2560-75	NLTDFAEQY ²⁵⁶⁸ SIQDWAK	+2	4.40	0.07
P+16	1965	3277-91	AVSM ³²⁷⁹ P ³²⁸⁰ SFSILGSDVR	+2	3.87	0.65
K+120	2732	2195-2210	K ²¹⁹⁵ IAIANIIDEIIEK ²²⁰⁸ LK [2208Lys]	+3	3.05	0.01
K+138	2732	2195-2210	K ²¹⁹⁵ IAIANIIDEIIEK ²²⁰⁸ LK [2195Lys]	+3	3.05	0.01

Appendix B

Table B-1 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after low, intermediate and high peroxynitrite exposure. All matched peptides were listed in the following table.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
Y+45	4151	1830-52	AY ¹⁸³¹ QNNEIKHIY ¹⁸⁴⁰ AISSAALSASYK [1831 hydroxylation of Y]	+3	3.53	0.09
W+16	4569-76	577-90	IVQILPW ⁵⁸³ EQNEQVK	+2	3.32	0.55
	4413-18	3953-73	DFSAEYEEDGKFEGLQEW ³⁹⁷⁰ EGK	+3	3.45	0.76
	4120	577-90	IVQILPW ⁵⁸³ EQNEQVK	+2	3.36	0.49
	8726-30	3953-73	DFSAEYEEDGKFEGLQEW ³⁹⁷⁰ EGK	+3	3.28	0.68
	8846-50	3953-73	DFSAEYEEDGKFEGLQEW ³⁹⁷⁰ EGK	+3	3.28	0.70
	5398-5405	2241-55	SGSSTASW ²²⁴⁸ IQNVDTK	+2	3.62	0.64
	8158-62	1451-74	GLLIFDASSW ¹⁴⁶¹ GPQMSASVHLDSK	+3	3.54	0.66
	5562	3953-73	DFSAEYEEDGKFEGLQEW ³⁹⁷⁰ EGK	+2	2.60	0.71
C+48	8571-76	1499-1513	GTYGLSC ¹⁵⁰⁵ QRDPNTGR	+2	3.08	0.66

Table B-2 List of peptides depicting various modifications observed in tryptic peptides (unique positive match) obtained from apo B-100 after another high peroxy nitrite exposure. Unlike the last time four nitrotyrosines were nitrated. Relevant peptides were listed below.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
Y+45	1382	2523-34	M ²⁵²³ Y ²⁵²⁴ QM ²⁵²⁶ DIQQELQR	+2	4.52	0.69
	2213-19	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	6.34	0.61
	2807-13	401-27	VHANPLLIDVVTY ⁴¹³ LVALIPEPSAQLR	+3	4.29	0.31
	1328	1600-10	SEY ¹⁶⁰² QADYESLR	+2	2.68	0.50

Table B-3 List of peptides depicting various modifications observed in pronase digest (unique positive match) obtained from apo B-100 after low, intermediate and high hypochlorite exposure. All positive matches from duplicate experiments were shown below.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
Y+34	2300-06	1051-67	FKY ¹⁰⁵³ NRQSM ¹⁰⁵⁸ TLSEVQIP	+3	3.57	0.29
	2619-34	1193-1202	FPVDLSDY ¹²⁰⁰ PK	+2	3.04	0.47
	2492-95	1193-1204	FPVDLSDY ¹²⁰⁰ PKSL	+2	3.26	0.43
W+16	2405	4010-22	GM ⁴⁰¹¹ DM ⁴⁰¹³ DEDDDFSKW ⁴⁰²²	+2	3.63	0.57
	2268	4385-98	REEYFDPSIVGW ⁴³⁹⁶ TV	+2	3.09	0.66

Table B-4 List of matched peptides after low hypochlorite treatment followed by a trypsin digestion of apo B-100. All peptides from duplicate analyses were shown.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
Y+34	2440-43	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	3.26	0.34
W+16	1702	3559-68	IYSLW ³⁵⁶³ EHSTK	+2	3.06	0.38

Table B-5 List of matched peptides after intermediate hypochlorite treatment followed by a trypsin digestion of apo B-100. All peptides from duplicate analyses were shown.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
W+16	2026	2118-33	LPQQANDYLNSFNW ²¹³¹ ER	+2	4.84	0.61
	2098	3587-3603	ATLELSPW ³⁵⁹⁴ QMSALVQVH	+2	4.89	0.54
	2188	4056-71	VNW ⁴⁰⁵⁸ EEEEASGLLTSLK	+2	4.40	0.62
	2271	2675-94	TIDQMQNSQLQW ²⁶⁸⁶ PVPDIYLR	+2	2.70	0.42
	3031	2489-2509	ITLIINW ²⁴⁹⁵ LQEALSSASLAHMK	+3	3.04	0.17
	3405-23	2535-59	YLSLVGQVYSTLVTYISDW ²⁵⁵³ W ²⁵⁵⁴ TLAAK [could be either]	+2	4.33	0.04
	4768	1451-74	GLLIFDASSW ¹⁴⁶¹ GPQMSASVHLDISK	+3	3.43	0.54
	1690-95	720-32	YW ⁷²¹ VNGQVPDGVSK	+2	3.33	0.61
C+48	1537	1102-18	ITEVALM ¹¹⁰⁸ GHLSC ¹¹¹² DTKEER	+3	5.22	0.68
	1621	1101-18	KITEVALM ¹¹⁰⁸ GHLSC ¹¹¹² DTKEER	+3	5.05	0.60
	1820-27	4214-22	EELC ⁴²¹⁷ TM ⁴²¹⁹ FIR	+2	2.51	0.55

Table B-6 List of matched peptides after high hypochlorite treatment followed by a trypsin digestion of apo B-100. All peptides from duplicate analyses were shown.

AA modification	Dta #	Peptide	AA sequence for the peptide	Chg. State	X _c	ΔC _n
Y+34	1834-41	276-87	Y ²⁷⁶ GM ²⁷⁸ VAQVTQTLK	+2	3.85	0.48
	1877-83	440-54	ATLY ⁴⁴³ ALSHAVNNYHK	+3	4.31	0.62
	1925	1985-2002	TQFNNNEYSQDLDAY ¹⁹⁹⁹ NTK	+2	5.44	0.22
	1929-46	3763-72	LDFREIQIY ³⁷⁷¹ K	+2	2.71	0.25
	2127	4499-4512	LQDFSDQLSDY ⁴⁵⁰⁹ Y ⁴⁵¹⁰ EK [could be either one]	+2	4.97	0.11
	2145	1189-1202	M ¹¹⁸⁹ TSNFPVDLSDY ¹²⁰⁰ PK	+2	3.61	0.65
	2249-53	3137-48	LPY ³¹³⁹ TIITTPPLK	+2	3.77	0.59
	2299-2307	140-57	QVFLY ¹⁴⁴ PEKDEPTYILNIK	+3	4.13	0.21
	2441-50	655-69	IEGNLIFDPNNY ⁶⁶⁶ LPK	+2	3.57	0.48
	2693-97	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+2	4.51	0.58
	3009-14	4520-40	LIDLSIQNYHTFLIY ⁴⁵³⁴ ITELLK	+3	5.72	0.49
2202-08	3137-48	LPY ³¹³⁹ TIITTPPLK	+2	3.95	0.58	
Y+68	2195-97	4499-4512	LQDFSDQLSDY ⁴⁵⁰⁹ Y ⁴⁵¹⁰ EK	+2	5.16	0.09
	2345	140-57	QVFLY ¹⁴⁸ PEKDEPTYILNIK	+3	3.73	0.19
	2588	4447-61	NIQEY ⁴⁴⁵¹ LSILTDPDGK	+2	4.04	0.69
	2734	3292-3311	VPSY ³²⁹⁵ TLILPSLELPVLHVPR	+3	4.85	0.60
W+16	2459	3137-55	LPYTIITTPPLKDFSLW ³¹⁵³ EK	+3	3.95	0.51
	3398	2335-59	YLSLVGQVYSTLVTYISDW ²⁵⁵³ W ²⁵⁵⁴ TLAAK [both oxidized]	+3	4.28	0.25
	1901	4056-66	VNW ⁴⁰⁵⁸ EEEEASGL	+2	3.02	0.65
C+48	1958	3754-66	TDLQVPSC ³⁷⁶¹ KLDFR	+2	3.06	0.44

Vita

Sourav Chakraborty was born in a small town near Calcutta in eastern part of India. He graduated with a B.Sc. (Hons) in Chemistry in the year 1999 from Rama Krishna Mission Residential College under University of Calcutta. He went to Forest Research Institute Deemed University at Dehra Dun to get a Master's degree in Wood Science and Technology in 2001. He joined Department of Chemistry at University of New Orleans in spring 2004. Further, he joined Dr. Matt Tarr's group in the summer of 2004 and continued his research in analytical and bioanalytical chemistry. Apart from chemistry, Sourav is an avid photographer and a proud owner of more than one Nikon and Olympus SLR and DSLR. His work was displayed in UNO Flambeau room in 2006, 2007 and 2009 during INST international photo competition.