1.17 Mass Spectrometry

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Abbreviations

2-D	two-dimensional
CAD	collisionally activated dissociation
CID	collision-induced dissociation
DC	direct current
ED	electron dissociation
ECD	electron capture dissociation
EI	electron impact ionization
EM	electron multipliers
ESI	electrospray ionization
ETD	electron transfer dissociation
FT	fourier transformed
FWHM	full width half maximum
HPLC	high-performance liquid chromatography
ICR	ion cyclotron resonance
IEM	ion evaporation model
IF	isoelectric focusing
IM	ion mobility
LIT	linear ion trap
MALDI	matrix-assisted laser desorption ionization
MCP	multichannel plates
MS	mass spectrometry
NMR	nuclear magnetic resonance
PTM	post-translation modification
Q	quadrupole
RF	radio frequency
SRM	single reaction ion monitoring mode
Th	Thompson
UPLC	ultra-high-pressure liquid chromatography

Glossary

Analyzer The main component of the mass spectrometer responsible for measuring the mass-to-charge ratio (m/z) of the analyte ions.

Bottom-up An approach in mass spectrometry based proteomics where the proteins are digested to yield peptides prior to mass analysis.

Chemical labeling A collection of methods that use covalently attached reporter probes to amino acid residues

to gather the information beyond the primary sequence of the protein.

Chromatographic separation A technique in analytical chemistry where the components of the mixture in mobile phase are separated within the stationary phase according to their physico-chemical properties.

ESI A process pioneered by John Fenn that creates ions by dispersing a liquid stream into a collection of fine droplets (50–100 microns diameter) through application of a high voltage to the liquid.

Fragmentation A physical fragmentation of ions inside the mass spectrometer used to gather the sequence information.

Functional proteomics The term usually applied to describe the studies where the overall focus is to comprehensively identify and quantify proteins in large-scale experiments.

Hydrogen-deuterium exchange (HX or H/D or HDX) A structural mass spectrometry method that studies the chemical exchange reaction of the main chain amide hydrogen atoms.

Ion mobility mass spectrometry A method in structural mass spectrometry that investigates the structural properties of the proteins (size, shape, charge state) via their behavior in the drift tube mass analyzer.

Ionization A process of transition of an uncharged polar molecule into an ion.

Mass accuracy An intrinsic characteristic of the mass spectrometer analyzer to accurately measure the mass-to-charge of the analyte ions.

Mass spectrometry A study of the mass-to-charge ratios of the analyte ions.

Oxidative labeling A technique where the specific amino acid residues (cysteine, methionine, etc.) are selectively oxidized and serve as a structural probe of the protein conformation.

Peptide A short amino acid oligomer, in most cases part of a larger protein.

Post translational modifications A fundamental cellular mechanism or regulation and signal transduction where specific amino acid residues are covalently labeled to convey biological information.

Protein An amino acid polymer, a cellular building block.

Resolution An intrinsic characteristic of the mass spectrometer analyzer to precisely measure the mass-to-charge of the analyte ions.

Structural mass spectrometry A collection of methods aimed to study structural properties (mass, size, shape, composition) of biological molecules.

Top-down An approach where the intact (or undigested) proteins are analyzed with a mass spectrometer.

1.17.1 Introduction

1.17.1.1 Mass Spectrometry

Mass spectrometry is an analytical technique used for characterization of chemical compounds and biological molecules. Mass spectrometers measure masses of ions (mass-to-charge ratios) in gas phase. They are essentially miniature and very accurate molecular scales that measure masses of individual ions. British scientists Sir J. J. Thompson and Francis W. Aston are credited with the invention of the mass spectrometry technique. Sir Thompson discovered electrons while studying the properties of gases in electric field, and Francis Aston developed the first mass spectrometer used to discover and separate the isotopes of chemical elements. Both Thompson and Aston are Nobel Prize laureates, and the mass-to-charge unit of the measurement is named after Thompson (Th). Instruments have evolved dramatically since the days of Thomson and Aston to become essential tools for scientific measurements. However, for several decades, mass spectrometry was used primarily for elemental analysis or for characterization of small molecules only. One of the principal limitations of adopting mass spectrometry to biological molecule analysis was the poor ionization efficiency of the mostly polar biological molecules. Ionization of molecules was accomplished primarily with electron impact ionization (EI), which required that molecules be volatilized into an electron beam. Most organic molecules have sufficient vapor pressure, especially at the low pressures of the mass spectrometer, but biomolecules, because they are polar and often charged, have a negligible vapor pressure. A solution to the problem was to derivatize molecules with the molecular moieties that mask charged or polar groups.[1,2] Vapor pressure is increased but at a cost of lower limits of detection due to the extra sample handling steps, reaction efficiencies, and side reactions. Such problems triggered vigorous research into methods to ionize polar and charged molecules such as proteins, DNA, and carbohydrates. It was not until the 1980s that the ionization techniques called electrospray ionization (ESI)[3] and matrix-assisted laser desorption ionization (MALDI)[4] made biological analysis possible and greatly broadened the applicability of mass spectrometry to biology.

1.17.1.2 Biological Mass Spectrometry

Protein analysis has been a cornerstone of biology. Proteins are the primary functional agents in biological processes and are involved in a wide variety of activities, such as enzymatic catalysis, signal transduction, molecular transport, and structural support functions within the cell. Although proteins are the products of genes, they do not always have the exact amino acid sequence predicted from the gene nucleotide sequence. Transcribed gene sequences are often spliced or modified through exon shuffling to produce isoform variants of the expected sequence. In addition, proteins are frequently modified post-translationally by the addition of other types of molecules or through a proteolytic trimming of the sequence. Not only do such structural changes have effects on the activity or function of a protein but also the modifications convey additional information within the cellular system. In addition to the modifications of a monomeric protein chain, higher order structures may form within cells as proteins come

together to form complexes. For example, groups of proteins can form complicated channels in lipid bilayers (e.g., nuclear pores), large structures to move DNA (e.g., kinetochores), or signaling complexes such as tyrosine kinase cascades. Protein complexes are held together via polar noncovalent interactions, which involve hydrogen bonding (H-bond), ionic interactions such as salt bridges, and hydrophobic interactions. The composition of protein complexes is dynamic, and covalent modifications (disulfide bonds, phosphorylation, acetylation, etc.) within individual subunits are often used to control the activity or stoichiometry of the components in the complex. Deciphering the components, stoichiometry, and modifications of the proteins in complexes helps to shed light on the mechanisms of physiological processes and on the functions of the individual proteins. Thus, the analysis of protein sequence, structure, and interactions has been a driving force of the mass spectrometry technology development for many decades.

Ideally, DNA sequencing provides the nucleotide sequences of genes from which a protein sequence can be predicted. However, this is not always possible because gene prediction is not perfectly accurate, and transcribed genes and the proteins translated from them can be processed in ways that may not be obvious from the gene sequence. Consequently, when studying the function of a gene, it is essential to understand the exact sequence of the protein creating the activity. Protein analysis has undergone a revolution during approximately the past 20 years as mass spectrometers have developed increasingly sophisticated capability for the ionization, detection, and characterization of proteins.[5] Introduction of robust multidimensional high-performance liquid chromatography (HPLC) separation methods[6] and direct interface of the separation with the mass spectrometers[7,8] has also contributed to the development of mass spectrometry (MS)-based proteomics. Finally, the task of interpreting mass spectrometry data for proteins has been simplified by the resources created through large-scale sequencing of genomes.[9,10]

1.17.1.3 Functional Versus Structural Proteomics

Mass spectrometry-based proteomics can generally be described as either functional or structural without a well-defined boundary between the two categories. The distinction between the two is instead based on the overall goal of the investigation. The term functional mass spectrometry is usually applied when the focus is to comprehensively identify and quantify proteins in large-scale experiments. Structural mass spectrometry is charged with the task of characterizing the proteins in biological systems. Therefore, investigation of protein structure, protein folding properties, and protein dynamics is considered structural MS-based proteomics. However, the distinction between the two categories is blurred when mass spectrometry is used to study post-translational modifications of proteins or protein-protein interactions because the goals are both a structural characterization and an understanding of the larger framework of cellular signaling and organization.

Two complimentary methods of MS-based proteomics have emerged. The first method is based on the traditional approach of protein sequencing that uses proteases to digest the protein into peptides for analysis. This method is often referred to as shotgun proteomics or 'bottom-up' analysis. When the protein is analyzed intact, it is referred to as 'top-down' analysis. With some exceptions, the bottom-up approaches are considered functional proteomics, whereas the top-down approaches seek to answer structural questions about individual proteins and protein complexes. The bottom-up approach is the most popular method when dealing with high-complexity samples for large-scale analyses.[11] The term 'shotgun proteomics'[6,12,13] reflects the method's similarity to the genomic shotgun sequencing in which DNA is sheared, sequenced in small fragments (contigs), and assembled into a complete sequence. In bottom-up applications, proteins are usually digested with a protease, chromatographically separated, and analyzed via a mass spectrometer. Mass spectrometers usually operate in tandem mode, in which the peptide ions are not only measured but also fragmented in the mass spectrometer. Fragmentation of peptide ions reveals their amino acid sequence. The scope of a top-down approach is limited to characterization of individual proteins or simple protein mixtures rather than the entire proteomes; however, the amount of structural insight learned about the target protein is much higher compared to the bottom-up method. The two methods are described in **Table 1**. Both approaches have certain limitations in terms of the ability to achieve complete coverage of the amino acid sequence of a protein. In the case of the bottom-up analysis, proteolytic peptides may be too small or too large for analysis, which results in limited sequence coverage for the target protein. Another drawback to the bottom-up analysis is the loss of spatial information about potential post-translational modifications. In contrast, although the top-down approaches provide much higher sequence coverage, they lack from poor sensitivity, large sample quantity requirements, and the low complexity mixture requirements. Ideally, it is desirable to create experimental data for all amino acids within a protein, and this goal, of course, could be difficult to achieve. When complete sequence coverage can be obtained, it becomes possible to comprehensively characterize the protein sequence, including modifications, mutations, and sequence variations.[14,15]

The development of mass spectrometers for the analysis of proteins began in earnest in the 1960s. Substantial progress was made considering the technological limitations of then-available ionization methods and mass analyzers. Ionization of molecules was accomplished primarily with EI, which required molecules to be volatilized into an electron beam. Most organic molecules have sufficient vapor pressure at the low inlet pressures of the mass spectrometer for effective ionization. However, because biomolecules are polar and often charged, they have a negligible vapor pressure. A solution to the problem was to derivatize molecules with the moieties that mask charged or polar groups.[1,2] In this way, the vapor pressure is increased but at a cost of lower limits of detection and higher sample amount requirements due to the extra sample handling steps, reaction inefficiencies, and side reactions. Such problems triggered vigorous research into methods to ionize polar and charged molecules such as proteins, DNA, and carbohydrates. Biological mass spectrometry can be viewed as an umbrella discipline that brings together analytical sciences,

Table 1 Comparison between the bottom-up and top-down approaches

Approach	*Prerequisite*	*Advantages*	*Disadvantages*	*Applications*	*Front end*	*Analysis*
Top-down	High mass accuracy instruments: LTQ-Orbitrap, LIT-FTICR, Q-TOF	High sequence coverage	Precursor ion charge state limitations	Single protein characterization	Ion exchange chromatography	EST
	Large sample amount	Comprehensive PTM and protein-protein complexes information	Separation limitations	Low complexity proteomic analysis	RP chromatography	*De novo*
	Low sample complexity	Multiple PTM identification	Low sensitivity	Alternative splicing information	ESI ionization	
		'Soft' fragmentation with ECD, ETD	Protein identification issues related to the charge state ambiguity	Multiple PTM analysis	Sample infusion	
		Better quantification compared to bottom-up	Poor ionization and transmission of large macromolecular ions	ESI-MS analysis		
		Structural approaches such as ESI-MS, native MS, ion mobility MS		Native MS analysis Ion mobility analysis		
Bottom-up	Wide variety of instruments: QT, LIT, TOF, etc.	Large-scale data acquisition	Narrow mass range	Protein identification via peptide analysis	Gel-based	PMF (simple mixture)
	Sample digest prior to analysis	High-complexity samples	Front-end separation required	Protein quantification, PTM analysis	Gel-free	Database search
		High sensitivity	Oversampling of high-abundance peptides	Deuterium exchange	RP chromatography	*De novo*
		Good front-end separation	Mass of the intact protein not directly accessible	Oxidative labeling	Ion exchange chromatography	Library search
		Chemical derivatization of peptides for quantification	Loss of labile PTMs		Affinity chromatography ESI, MALDI	

mass spectrometry, informatics, and biology. Thus, before any successful protein identification, the following processes have to take place: chromatographic separation, analyte ionization, mass analysis, and experimental data analysis. The following sections highlight developments in the previously mentioned areas that enable the routine use of the mass spectrometry in biology.

1.17.2 Mass Spectrometry Technology

1.17.2.1 Ionization Techniques

The emergence of two new ionization techniques revolutionized the analysis of biomolecules including proteins. The first, electrospray ionization (ESI), is a process that creates ions by dispersing a liquid stream into a collection of fine droplets (50–100 μm diameter) through application of a high voltage to the liquid.[3] ESI was pioneered by the 2002 Nobelist laureate John Fenn. The droplets are created at atmospheric pressure and directed through an inlet into the mass spectrometer. Through a combination of heat and/or gas, the droplets are reduced in size, subsequently building the charge density on the droplet. As the Rayleigh limit is reached – the point at which charge density exceeds droplet surface tension – the droplet fractures or ejects molecular ions via coulombic fission. This process of gradual droplet desolvation within the inlet and later in the source optics of the mass spectrometer yields a solvent-free 'naked' molecular ion. There are two schools of thought on the physical mechanism and the sequence of events leading to the droplet desolvation. The ion evaporation model (IEM)[16] postulates that as a droplet approaches the Rayleigh limit, a desolvated analyte ion gets ejected from the droplet, whereas the charge residue model[17] presumes that the solvated droplet undergoes evaporation and coulombic fission, yielding a naked molecular ion. An important application of ESI is to interface HPLC to mass spectrometers in real time and allow for simultaneous separation and mass spectrometric analysis of complex mixtures. ESI can be used over a range of flow rates from 1 ml/min to 10–20 nl/min, and it can ionize a broad range of molecules from low-molecular-weight organic molecules to very large-molecular-weight protein complexes (50 MDa). The ESI operated at nanoliter per minute flow rates is called nano-ESI.[7,18] Nano-ESI has several advantages over the regular (high-flow) ESI, including lower sample amount requirements, higher sensitivity, and higher tolerance to salts and high ionic strength buffers. Nano-ESI also produces smaller initial droplets that undergo fewer fission events and absorb less energy during droplet desolvation. The nano-ESI conditions are 'gentler' than those of ESI, which allows for analysis of noncovalent protein assemblies and complexes such as the GroEL complex (discussed later).[19] Another property of ESI is the generation of multiply charged ionic species. The number of these unique charge states varies among the molecules and usually scales as a square root of the molecular weight. For example, a 10-kDa protein could hypothetically have up to 100 unique charge states from $+1$ to $+100$. A charge state of $+10$ is recorded in the mass spectrometer as a mass-to-charge ratio (m/z) of 1000 Th. One of the drawbacks of ESI is the 'dilution' effect that multicharging creates, reducing the effective concentration of a particular charge state of the analyte compared to the non-ionized molecule. In practice, however, most analyte ions are distributed among a few predominant charge states instead of the theoretical maximum. Several interesting variations of ESI have been developed. Typically, the analyte molecules are in the liquid stream, but Cooks et al. showed that the ESI plume can entrain molecules off a surface and carry them into the mass spectrometer.[20] The technique is called 'direct electrospray ionization,' and it has been used in a variety of applications including tissue imaging.[21]

A second ionization technique developed for biomolecules is MALDI. Koichi Tanaka received the Nobel Prize in Chemistry in 2002 for his contribution toward the laser desorption ionization technique.[4] This technique uses the energy of a laser to desorb ions from a surface. Analyte molecules are mixed with a 'matrix' that consists of low-molecular-weight organic acid molecules that absorb electromagnetic energy at the wavelength of the laser. The rapid deposition of energy into the matrix causes the matrix to desorb into the gas phase, allowing for ion-molecule reactions between ionized matrix and analyte to result in ionization of the analyte. A variety of laser wavelengths have been used in MALDI, including infrared lasers. Typically, peptides or proteins are mixed with a 1000-fold molar excess solution of matrix on a surface and then allowed to dry. Mass spectrometer ion sources have been designed so that large plates can be used with potentially hundreds of samples placed on a plate for parallel analysis. Matrix-free MALDI techniques such as surface-assisted laser desorption/ionization[22] and desorption/ionization on silicon[23] substitute matrix lattice for porous graphite and silicon, respectively, have higher tolerance toward detergents and salts, and do not suffer from matrix effects. An important development in MALDI ionization is atmospheric pressure MALDI.[24] This interface allows easy interchange between MALDI and ESI sources. The concept of MALDI has led to techniques such as surface-enhanced laser desorption ionization[25] that introduced surface affinity toward various protein and peptide molecules.[11] Because MALDI is used with pulsed lasers, this restricts its use to mass spectrometers that can handle the production of a pulsed packet of ions, such as time-of-flight (TOF) and ion trap (IT) mass spectrometers.

1.17.2.2 Mass Spectrometry Instrumentation

Mass spectrometers have been used in analytical science for many years. J. J. Thomson built a crude mass spectrometer to determine the mass-to-charge value of electrons. He was awarded the 1906 Nobel Prize in Physics for this work. Mass spectrometry instruments have evolved dramatically since the days of Thomson to become essential tools for scientific measurements. A mass spectrometer consists of the three basic components: an ion source, a mass analyzer, and an ion detection system. The components of the mass spectrometer are housed within a vacuum manifold to minimize interferences caused by the analyte ions colliding with air molecules. Typically, there are several compartments of different vacuum levels within the instrument. The source region and the ion guiding optics have higher pressure than the mass analyzer

and the detector components. Vacuum requirements vary depending on the type of mass spectrometer and range from 1×10^{-3} to 1×10^{-10} torr. The ion source is used to convert solids, liquids, or gases into gas-phase ions and transmit the ions into the analyzer. In the case of the ESI ion source, the ionization is performed at atmospheric pressure, and the electrospray plume is directed to a small opening (~ 400 μm in diameter) in the ion source. The charged solvent droplets are further desolvated within the source region (high-pressure region) and transported via a series of electrostatic guiding lenses into lower vacuum regions of the instrument. Determination of the m/z values of ions is accomplished in the mass analyzer. A variety of mass analyzers have been developed (**Table 2**), with each type taking advantage of a different physical property of the ion motion for mass-to-charge-based separation.

Wolfgang Paul, winner of the Nobel Prize in Physics in 1989, has greatly contributed to the development of quadrupole (Q) and IT mass analyzers. Quadrupoles and ion traps are multipole analyzers that consist of four circular parallel rods, a ring electrode, and a trapping electrode (ID). A quadrupole uses a fixed direct current (DC), while the alternating radio frequency (RF) currents are applied to the rods. When the analyte ions are sent into the quadrupole, only the ions with stable trajectories (for a particular DC and RF settings) are able to pass through the field. Ions of different m/z values have different trajectories; thus, the field inside of the quadrupole is used for the m/z-based separation. Ion traps feature additional charged end caps that allow for trapping of the ions along the axis of the ring electrode. Quadrupole and ion trap analyzers are very well suited for biological mass spectrometry because they do not require ultra low vacuum (lower than 1×10^{-6} torr) and therefore can be interfaced with atmospheric pressure ionization techniques such as ESI. One advantage of quadrupoles is a mass range up to 4000 Th, which allows for analysis of peptides and multiply charged small proteins. As such, these mass analyzers are used heavily in bottom-up proteomic applications. At the same time, the fast scan rates of the ion trap mass analyzers allow for seamless interfacing of real-time LC separation with data collection. The two examples of the commercial quadrupole and ion trap instruments are the triple quadrupole mass spectrometer TSQ and the linear ion trap (LIT) known as the LTQ[26] from Thermo Scientific. These analyzers can be used 'stand alone' or as a front end of the hybrid mass spectrometer.

TOF mass analyzers are conceptually the simplest type of analyzers in which ions are separated based on the arrival time at a detector. Lighter ions have higher kinetic energy and higher velocity than the heavier ions, and they arrive earlier to a detector given synchronized pulsed extraction (starting point). Stand-alone TOF analyzers are used with pulsed ionization methods such as MALDI for several reasons. TOF instruments are incompatible with ESI because they require lower vacuum than IT instruments and cannot be interfaced with the high-pressure ESI source. In addition, the ions have to be accumulated and synchronized before entering the flight tube, which favors pulsed ionization methods such as MALDI. Nonetheless, there are hybrid mass spectrometers such as Q-TOF and LTQ-TOF that feature either a quadrupole or an ion trap mass analyzer that precede TOF. This configuration is

Table 2 Common mass spectrometers used in biology and their specifications

Instrument	*Applications*	*Resolution*[a]	*Mass accuracy (ppm)*	*Sensitivity*	*Dynamic range*	*Scan rate*
LIT (LTQ)	Bottom-up protein identification in high-complexity, high-throughput analysis, LC-MS^n capabilities; structural approaches H/D exchange, chemical labeling	2000	100	Femtomole	1e4	Fast
TQ (TSQ)	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2000	100	Attomole	1e6	Moderate
LTQ-Orbitrap	Bottom-up protein identification, quantification, PTM identification; top-down protein identification with ETD, PTM characterization, H/D exchange	100 000	2–5	Femtomole	1e4	Moderate
LTQ-FTICR, Q-FTICR	Protein identification, quantification, PTM identification; top-down protein identification with ECD	500 000	<2	Femtomole	1e4	Slow
Q-TOF, LTQ-TOF	Bottom-up, top-down protein identification, PTM identification, ESI-MS, native MS, ion mobility MS	20 000	5	Attomole	1e6	Moderate/fast
Q-LTQ	Bottom-up peptide and protein quantification; medium complexity samples, peptide and protein quantification (SRM, MRM, precursor, product, neutral fragment monitoring)	2000	100	Attomole	1e6	Moderate/fast

[a] Resolution at full width half maximum.

essential for tandem mass spectrometry analysis. During the process of tandem mass spectrometry, the ions are filtered in the quadrupole, fragmented in a gas-filled collision chamber, and analyzed in the TOF mass analyzer. These instruments are routinely used with ESI because both the front-end quadrupole and the ion trap function at the vacuum level compatible with ESI. These hybrid instruments feature differential vacuum manifolds in which the front-end analyzers operate at higher pressure than the back-end analyzers. The advantage of TOF mass analyzers compared to quadrupoles and ion traps is their high mass accuracy, high resolution, and high, theoretically infinite, mass range. Mass analysis in the TOF can produce resolutions on the order of 40 000 and mass accuracies at the low parts per million level. However, the low scan speed and the stringent calibration requirements are two of the shortcomings of TOF analyzers. As a result of developments in Q-TOFs, ion mobility devices have been added to the hybrid instruments to provide another dimension of ion separation. Ion mobility will separate based on a combination of molecular attributes in addition to size, such as molecular shape. The ions are separated within the ion mobility drift tube according to their size, charge and the overall shape.[27]

A very important class of hybrid instruments, especially for the areas of structural proteomics and top-down analysis, feature either the ion cyclotron resonance (ICR)[28] or the Orbitrap[29] mass analyzers. These mass analyzers use strong magnetic ICR and electrostatic (Orbitrap) fields to trap the ions and record their orbiting motion, which is a function of an ion's m/z value. A pulsed RF is applied to excite the ions of certain m/z values that in turn produce a detectable image current. The image current is Fourier transformed (FT) to obtain component frequencies that correspond to m/z ratios of individual ions. The component frequencies can be calculated with high accuracy, allowing for high mass accuracy determination of m/z values. FTICR and Orbitrap mass analyzers provide the highest resolution and mass accuracies of any analyzers used in biological mass spectrometry. These analyzers are typically interfaced with an LIT as the front-end analyzer. The two common platforms are the LTQ-FTICR and the LTQ-Orbitrap.[29,30] The LTQ mass analyzer is used for ion accumulation, ion storage, and ion fragmentation in tandem mass spectrometry experiments. Both FTICR and Orbitrap analyzers feature high resolution and high mass accuracy in the range of 2 or 3 ppm.[31] Some of the advantages of the Orbitrap analyzer compared to FTICR include higher scan rate, lower cost, and lower maintenance requirements. However, the LIT-FTICR instruments offer higher resolution (**Table 1**) and slightly higher mass accuracy than the LTQ-Orbitrap. Historically, the LIT-FTICR instruments have been used predominantly for top-down structural analysis of proteins, whereas the LTQ-Orbitrap has become a universal solution when high mass accuracy is required in bottom-up experiments. A model of the LTQ-Orbitrap called Velos from Thermo Scientific (**Figure 1**) offers higher sensitivity and improved dynamic range due to the faster scan rates than those of a regular LTQ-Orbitrap instrument. The Velos Orbitrap scans at 33 300 m/z per second in the linear ion trap or, for a mass range of 1000 m/z, 33 scans per second. Such fast scan speeds allow the collection of tandem mass spectra for a large number of peptide ions.

Finally, the magnetic sector mass analyzers (Sector) separate the ions of different m/z values based on the circular motion of the ions within the static magnetic field. These mass analyzers are used with fast atom bombardment or EI ionization techniques, which are not usually compatible with biological molecules.

The final element of the mass spectrometer is the ion detection system. Once ions are separated, they must be detected and quantified. Detectors range from conversion dynode electron multipliers (EM) to multichannel plates (MCP) and Fourier transform methods. An EM is a vacuum tube that transforms a primary emission of an analyte ion striking the EM into a secondary emission of electrons from within the tube. This incidental electron current is proportional to the number of analyte ions reaching EM. EM detectors are usually coupled to quadrupole and ion trap (IT and LIT) mass analyzers. An MCP detector uses similar principles of secondary electron emission and is often used with TOF analyzers. The FT detection methods are used with high mass accuracy analyzers such as the ICR and the Orbitrap. The principle behind

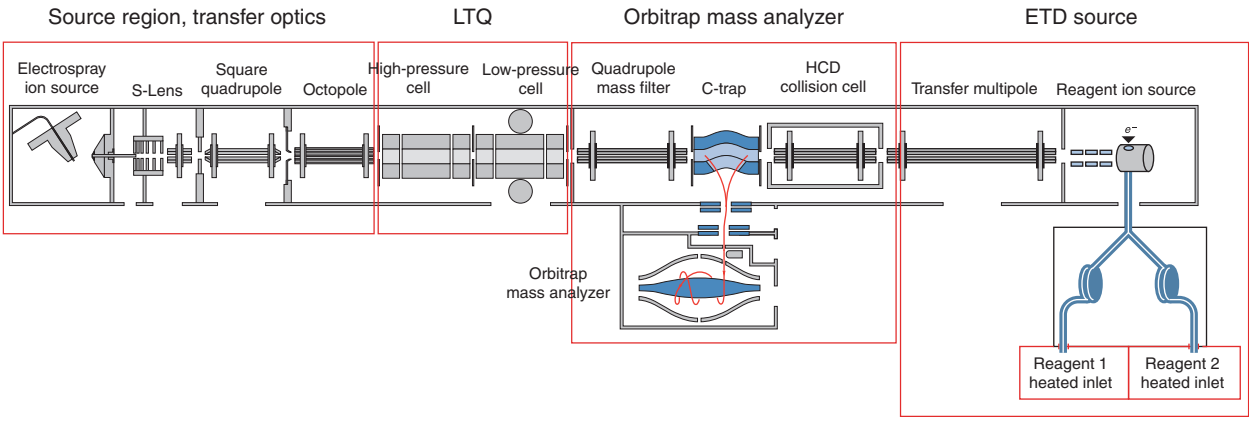

Figure 1 LTQ-Orbitrap Velos ETD (Thermo Scientific) is a hybrid instrument that combines the high mass accuracy of Orbitrap and the high throughput of the LTQ Velos. Highlighted are the source region, the LTQ mass analyzer, the Orbitrap mass analyzer, and the ETD source. Reprinted with permission from Hu, Q.; Noll, R. J.; Li, H.; Makarov, A.; Hardman, M.; Graham Cooks, R. The Orbitrap: A new mass spectrometer. *J. Mass Spectrom.* **2005**, *40*(4), 430–443. Copyright by Wiley.

the FT detection is to convert (transform) the unique oscillation orbits of the ions into their corresponding m/z values.

As can be seen from the previous examples, the mass spectrometer is not a single entity but, rather, a collection of components that perform ion storage, mass analysis, and ion detection. Thus the capability and the application of a mass spectrometer are often determined by a combination of these different components. However, it is the mass analyzer (mass accuracy and resolution) that ultimately shapes the instrument's performance.

1.17.2.3 Resolution Versus Mass Accuracy

Mass spectrometers are used to measure the molecular weight of molecules, often in an attempt to identify or characterize a molecule's structure. The more accurate the m/z measurement, the more useful it is for molecular structure determination[31]; thus, mass accuracy is very important. Mass accuracy is generally expressed in parts per million, which is calculated as (observed MW – exact MW)/exact MW $\times 10^6$, where MW is molecular weight. Measurements with the mass accuracy below 5 ppm are considered outstanding, and this level of measurement for peptides can significantly reduce the number of alternative peptide identifications.[31] Another important parameter is the mass resolution, which is defined as $M/\Delta M$. When ΔM of the ion peak is measured at 50% of the peak height, the resolution is defined as the full width half maximum (FWHM) resolution. Whereas mass accuracy is the measure of closeness of the experimental m/z value to the theoretical value, resolution reflects the number of distinct chemical species that can be detected within an arbitrary window of m/z. The higher the resolution, the narrower the peaks and the greater the distance between peaks, allowing for detection of a larger number of different peaks. These concepts are illustrated in **Figure 2**, which demonstrates the difference between a mass accuracy of 100 ppm and a mass accuracy of 2–5 ppm for the same analyte ion. Therefore, high resolution is a prerequisite for high mass accuracy measurements, and mass spectrometers that can measure m/z value with high accuracy and high resolution are useful to distinguish different types of modifications (e.g., phosphorylation PO_4 ($+79.966$ Th) vs. *O*-sulfonation SO_4 ($+79.956$ Th)) as well as provide more confidence in amino acid sequence, post-translational modification assignment, and quantitative information.

1.17.3 Functional Proteomics

1.17.3.1 Sample Preparation and Chromatographic Separation

The strength of a tandem mass spectrometer lies in the ability to analyze complex mixtures of molecules. This powerful capability has been harnessed for studies of proteins and has been a key technology in proteomics.[32] Protein mixtures that may represent protein complexes, organelles, subcellular compartments, whole cells, or even tissues have been analyzed using tandem mass spectrometry in a process referred to as shotgun proteomics. Thus, sample preparation and chromatographic separation techniques play an important role in the overall analysis. As mentioned previously, top-down analysis requires high concentrations of low-complexity pure samples of target proteins. Most top-down studies are performed without chromatographic separation. In bottom-up analysis, however, obtaining complete protein digestion is often difficult, so combinations of detergents and different proteases have been used to increase protein solubilization and digestion efficiency. Even more problematic is the analysis of membrane proteins because they are insoluble in aqueous buffers.[33] A variety of methods have been developed to digest membrane proteins and analyze the transmembrane domains of the protein.[34]

Chromatographic separation is a very important step within the overall process of biological mass spectrometry. As previously mentioned, bottom-up experiments are usually performed using highly complex samples, such as a total cellular protein fraction or an isolated organelle. When proteolytically digested, the complexity of the mixture increases further because each protein yields many proteolytic peptides. Chromatography is therefore a necessary step during a bottom-up proteomic analysis. Electrospray ionization provides an interface to the mass spectrometer for chromatographic separations and thus a variety of separation methods have

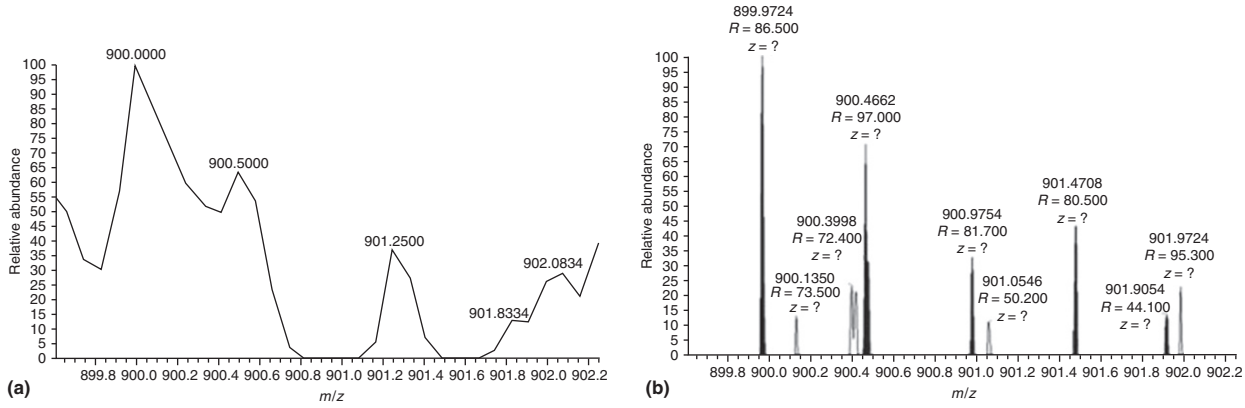

Figure 2 Resolution and mass accuracy of the low mass accuracy (100 ppm) LTQ mass analyzer (a) and the high mass accuracy (2–5 ppm) Orbitrap mass analyzer (b).

been developed. To achieve the best sensitivity and the lowest limits of detection, microscale capillary chromatography is interfaced with a nano-ESI source in line with the mass spectrometer. Capillary chromatography features columns with interior diameters less than 100 μm. Reverse phase material (RPLC or RP) is almost exclusively used to separate peptides based on their size and hydrophobicity, and a significant advantage of RPLC is that the buffers used are compatible with ESI.[35] Given the high resolution, efficiency, reproducibility, and mobile phase compatibility with ESI, the analytical RPLC is used as the single phase (the only column for separation) or as the last dimension of multidimensional separation before mass analysis.[11]

However, in general, single phase chromatography has limited peak capacity and resolution for a high-complexity sample such as proteolytic digests of whole cell protein. High-complexity large-scale proteomic samples contain thousands of proteins that can range upward of five orders of magnitude in their abundance, with the complexity of the shotgun proteomic samples being even higher after each proteolytically digested protein yields multiple peptide products.[36] One of the ways to address limited peak capacity is to use long RP columns (100 cm) with small particle size (<2 μm) operated under high-pressure conditions with ultra-high-pressure liquid chromatography (UPLC) pumps. Several research groups have reported a peak capacity of more than 1500 (theoretical number of unique peptides resolved) per single phase run.[37,38] An additional method by which to increase the resolution and address limited peak capacity is to integrate RPLC as part of a multidimensional separation approach. The multidimensional separation approach combines several separation techniques coupled to improve the resolving power. Orthogonality of the individual separation methods[39–41] is required for multidimensional separation in which each dimension uses different (orthogonal) molecular properties of molecules as a basis for separation. Such methods include isoelectric focusing (IF) or ion exchange chromatography at the front end followed by a reversed phase analytical chromatography. The front-end strong cation exchange chromatography followed by RPLC has become a popular method in shotgun proteomics known as multidimensional protein identification technology.[6,12,36]

1.17.3.2 Data Analysis

Given the potential to collect very large numbers of tandem mass spectra in bottom-up experiments, high-throughput methods for data analysis are needed. Large-scale sequencing of genomes has provided a sequence infrastructure that aids in the interpretation of tandem mass spectra of peptides.[32,42–44] Because a tandem mass spectrum represents the amino acid sequence of a peptide, predicted peptide sequences from a database can be compared to the spectrum and evaluated with a variety of mathematical techniques. The method used by the SEQUEST algorithm creates a theoretical fragmentation pattern for each database sequence and compares it to the experimental spectrum using a cross-correlation analysis. The highest scoring match is frequently the correct sequence. Many search engines are built on statistical frameworks that also take into account the in silico database composition to generate the likelihood of the correct match. These algorithms have enabled large-scale identification of proteins as well as post-translational modifications such as phosphorylation.[45,46]

1.17.3.3 Quantification Analysis

Measuring changes in biological systems requires the use of quantitative methods. Mass spectrometry is considered a quantitative method because the relative quantities of different ions may be measured by the detector in addition to the masses and identities of the peptide and protein samples. However, because the process of ionization is dependent on the physico-chemical properties of molecules as well as the matrix, large variability does occur during the creation of ions. From the onset of cell lysis, the original level of a protein is perturbed by digestion, fractionation, ionization efficiency, the overall ion population (signal suppression), and detection within the mass spectrometer. As such, the protein quantification approaches include relative quantification (label-free and chemical labeling) and absolute quantification. The relative approaches rely on a comparative measurement of the protein abundance under several conditions without reference to an external standard, whereas the absolute quantification methods require addition of an external standard. Because the detailed coverage of protein quantification techniques is beyond of the scope of this chapter, the reader should consult review articles in this field.[47–50] Some of the more common quantification methods, such as the use of stable isotopes to create labeled peptides used for comparisons, are discussed next.[51]

Stable isotopes are used to create internal standards because they introduce the mass shifts between the light and the heavy labeled ions that can be simultaneously measured in the mass spectrometer. Stable isotope labels can be added through metabolic labeling or through covalent labeling. Metabolic labeling is achieved by replacing amino acids in growth media with stable isotope-labeled versions of either all amino acids or specific amino acids.[52] In microorganisms, metabolic labeling can be achieved by simply adding salts of ^{15}N, and the organisms use the ^{15}N to make new amino acids and proteins. Heavy isotopes have been incorporated into a wide variety of organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, mice, and rats. The main challenge to labeling any organism is obtaining or creating a labeled food source.[53,54] Frequently, stable isotopes are incorporated into a control state, and then proteins from the control state are mixed with the experimental state (perturbed or activated in some manner). Changes in specific peptides can then be measured in the mass spectrometer. A second strategy to introduce labels is through covalent labeling with a molecule that contains stable isotope labels. As with the isotope-coded affinity tags, the label can be introduced at the protein level through cysteine amino acids, or tags can be introduced at the peptide level as done with the isobaric tag for relative and absolute quantitation and tandem mass tags reagents.[55–57] Both of these reagents label the N termini of peptides, and they do not reveal their mass differences until fragmentation in collisionally activated dissociation (CAD). The previously

discussed methods are used for relative quantification, in which changes are determined relative to a control state. Specific software tools are required to analyze large-scale data sets for quantification because peptide identifications must be combined with peak heights for the isotopomers to calculate abundance changes.

To determine absolute changes, measurements must be made against a known quantity of the internal standard or, more accurately, a standard curve should be created with the internal standard.[58] When measurements are made on many molecules in the same analysis, the assumption is that the standard curve is linear and measurements are made in reference to a single value.[59] These types of measurements are often performed using a triple quadrupole mass spectrometer in a single reaction ion monitoring mode (SRM). This method selects a precursor ion in the first quadrupole, which is fragmented in the collision cell (second quadrupole), and only the intensities of select fragment ions are measured in the third quadrupole, as opposed to scanning the entire mass range. The benefits of this approach are excellent limits of detection (low femtomolar analyte amount), high precision due to the two-stage filtering, and the increased linear dynamic range of 1×10^6.

1.17.4 Structural Proteomics

Some of the general strategies in functional and structural MS-based proteomics are listed in **Table 2**. As mentioned previously, functional proteomics usually requires the bottom-up approach of shotgun proteomics,[6,13,36] in which proteins are proteolytically digested to yield peptides prior to analysis. Peptides are much more amenable to chromatographic separation than are proteins due to the narrower distribution of their masses, charges, and hydrophobicities, thus allowing for analysis of complex biological samples. Peptides are also more compatible with the process of ESI than are proteins. Peptides lack the sophisticated tertiary structure of proteins and present fewer potential basic side chains (proton acceptors), thus reducing the number of differentially charged species. However, because most of the information about the protein is lost after proteolytic digestion, structural proteomics is almost always (with the exception of chemical labeling experiments) carried out as a top-down approach in which intact proteins or even protein complexes are subject to mass analysis. Next, the top-down strategy is described, and specific approaches in the structural characterization of proteins are discussed.

1.17.4.1 Top-Down Mass Spectrometry

Top-down mass spectrometry involves analysis of intact proteins without prior proteolytic digestion.[28,60,61] Mass analysis of intact proteins offers the following advantages over the bottom-up approach: (1) Abundance of discrete protein forms can be measured directly,[62] and (2) the top-down method offers higher protein sequence coverage, leading to identification of multiple isoforms, splice variants, and point mutations.[63,64] Additional advantages over the bottom-up approach include improved protein quantification derived directly from the protein spectra instead of being reconstructed from peptide data[65–67] and more comprehensive characterization of post-translation modifications (PTMs).[68] Comparisons between the top-down and the bottom-up methods are highlighted in **Table 1**.

Several technological challenges have impacted the overall development of the top-down method compared to the bottom-up approach. As mentioned previously, MS-based analysis consists of the following steps: front-end fractionation, sample ionization, data acquisition, and data analysis. Front-end fractionation of intact proteins does not offer the resolution and the sensitivity of proteolytic digest fractionation. Whereas the gel-free fractionation using either a single phase RPLC[69] or multidimensional orthogonal separation[6,12,36,70] of complex samples is mainstream in bottom-up proteomics, there is not a dominant chromatographic platform for intact protein separation. Due to their large size and tertiary structure, proteins as a rule do not separate as well as peptides by HPLC. Therefore, a two-dimensional (2-D) gel-based separation is often used for large-scale top-down analysis. Gel-based separation, however, does not interface with mass spectrometry in real time and presents a bottleneck for a large-scale analysis because the gel regions of interest or individual gel spots usually have to be cut out and digested prior to chromatographic separation and mass analysis, Both MALDI and ESI are used for ionization in top-down proteomics, but both methods are less efficient when applied to larger molecules. MALDI ionization of intact proteins suffers from low sensitivity due to so-called 'matrix effects,'[23] whereas the top-down ESI suffers from lower ion transmission efficiency compared to ionization of peptides. Another restrictive requirement in top-down analysis is the use of high mass accuracy instruments. Top-down experiments are usually performed with FTICR instruments,[28] Q-TOF instruments,[71,72] and the LTQ-Orbitrap hybrid instruments.[73,74] Unfortunately, high mass accuracy instruments are expensive to purchase and to maintain (Q-TOF and FTICR) compared to Q and IT instruments commonly used in bottom-up proteomics. See **Table 2** for a comparative chart of the instruments commonly used in structural mass spectrometry.

Data acquisition modes are also different for bottom-up and top-down approaches. As mentioned previously, tandem mass spectrometry consists of two types of scans – the full MS scan and the fragmentation MS/MS scan. Protein or peptide sequence information is derived from fragmentation MS/MS scans. A classical method of collision-induced dissociation (CID) or CAD has traditionally been used in MS/MS. CID fragmentation involves colliding target ions with helium or argon gas. The process transfers kinetic energy of the collision into an internal energy and increases the intramolecular oscillation energy of an ion, which results in cleavage of amide bonds to yield *b*- and *y*-type ions (**Figure 3(a)**). Although CID is an excellent method for peptide fragmentation,[75,76] it provides limited sequence coverage and loss of labile and biologically important PTMs when applied to larger peptides and proteins. As a result, 'soft fragmentation' techniques of electron capture dissociation (ECD)[77] and electron transfer dissociation (ETD)[78,79] were developed for use with top-down mass spectrometry. ECD and ETD yield a more complete fragmentation pattern than CID, which aids fragment identification.

Finally, in terms of data analysis, either a database search[80] or a *de novo* method[70] is used in the bottom-up and top-down

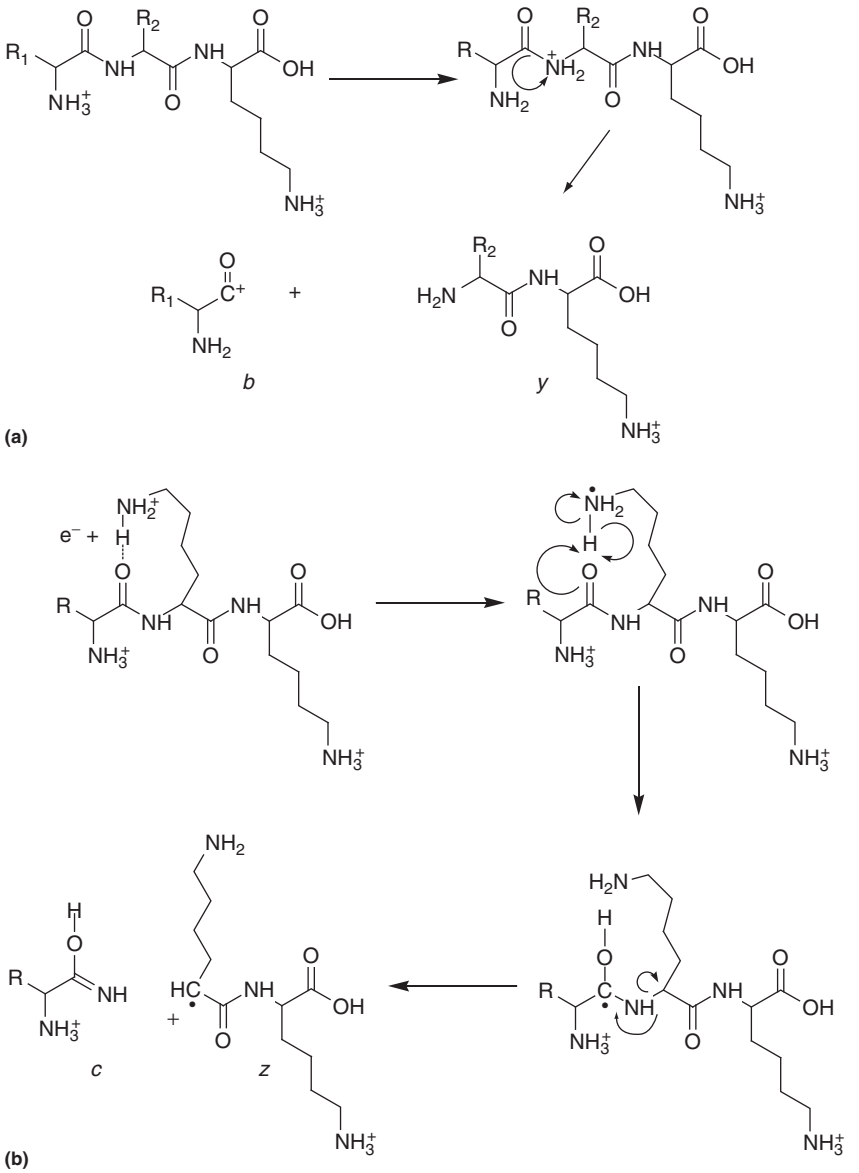

Figure 3 Peptide fragmentation products using CID (a) and ETD (b) fragmentation. CID fragmentation produces *b* and *y* ion series, whereas the ETD fragmentation produces *c* and *z* ion series. Reprinted with permission from Syka, J. E.; Marto, J. A.; Bai, D. L.; Horning, S.; Senko, M. W.; Schwartz, J. C.; Ueberheide, B.; Garcia, B.; Busby, S.; Muratore, T.; Shabanowitz, J.; Hunt, D. F. Novel linear quadrupole ion trap/FT mass spectrometer: performance characterization and use in the comparative analysis of histone H3 post-translational modifications. *J. Proteome Res.* **2004**, *3*(3), 621–626. Copyright by American Chemical Society.

approaches. Given the limitations of the top-down method, including front-end separation, high sample quantity demand, and poor ionization efficiency compared to that of the bottom-up approach, most top-down applications are targeted studies of purified proteins. However, several studies have shown that large-scale profiling of intact proteins is achievable. Neil Kelleher and co-workers, for example, carried out a large-scale top-down MS analysis (without MS/MS fragmentation) and quantification in yeast.[65] They also conducted a series of large-scale tandem analyses (with MS/MS fragmentation of intact proteins) during which they identified and quantified respectively 22 and 221 proteins using 1-D and 2-D chromatography. Don Hunt's group has also been successful in implementing ETD fragmentation to carry out large-scale top-down analyses.[81–83] Regarding the target protein size limitations, the top-down approach has been successfully applied to characterize proteins less than 100 kDa because larger proteins do not transmit well inside the mass spectrometer. However, Fred McLafferty's laboratory carried out a tandem top-down characterization of a 229-kDa protein.[84]

1.17.5 Characterization of Post-Translational Modifications

In biological systems, signaling proteins are considered the cellular information carriers. They shuttle information vital for cell motility, division, gene expression programs in various

cellular compartments, and membrane permeability, along with a myriad of other cellular processes. Much of this information in signaling proteins is carried in the form of PTMs. Among the most studied PTMs are phosphorylation, acetylation, methylation, carboxylation, and myristoylation. Knowledge of PTMs not only provides further structural information about the protein but also helps us to understand the larger picture of cellular regulation. This section discusses the MS-based top-down approach applied to the characterization of PTMs and the soft fragmentation techniques of ECD and ETD that have made this approach feasible.

1.17.5.1 Soft Fragmentation with ECD and ETD

ECD and ETD are related techniques for generating ions while maintaining PTMs. ECD was originally developed to interface with FTICR mass spectrometers,[77] and ETD was later optimized as a modified version of ECD in order make the electron transfer reaction compatible with IT instruments.[79] The main difference between ECD and ETD is the source of electrons. The source for ECD is a heated filament source, whereas ETD employs negatively charged polyaromatic hydrocarbons such as fluoranthene as electron carriers. Whereas CID dissociation is based on the energy generated via collision, electron dissociation (ED) proceeds via electron capture by the protonated target ion. The reaction results in backbone cleavage along the N-Cα bonds and yields the c and z ion series (**Figure 3(b)**). The ED reaction starts with the electron capture by amide carbonyl group. When the carbonyl group is hydrogen-bonded to a protonated downstream basic side chain (lysine, arginine, or histidine), electron capture yields an amino-ketal radical site. The unstable radical site directs dissociation of the N-Cα bond, which results in c^+ c-type ions and $z^{+\bullet}$ z-type odd electron ions. This process is influenced by the location and distribution of proton donors, which are the positively charged basic amino acid side chains. Large-scale proteomic studies have shown that ED methods (ETD in this case) perform better than CID when applied to longer peptides at higher charge states.[85,86] ED methods belong to the category of soft or 'cold' ionization because unlike the CID fragmentation in which increased internal energy of the ions leads to dissociation, ED fragmentation is based on the formation of unstable radical ions. For a detailed review of ED fragmentation mechanisms, see Wiesner et al.[87]

Given the improved performance of ETD/ECD compared to CID in top-down and PTM analysis, it is not surprising that ED methods gained popularity for structural proteomics applications. Some of the first top-down studies involving ECD fragmentation[88] and its application to PTM characterization were performed by Fred McLafferty's research group, which localized a labile and otherwise undetectable modification carboxylation without the loss of CO_2.[68] Since then, ECD fragmentation has been applied to the characterization of glycosylation,[89] phosphorylation,[89] oxidation,[90] as well as multiple and rare PTMs on several *Mycobacterium tuberculosis* secreted proteins.[91] A top-down ECD method was also applied to the study of protein folding kinetics in which ubiquitin[92] or cytochrome *c*[93] were unfolded via infrared photoexcitation and studied via ECD for fragments held together by noncovalent interactions. Top-down ECD was also employed to study DTT reductive unfolding of ribonuclease homologs[94] as well as to characterize multiple protein forms.[95] In addition, top-down ECD is routinely used to investigate conformation of protein ions in gas phase.[92,96,97] Despite the advantages of ECD for 'cold fragmentation' over CID, it is not a mainstream technique because it requires expensive FTICR instrumentation.

As mentioned previously, ETD-capable instruments are much more widely available than the ECD fragmentation instruments interfaced to FTICR mass spectrometers. The ETD source can be coupled to either a linear trap such as the LTQ-ETD or a hybrid instrument such as LTQ-Orbitrap-ETD. One of the first top-down analyses using online separation followed by the ETD fragmentation was performed by Don Hunt and coworkers, who identified a co-eluting isoform of chicken histone H2A.Z (originally thought to be a PTM)[78] via tandem mass spectrometry (**Figure 4**). Currently, histones are of particular interest in cellular biology due to a relationship between the methylation of histones and the so-called 'histone code.' Multiple patterns of methylation of histone proteins are assumed to dictate epigenetic regulation of chromatin and gene transcription. A study carried out on an LTQ-Orbitrap-ETD instrument describes top-down characterization of four core histones (H2A, H2B, H3, and H4) and their methylation.[98] **Figure 5(a)** shows the annotated ETD fragment ion spectrum of intact H4 histone charge +18. ETD fragmentation results in a high coverage of the protein, enabling the authors to determine a methionine cleavage at the N terminus and two PTMs – acetylation and dimethylation (**Figure 5(c)**). One of the major advantages of ECD/ETD methods compared to CID is a near-complete fragmentation coverage of large peptides and small proteins, as evident from **Figure 5(c)**. Extensive work of histone characterization has also been carried out by Neil Kelleher's group.[66,99,100]

1.17.6 Chemical Labeling Approaches

Within the scope of structural proteomics, this chapter has covered the top-down mass spectrometry and the application of mass spectrometry to the characterization of PTMs of proteins. The identity and location of PTMs provide the functional information such as signaling cascades and place the studied protein in the context of the overall cellular organization. However, the nature and location of PTMs provide limited information about the overall protein structure, its subunit organization, folding, and dynamics. A collection of MS-based approaches called chemical labeling aims to probe protein structure and dynamics. Chemical labeling involves the introduction of a non-native moiety into a protein either by covalently labeling certain amino acids or by creating the 'reporter groups' from the existing amino acids by placing the protein into a different chemical environment. Mass spectrometry is then used to find the location of these altered groups within the overall sequence and gain structural insight with regard to the protein conformation in solution.

Traditionally, many different non-mass spectrometry techniques have been used to study structural properties of proteins. These studies include mass spectroscopic methods such

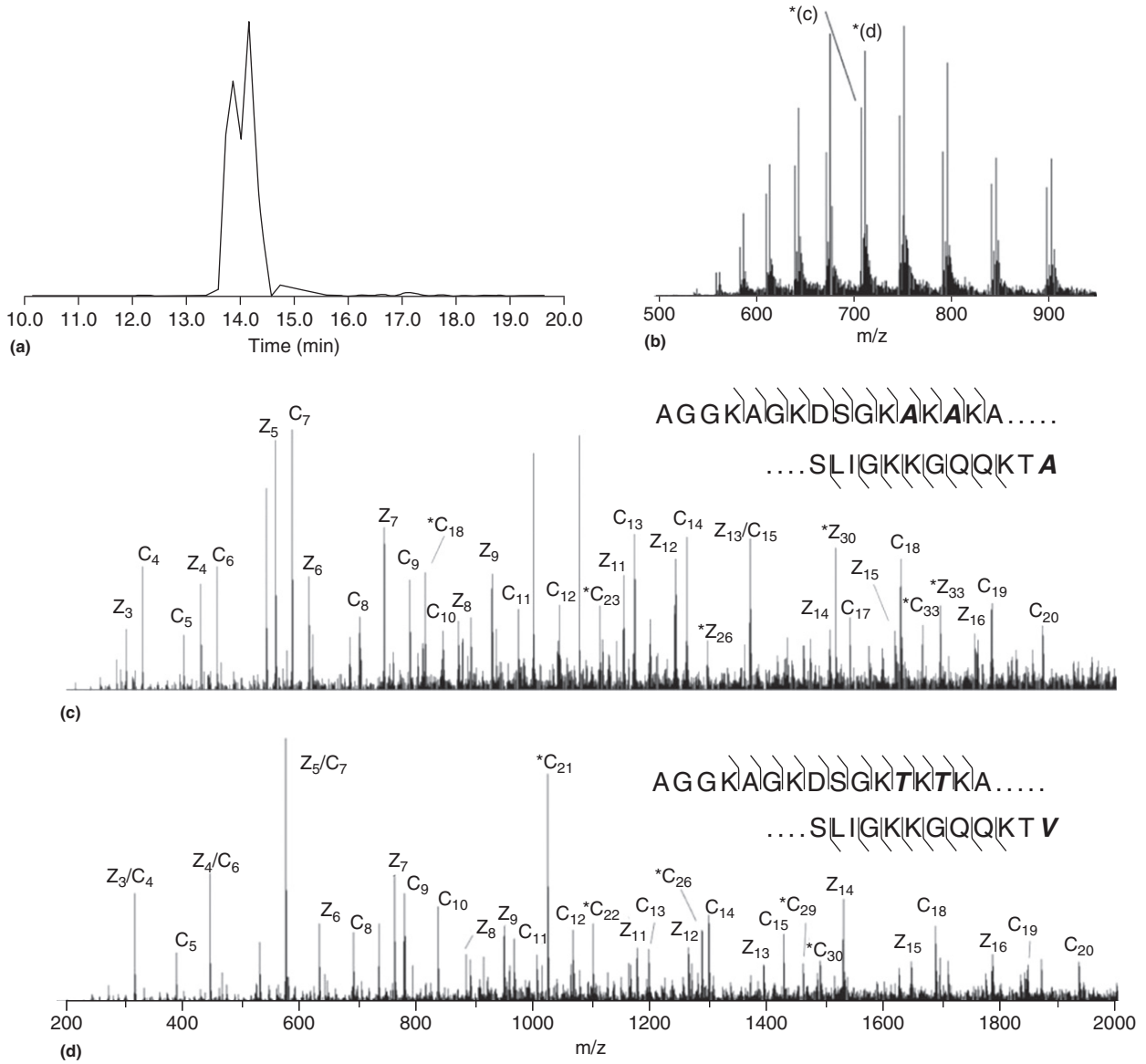

Figure 4 Analysis of histone H2A.Z isoforms by online chromatography and electron transfer dissociation reactions. (a) Chromatogram of the protein separation. (b) The protein isotope envelopes of two coeluting isoforms, the corresponding *m/z* values (c) and (d), were selected for fragmentation. (c) The resulting tandem mass spectrum after dissociation of the (c) species, unmodified histone H2A.Z. (d) The resulting tandem mass spectrum after dissociation of the (d) species. Comparison of the *c*- and *z*-type fragment series indicates the presence of a new H2A.Z isoform with four amino acid substitutions. Reprinted with permission from Coon, J. J.; Ueberheide, B.; Syka, J. E.; Dryhurst, D. D.; Ausio, J.; Shabanowitz, J.; Hunt, D. F. Protein identification using sequential ion/ion reactions and tandem mass spectrometry. *Proc. Natl. Acad. Sci. USA* **2005**, *102*(27), 9463–9468. Copyright by Proceedings of the National Academy of Sciences.

as fluorescence, UV-VIS, and infrared spectroscopy and circular dichroism. Conformational changes upon ligand binding or folding/unfolding events due to solvent perturbations can also be measured with calorimetric methods such as differential scanning calorimetry. Finally, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy provide the highest level of resolution and highest detail of protein structure. Both methods are the pillars of protein structural studies. However, X-ray crystallography provides limited information regarding protein dynamics and is also limited to probing protein conformations that exist in crystal lattices and not in solution. NMR methods require large sample quantities and present challenges when studying proteins larger than 50 kDa.[101] One could question the applicability of mass spectrometry for structural studies because the method measures *m/z* ratios of protein and peptide ions in gas phase. These conditions are quite removed from the native environment of biomolecules. However, the *m/z* values of protein and protein segments recorded in the mass spectrometer report the chemical environment they were exposed to when in solution. Thus, structural mass spectrometry, especially labeling mass spectrometry, is similar to developing a frame of film that has

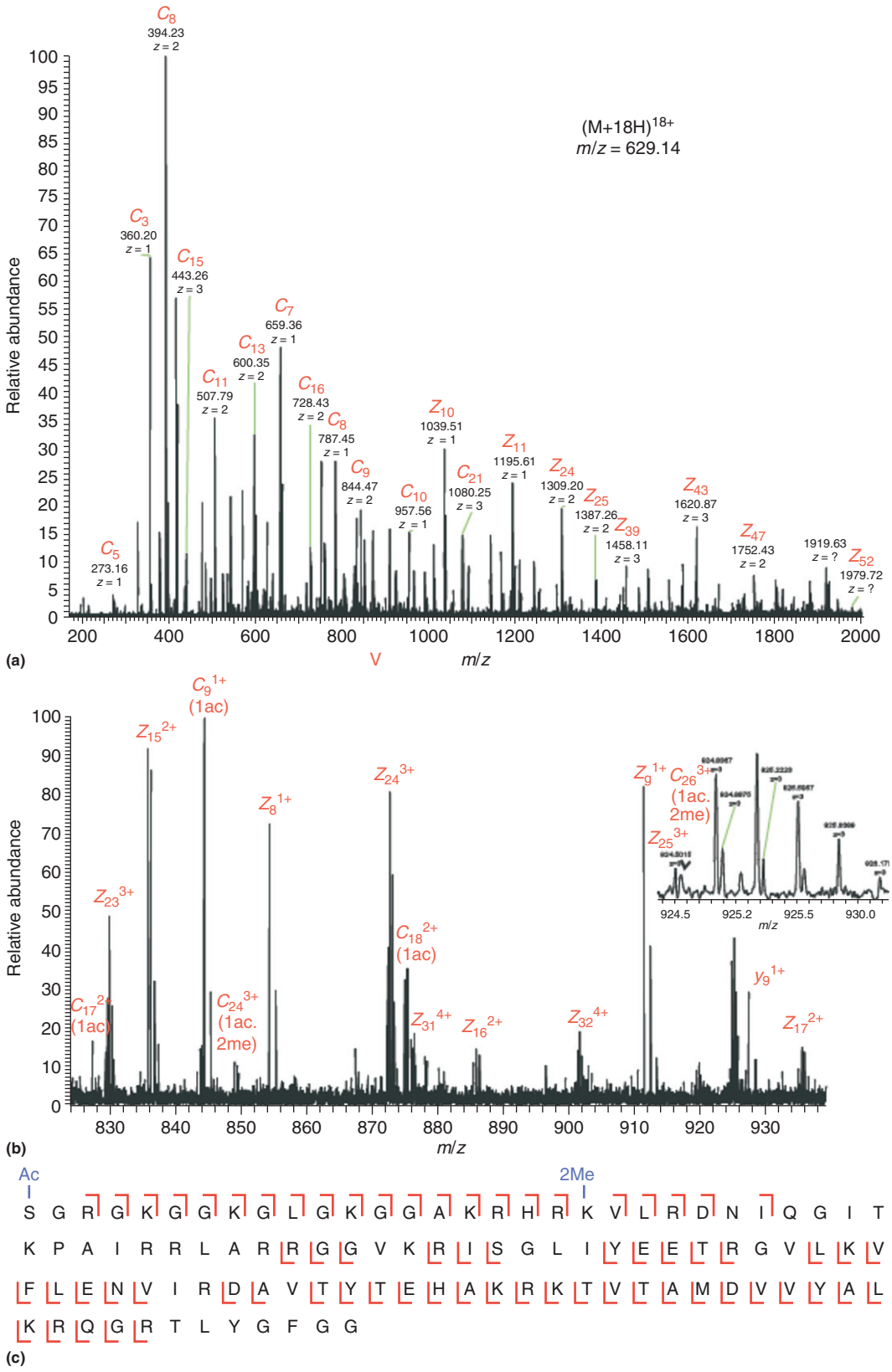

Figure 5 Online LC-MS/MS analysis of intact histone H4 using ETD. (a) ETD fragmentation spectrum of the +18 charge state with the most abundant fragment ions labeled. (b) Labeled ETD fragments in the *m/z* range of 820–940. (c) Fragment ion assignment and localization of the acetylation and dimethylation PTMs. Reprinted with permission from Eliuk, S. M.; Maltby, D.; Panning, B.; Burlingame, A. L. High resolution electron transfer dissociation (ETD) studies of unfractionated intact histones from murine embryonic stem cells using online capillary LC separation: Determination of abundant histone isoforms and post-translational modifications. *Mol. Cell. Proteomics* **2010**, *9*, 824–837. Copyright by American Society for Biochemistry and Molecular Biology.

recorded ('exposed') the native environment and its perturbation prior to being 'developed' via mass spectrometry. Protein chemical labeling methods have been developed to study protein structure, conformational changes, and dynamics by using covalent modifications. Some of the chemical labeling methods include hydrogen/deuterium exchange, covalent labeling, and oxidative labeling of proteins in solution. Next, these methods are described and compared, and specific applications are discussed.

1.17.6.1 Hydrogen/Deuterium Exchange

Hydrogen/deuterium exchange (HX, H/D, or HDX) methods study the chemical exchange reaction of the main chain amide hydrogens. The amide hydrogens are uniformly distributed along the protein main chain because every natural amino acid (with the exception of proline) features a primary amine at its N terminus. Thus, in a repeating segment of the protein main chain (…$C_\alpha HR_1$-C(O)-N**H**-$C_\alpha HR_2$…), the amide hydrogen (depicted in bold) is labile and subject to exchange for another hydrogen or a heavier hydrogen isotope, such as deuterium or tritium. Solvent-exposed amide hydrogens participate in hydrogen bonds (H bonds) that stabilize protein secondary and tertiary structure and are in turn protected by this extensive network of H bonds. The individual hydrogen/deuterium exchange rates vary greatly depending on the amino acid residue, its local environment (surrounding amino acids), its global location within the protein, and its solvent accessibility. The origin of HX dates back to the 1950s and is linked to Linus Pauling's discovery of α helices and β sheets, the elements of protein secondary structure. Kai U. Linderström-Lang and co-workers started probing proteins for their secondary structure by studying hydrogen/tritium exchange of insulin,[102] myoglobin,[103] and the pH dependence of deuterium exchange.[104] The proteins were exchanged into D_2O for a specific amount of time and transferred into the H_2O-based solvent for measurement. The measurements were carried out via a density gradient column[102,105] or via scintillation counting (tritium exchange).[106]

Amide hydrogen exchange is both acid (H_3O^+) and base (OH^-) catalyzed; therefore, the overall rate of hydrogen exchange is the sum of both:

$$k_{ch} = k_{int,A}[H_3O^+] + k_{int,B}[OH^-] \quad [1]$$

where k_{ch} is the unprotected HX rate, and $k_{int,A}$ and $k_{int,B}$ are intrinsic second-order reaction constants for the acid (A) and base (B) catalyzed reaction.[105] Unprotected HX rate is the exchange rate at the residue that is located at the surface of the protein and is fully accessible to solvent molecules. Many of the HX parameters, such as HX rates of individual amino acids, their local environment, pH, and temperature, have been calibrated and tabulated by Walter Englander and colleagues,[107,108] and the minimum HX rates for unstructured polypeptides were found to be between pH 2 and pH 3 at approximately 0 °C. The physical model of hydrogen exchange assumes that several specific events occur in order for the exchange to take place. First, transient separation of the H-bond donor and acceptor, which may be due to the dynamic breathing of the protein, is a conformational change within the protein upon denaturation or an environment change caused by the variation in solvent conditions. Once the separation occurs, the amide hydrogens are solvent accessible and susceptible to exchange for the deuterium. Although all hydrogens exchange relatively slowly due to hydrogen bonding, protected hydrogens at the core of the protein exchange at much slower rates than the hydrogens at the surface. Depending on the level of protection, some hydrogens must first be brought into contact with the solvent, which may require conformational changes and accounts for the slower exchange rates.[109,110] The severity of these conformational changes can range from the 'molecular breathing' to partial or complete unfolding of the protein for the buried core residues. Thus, the exchange rates of amide hydrogens provide information regarding the dynamics of the protein in solution. **Figure 6** shows a schematic in which a small region of a protein undergoes unfolding upon addition of a denaturant. The unfolding and refolding rate constants are k_1 and k_{-1}, respectively, whereas the hydrogen/deuterium exchange rate from the unfolded segment is k_2. Depending on the values of the rate constants, there are two mechanisms for the exchange in the unfolded region, EX1 and EX2. When the refolding rate

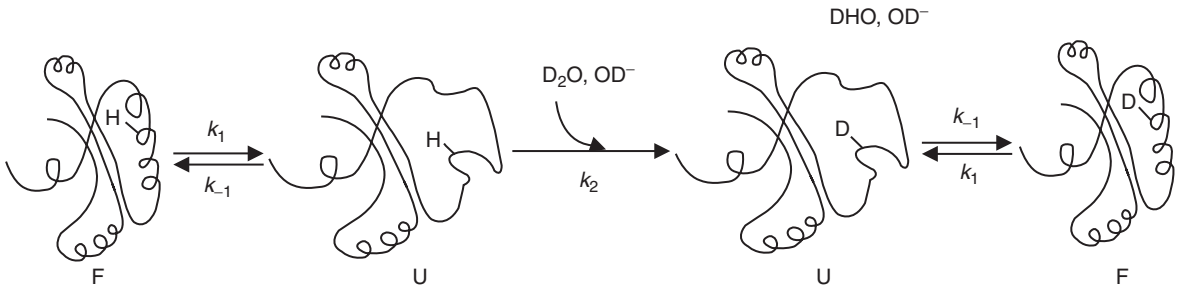

Figure 6 Pictorial representation of a folded (F) protein unfolding in a small region to facilitate partial or complete isotope exchange. The kinetics of the unfolding and refolding processes are described by rate constants k_1 and k_{-1}, respectively, and exchange from the unfolded polypeptide is described by k_2. Reprinted with permission from Deng, Y.; Zhang, Z.; Smith, D. L. Comparison of continuous and pulsed labeling amide hydrogen exchange/mass spectrometry for studies of protein dynamics. *J. Am. Soc. Mass Spectrom.* **1999**, *10*(8), 675–684.

k_{-1} is much higher than the exchange rate k_2 ($k_{-1} \gg k_2$), the unfolding has to happen many times for the exchange to take place. The process is referred to as bimolecular exchange (EX2) and the overall exchange rate is controlled by the folding events. This mechanism yields several partially exchanged intermediates as the reaction progresses (Figure 7). Another extreme case is when the exchange rate is much higher than the refolding rate ($k_2 \gg k_{-1}$), leading to a unimolecular exchange EX1. The EX1 mechanism is in place when only two discrete populations (undeuterated and deuterated) are present during the exchange (Figure 7).

As mentioned previously, most HX experiments consist roughly of the following steps: (1) Protein is exchanged into a buffer containing D_2O; (2) protein conformation is altered via addition of a denaturant, a cofactor, or a binding partner for a period of time to allow for complete (continuous exchange) or partial exchange (pulse exchange) of the exposed sites; (3) the exchange reaction is stopped by placing the solution at acidic pH in an ice bath; (4) intact protein or chromatographically separated proteolytic fragments are analyzed by mass spectrometry; and (5) exchange sites are determined from the peptides that exhibit deuterium mass shifts.

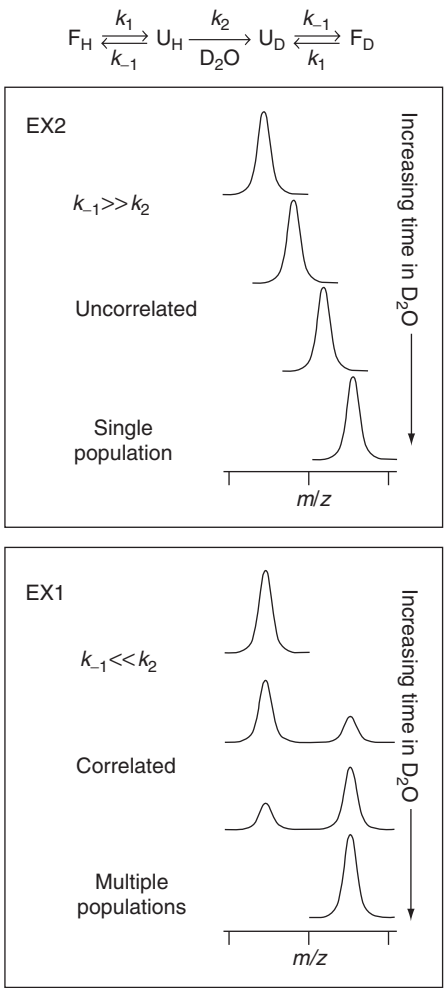

Figure 7 Protein unfolding and deuterium labeling in hydrogen exchange mass spectrometry, where the two kinetic extremes, EX1 and EX2, are depicted. Reprinted with permission from Weis, D. D.; Wales, T. E.; Engen, J. R.; Hotchko, M.; Ten Eyck, L. F. Identification and characterization of EX1 kinetics in H/D exchange mass spectrometry by peak width analysis. *J. Am. Soc. Mass Spectrom.* **2006**, *17*(11), 1498–1509.

There are two main strategies in terms of hydrogen exchange methodology, referred to as a continuous and a pulsed HX.[111] Figure 8 outlines these two strategies. In the process of continuous labeling, protein is exchanged into a D_2O-based solvent (15-fold dilution yields >95% deuterium concentration).[112] A denaturant or a cofactor is added, shifting the equilibrium toward the perturbed or unfolded state and exposing the previously protected residues to the solvent. The deuteration reaction is unidirectional because the concentration of deuterium in solution is much higher than that of hydrogen. Either the reaction is allowed to proceed until all of the recently unprotected protons have exchanged or discrete time points are selected for analysis to study the dynamics of the folding/unfolding events. Following exchange, the reaction is quenched by lowering the pH to 2.5 and lowering the temperature to 0 °C. The next step is the analysis of either the intact protein (global analysis) or proteolytically digested peptides (local analysis). The global HX analysis is usually carried out via the previously discussed top-down mass spectrometry, whereas the local HX requires protein digestion post labeling and the bottom-up approach.

1.17.6.1.1 Continuous deuterium exchange

The goal of the global analysis is to determine the total deuterium content of the protein as a result of the structural perturbations.[113] The mass spectra of the native protein are compared to the spectra of the labeled protein. Either ESI LC-MS or MALDI-MS may be used for analysis, but the ESI approach is more popular than MALDI for several reasons. During the ESI LC-MS analysis, labeled protein is transferred onto a trapping column, which serves to concentrate the protein for mass analysis and to 'wash off' the nonspecific labile deuterons at amino acid side chains and nonspecific amide deuterons (chromatography is carried out in 100% H_2O). However, although the so-called back-exchange (post-labeling deuterium to hydrogen exchange) at side chain positions is advantageous, the reaction also occurs at the informative backbone amide sites, albeit at a much slower rate. The back-exchange starts at the onset of the analysis, which has to be carried out as quickly as possible at 0 °C. An example of the information learned from global HX experiments is illustrated in Figure 9(a), in which the total deuterium level is plotted against the endpoint of the labeling reaction.[114] One can learn the total number of rapidly exchanging amide hydrogens (region a), the number of the denaturation-related exchanges due to the conformational change (region b), and the number of the exchange sites that do not exchange given the perturbation (region c). Figure 9(b) shows the HX results (using a tritium gel-filtration analysis) for the two forms of hemoglobin, oxy and deoxy.[115] The exchange-out of the deoxy form is slower than that of the oxy hemoglobin. However, addition of O_2 to the deoxy hemoglobin triggers the conformational change in the allosterically sensitive region. As a result, some of the previously protected side chains become exposed to the solvent and the

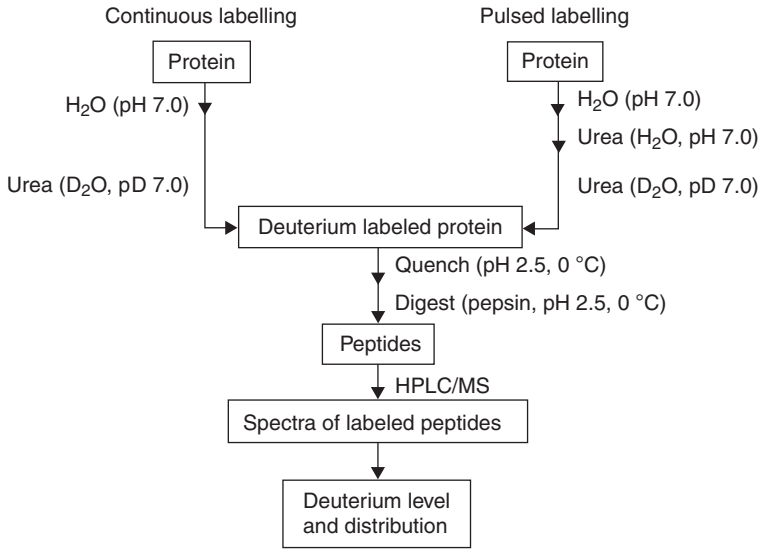

Figure 8 Schematic representation of the continuous and the pulsed deuterium exchange labeling. Reprinted with permission from Deng, Y.; Zhang, Z.; Smith, D. L. Comparison of continuous and pulsed labeling amide hydrogen exchange/mass spectrometry for studies of protein dynamics. *J. Am. Soc. Mass Spectrom.* **1999**, *10*(8), 675–684.

overall hydrogen exchange rate increases, as observed by the merge of the two curves past O_2 exposure.

The local analysis or bottom-up approach was first introduced by David Smith's research group[116] and involves proteolytic digestion of the labeled proteins prior to mass analysis. The local analysis, also called functional analysis, is able to reveal the exact position of the exchanged residues, potentially providing the structural details at the amino acid level. The most common way to carry out the local analysis is by passing the reaction mixture through a column-immobilized acid protease stable at low pH, such as pepsin, via HPLC prior to chromatographic separation and analysis. Pepsin is a nonspecific protease that cleaves at hydrophobic residues; however, the cleavage specificity is reportedly reproducible under identical conditions.[114] Often, multiple proteases that generate multiple overlapping peptides are used during digestion, which increases further label localization.[117] Improved label localization with overlapping peptides results in a higher protein coverage, a larger number of identified deuterium incorporation, and a higher confidence of these incorporations.

Following pepsin cleavage, the peptides are separated via reversed phase chromatography interfaced online with the ESI mass spectrometer. Chromatography plays a much more important role in local HX compared to the global approach because a single protein species generates a complex mixture of peptides that require separation prior to mass analysis. Given the strict time constraints of the analysis to minimize the deuterium back-exchange, UPLC has been gaining popularity because it allows for faster analysis.[118] Whereas the amount of heavy label in individual peptides is estimated from either the full MS or the MS/MS fragmentation scans, peptides are usually identified via fragmentation MS/MS scans similar to a regular bottom-up approach. The mass difference between the unlabeled and the labeled peptides is calculated as the difference between their centroided masses, often with the help from specialized software packages.[119,120] An example of the level of structural information that can be learned from the local analysis is illustrated in **Figure 10**. The method was applied to characterize the wild type and the mutants of membrane protein bacteriorhodopsin.[121] The mutants T90A and D115A eliminate hydrogen bonding and were shown to decrease the overall stability of the protein. Bacteriorhodopsin is a membrane protein, and the regions that flank the mutation sites are believed to be buried in the detergent micelle instead of being solvent exposed as in wild type. Therefore, these regions in the mutant forms are expected to exhibit slower HX rates than in the wild type. **Figure 10(a)** shows how the mutations affect folding properties of protein segments in the vicinity of mutation. It is evident from **Figure 10(b)** that a number of exchanged protons decrease in both T90A and D115A mutants in the three regions that flank the mutations. This point is also illustrated in terms of the calculated exchange rate in **Figure 10(c)**. In case of bacteriorhodopsin, the affected H bonds were found to be energetically weaker than expected.

1.17.6.1.2 Pulsed deuterium exchange

Pulsed HX exchange methods differ from continuous exchange methods in the timing and duration of the labeling steps (**Figure 8**). A population of molecules is induced to undergo conformational changes in H_2O-based solvent, after which the reaction is exposed (pulsed) to deuterium for a brief period of time. Therefore, only the molecules that were unfolded or in any way affected by the denaturant undergo the exchange. Whereas continuous labeling is best suited for monitoring slow unfolding transitions, pulsed experiments are geared toward studying folding reactions, kinetic intermediates, and kinetic mechanisms. Pulsed HX experiments often require that the exchange reaction be carried out using stop-flow and quench-flow equipment traditionally involved in studying fast non-steady-state kinetics.[122] An example of

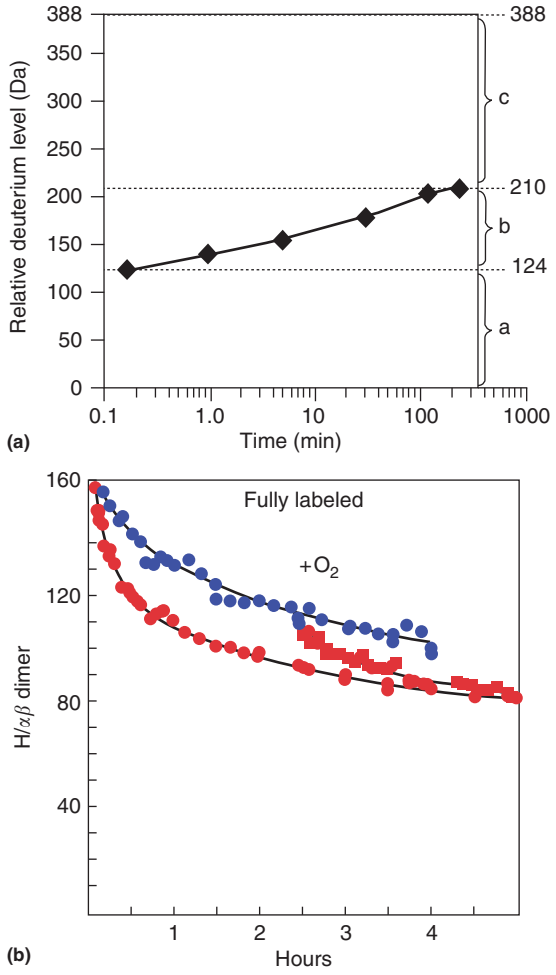

Figure 9 (a) Example data for deuterium exchange into an intact protein. The maximum of the *y* axis is equal to the maximum number of exchangeable backbone amide hydrogens. Region a represents those amide hydrogens that exchanged by the first time point. In region b, the amide hydrogens that become unprotected become deuterated. Region c contains those amide hydrogens that did not exchange even after the longest exchange time point. Reprinted with permission from Morgan, C. R.; Engen, J. R. Investigating solution-phase protein structure and dynamics by hydrogen exchange mass spectrometry. *Curr. Protoc. Protein Sci.* **2009**, Chapter 17, Unit 17.6.1–17. Copyright by Wiley. (b) HD results for hemoglobin. Whole Hb initially labeled to exchange equilibrium in tritiated water, then exchanged out in oxy (red) and deoxy (blue) forms, measured by the early tritium gel filtration method. Some allosterically sensitive sites are faster in oxy-Hb. When O_2 is added to exchanging deoxy-Hb, the sensitive sites are made faster and the separate curves merge. Reprinted with permission from Englander, S. W. Hydrogen exchange and mass spectrometry: A historical perspective. *J. Am. Soc. Mass Spectrom.* **2006**, *17*(11), 1481–1489.

pulsed experiments is illustrated in **Figure 11**, in which a fast time series of folding intermediates of cytochrome *c* is characterized by pulsed HX and ESI mass spectrometry. After unfolding in the presence of a denaturant (**Figure 11(a)**), the protein was pulsed into a deuterated solvent and then allowed to refold for 5, 31, and 506 ms (**Figure 11(b)–11(d)**) under nondenaturing conditions. The unfolded state of cytochrome *c* (**Figure 11(a)**) has a lower *m*/*z* than the refolded state (**Figure 11(e)**) because the unfolded protein usually has a more extended conformation, higher surface-accessible area, and a larger number of potential deuterium label targets. Once en route to refolding, some of the previously exposed internal residues become protected once again and trap the label. Thus, the refolded protein would accumulate the heavy label that cannot be readily back-exchanged. It is apparent that the exchange reaction proceeds via the previously discussed EX1 mechanism (the rate of exchange is much greater than the refolding rate). The dashed lines in **Figure 11(b)–11(d)** represent the contribution of the two states (unfolded and refolded) toward the observed signal. By deconvoluting the observed mixed population envelope, one could estimate the refolding rate of cytochrome *c*. In addition, the research group of Lars Konermann has carried out several pulse labeled HX studies, including a characterization of transient myoglobin folding intermediates.[123]

A wide range of instruments are used for HX experiments, including ion traps, triple quadrupole analyzers, TOF instruments, and the high mass accuracy LTQ-FTICR and LTQ-Orbitrap instruments. Top-down global HX analysis usually requires high mass accuracy instruments (TOF, FTICR, and Orbitrap) and availability of either ECD or ETD fragmentation methods geared toward intact protein fragmentation. An FTICR instrument was used in a top-down global HX study of a 17-kDa protein,[124] a Q-TOF instrument was used in a global study of the phosphorylation-induced conformation change of the response regulator vancomycin resistance-associated regulator,[125] and an LTQ-Orbitrap instrument was used to characterize a human apolipoprotein.[126] A novel HX method of gas-phase deuterium labeling[127] involves mobility separation of ions within the mass spectrometer using an ion mobility/TOF instrument.[128] The bottom-up local analysis, on the other hand, does not require a high mass accuracy instrument. The authors of a study describing local HX with ETD fragmentation used a 3-D ion trap instrument.[129] In addition, the analysis of the bacteriorhodopsin mutants mentioned previously also involved the use of a 3-D ion trap instrument.[121]

Overall, hydrogen exchange is the most versatile chemical labeling method in structural MS-based proteomics. It is used to study protein behavior under various conditions and at various timescales. Hydrogen exchange reaction is the least disruptive to the native structure of the protein molecule compared to other methods of chemical labeling, such as oxidation. However, one disadvantage of HX is the labile nature of the deuterium label. Thus, the postlabeling analysis has to be carried out quickly and under conditions that diminish the rates of back-exchange. Another phenomenon, called label scrambling, hinders the interpretation of results from HX experiments. In addition to back-exchange, the substituted amide deuterons scramble during the ESI ionization, during ion transmission in the gas phase, and during CID fragmentation.[130–132] One of the solutions to label scrambling during fragmentation is to use soft fragmentation techniques such as ETD.[129] The increased deuterium scrambling due to the back-exchange and label scrambling within the MALDI matrix is one of the reasons why MALDI is not as suitable for HX as ESI.[133,134] Another reason MALDI is not as popular for

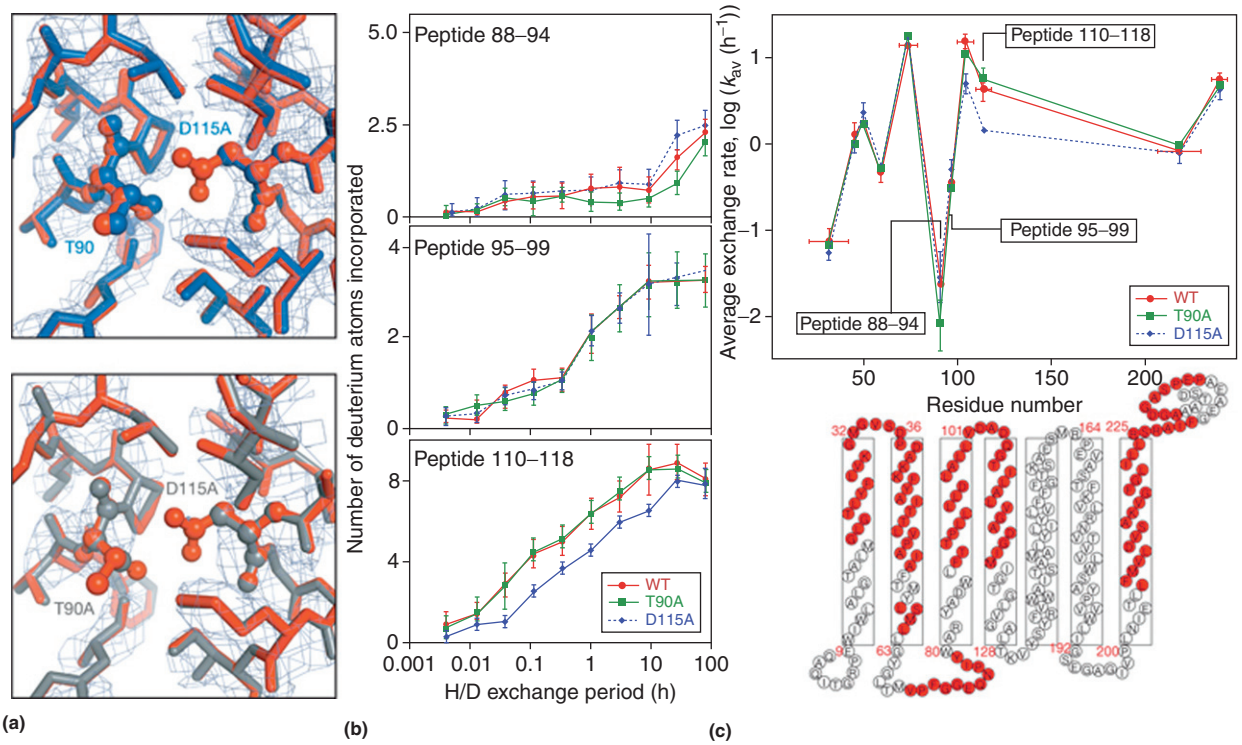

Figure 10 (a) Electron density maps and overlay of refined mutant and wild-type structures for D115A (top) and T90A/D115A (bottom) mutants. (b) Plot of the number of hydrogens exchanged in the denatured state against time for peptides overlapping the T90A mutation (top), a region between T90A and D115A mutation (middle), and the D115A mutation (bottom). (c) A plot of average exchange rates for peptides throughout the protein (top) and a schematic illustration of the bacteriorhodopsin structure (bottom) showing the sequences covered by the deuterium exchange experiment in light red. Reprinted with permission from Joh, N. H.; Min, A.; Faham, S.; Whitelegge, J. P.; Yang, D.; Woods, V. L.; Bowie, J. U. Modest stabilization by most hydrogen-bonded side-chain interactions in membrane proteins. *Nature* **2008**, *453*(7199), 1266–1270. Copyright by Nature.

HX is the fact that it cannot be interfaced online with HPLC. Chromatographic separation is often a necessary step that reduces complexity of the spectra because of the co-ionizing peptide species and washes away the side chain deuterium label.[116] However, a study using a MALDI in-source decay (higher energy laser ionization to allow for nonspecific fragmentation in the source region of the mass spectrometer) provides the conditions to minimize deuterium scrambling.[135]

Despite the phenomena of back-exchange and label scrambling, hydrogen exchange is a popular tool used in many applications of structural proteomics. It has been used in studies involving protein dynamics and conformational change,[136–138] protein-protein complexes and functional interactions,[139,140] protein-cofactor conformational changes,[141] and protein folding.[124] There are many excellent reviews on this topic.[105,112,114,142,143]

1.17.6.2 Oxidative Labeling

Oxidative labeling is another application of covalent labeling approaches to structural studies of proteins. The main advantage of oxidative labeling over the previously described hydrogen exchange is that oxidative labeling is not subject to back-exchange, so the oxidation-related modifications are stable and remain on the affected residues throughout the analysis. However, one drawback is that labeling is restricted to a few reactive residues, and if the residues are not present in the region of the protein studied, the chemical labeling method is uninformative.[144] Another difference between HX and oxidative labeling is that in the case of chemical labeling, the dose-response curve has to be generated to determine the extent of the modification as the function of the reagent concentration.

Oxidative labeling targets redox-sensitive residues such as tryptophan, tyrosine, phenylalanine, and histidine and sulfur-containing residues such as methionine and cysteine.[145] Here, the focus is on oxidative labeling using reactive oxygen species, particularly hydroxyl radical-mediated labeling. It has been known for some time that hydroxyl radicals modify side chains of proteins in addition to generating the backbone cleavage events,[146] in which the sulfur-containing and aromatic residues are the preferred targets of modification. Mark Chance and co-workers are credited with the development of hydroxyl radical labeling mass spectrometry analysis[147] using either radiolysis or Fenton reagents to generate the hydroxyl radical species. **Figure 12** shows the general flowchart of a hydroxyl radical labeling experiment. In this illustration, the reactive radicals are generated by radiolysis, followed by oxidative labeling of the native protein and of the protein-ligand complex. The protein is digested, and the identity of oxidation sites is determined by tandem mass spectrometry (**Figure 12**, bottom right).[144] At the same time, the dose-response curve

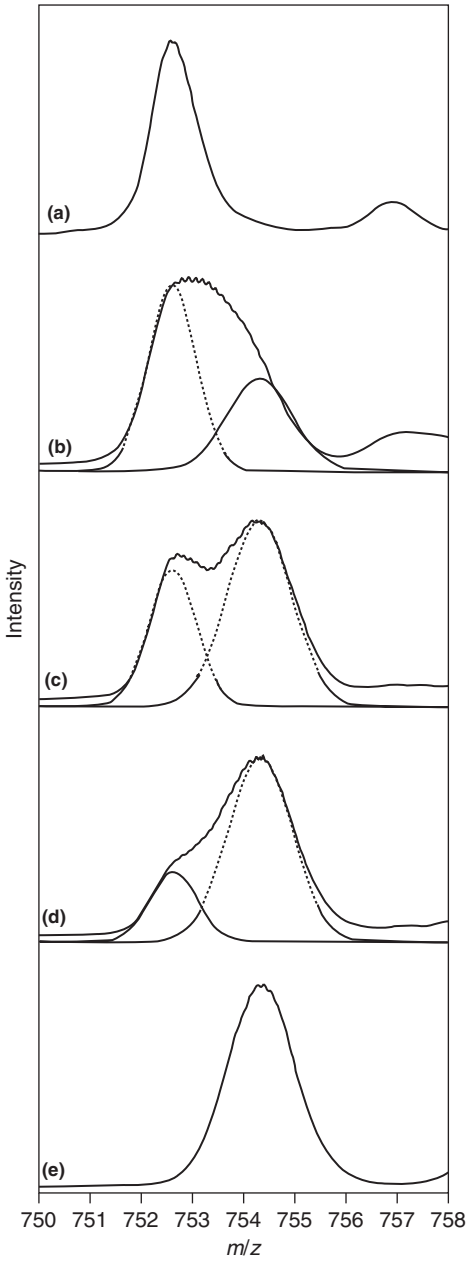

Figure 11 ESI mass spectra of the N-terminal segment of cytochrome *c*, including amide linkages 121, following folding for 5, 31, and 506 ms are presented in panels (b)–(d). The areas of the low- and high-mass envelopes of isotope peaks, indicated by dashed lines, represent the fraction of the cytochrome *c* population in which the N terminus was unfolded or folded, respectively. Panels (a) and (e) indicate isotope patterns of this segment representing unfolded and folded cytochrome *c*, respectively. Reprinted with permission from Yang, H.; Smith, D. L. Kinetics of cytochrome *c* folding examined by hydrogen exchange and mass spectrometry. *Biochemistry* **1997**, *36*(48), 14992–14999. Copyright by American Chemical Society.

(Figure 12, bottom left) is generated using the full-scan MS data. In this case, the protein-ligand complex provides a higher degree of protection to the complex interface surface and therefore exhibits a lower amount of added label (Figure 12).

There are several methods for generating the hydroxyl radicals ($^{\bullet}$OH): electron pulse radiolysis, synchrotron radiolysis of water (X-ray photoelectric effect),[148] laser photolysis of H_2O_2,[149] Fenton reactions,[150] tethered metal chelates,[151] and high-voltage electric discharge such as ESI ion source.[152] Reactive hydroxyl radicals attack saturated, unsaturated, and aromatic carbons of organic molecules. In the case of saturated carbon, the hydroxyl radical abstracts hydrogen, and in the case of unsaturated carbon, a hydroxyl addition to the electron-rich system takes place.[144] The ensuing rearrangements depend on the structure of the target molecule. Although used for side chain labeling, hydroxyl radicals may also cleave the protein main chain via a direct attack at the α-carbon of amino acid residues. Hydroxyl radicals may also trigger rearrangements following attachment onto a proline, glutamate (γ-carbon side chain radical transfer), valine, leucine, or aspartate (β-carbon side chain radical transfer). However, despite the unwanted cleavage or rearrangement, hydroxyl radical labeling is used for side chain labeling because the backbone cleavage rates are one to three orders of magnitude slower than the side chain oxidation rates (1×10^{7} to 1×10^{10}). For detailed molecular mechanisms of hydroxyl radical generation and substrate labeling, see a review article by Xu and Chance.[144] As mentioned previously, the approximate order of amino acid side chain reactivity toward $^{\bullet}$OH is Met and Cys, followed by the aromatic side chains.[153] The second-order rate constant k_i for the reaction with $^{\bullet}$OH can be expressed using the following equation:

$$k_i = \alpha_i \times k_i^{\text{int}} \qquad [2]$$

where k_i^{int} is the intrinsic rate constant of fully unprotected side chain (tabulated for each amino acid[144]), and α_i is the dimensionless parameter ($0<\alpha_i<1$) that reflects the protein conformation.[101]

Similar to hydrogen-deuterium exchange, oxidative labeling generates spatial oxidation patterns consistent with the solvent exposure of reactive residues. The exposure principles for oxidative labeling are the same as those that govern HX methods: Compared to protected residues, the unprotected residues accumulate the label at faster rates because of their exposure to the solvent. Thus, quantification of the oxidative changes serves as the means of characterizing protein structure. As mentioned previously, general considerations used in oxidative labeling are similar to those for hydrogen exchange experiments; however, the critical stages of the experiment are different between the two approaches. Because hydrogen exchange is considered a noninvasive method, no major structural changes are attributed to the presence of the label. However, because of the labile nature of the label and ensuing back-exchange and deuterium scrambling phenomena, the analysis is confined to a strict set of rules (pH, temperature, protease, duration, ionization, etc.). Oxidative labeling methods, on the other hand, benefit from a wealth of preparative, chromatographic, and analytical methods used in protein mass spectrometry. Because the oxidative label is permanent, a wide range of temperatures, digestion conditions, chromatographic conditions, and mass spectrometry techniques are applicable to the analysis. One of the potential

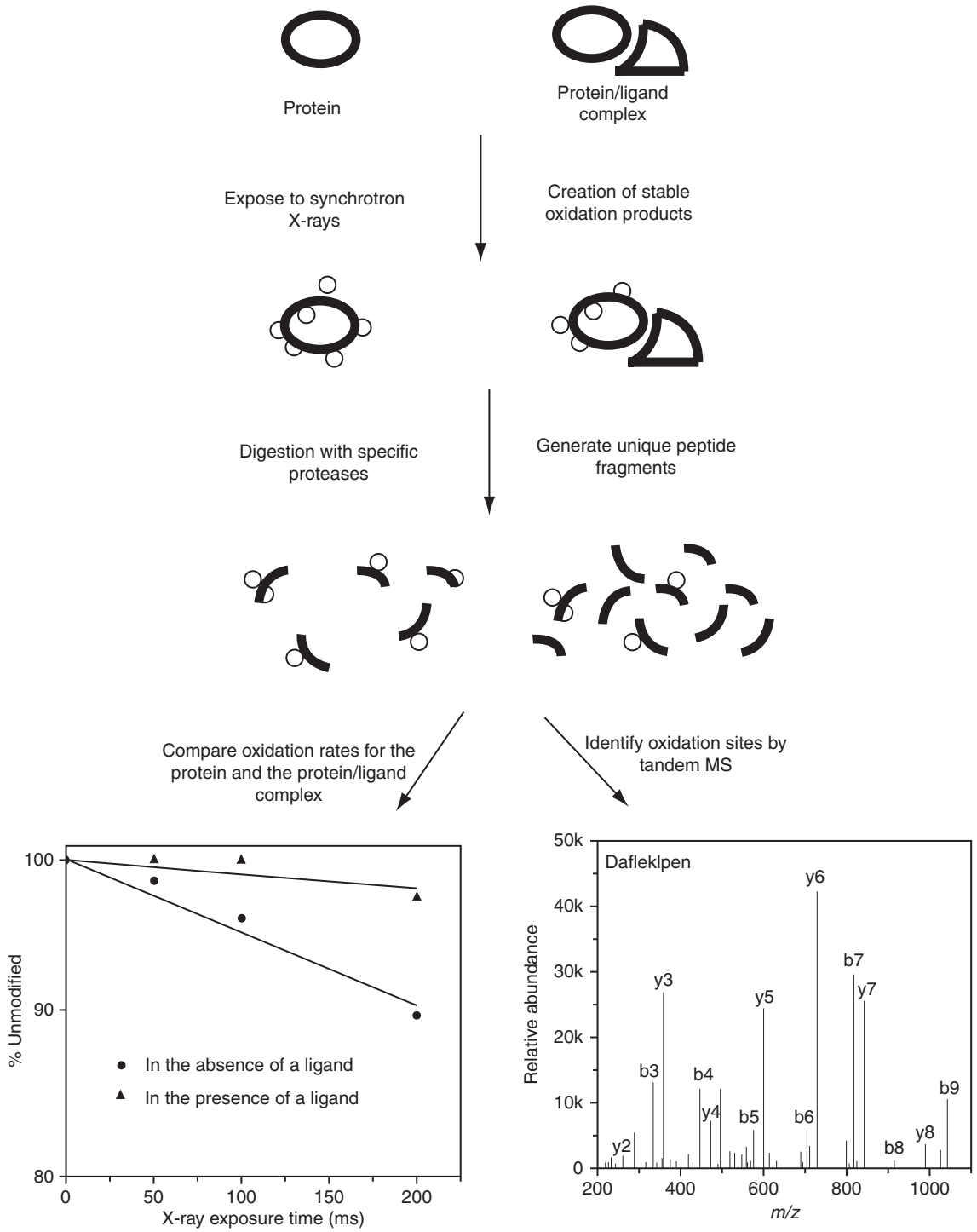

Figure 12 Schematic representation of hydroxyl radical footprinting. Reprinted with permission from Xu, G.; Chance, M. R. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. *Chem. Rev.* **2007**, *107*(8), 3514–3543. Copyright by American Chemical Society.

pitfalls of oxidative labeling compared to hydrogen exchange is the labeling step. Covalent modifications including oxidative labeling are known to induce structural changes.[154,155] Lars Konermann and co-workers performed a study using holomyoglobin that showed the time-dependent nature of the structural changes due to oxidative labeling (**Figure 13**).[156] In **Figure 13**, the panel on the left shows a time series of oxidative labeling and the extent of label incorporation using ESI LC/MS of intact holomyoglobin at pH 7. As the exposure to hydroxyl radicals increases from 0 to 320 s, both the average mass (center of the mass distribution) and the distribution of the masses (width of the envelope) of the protein increase. The

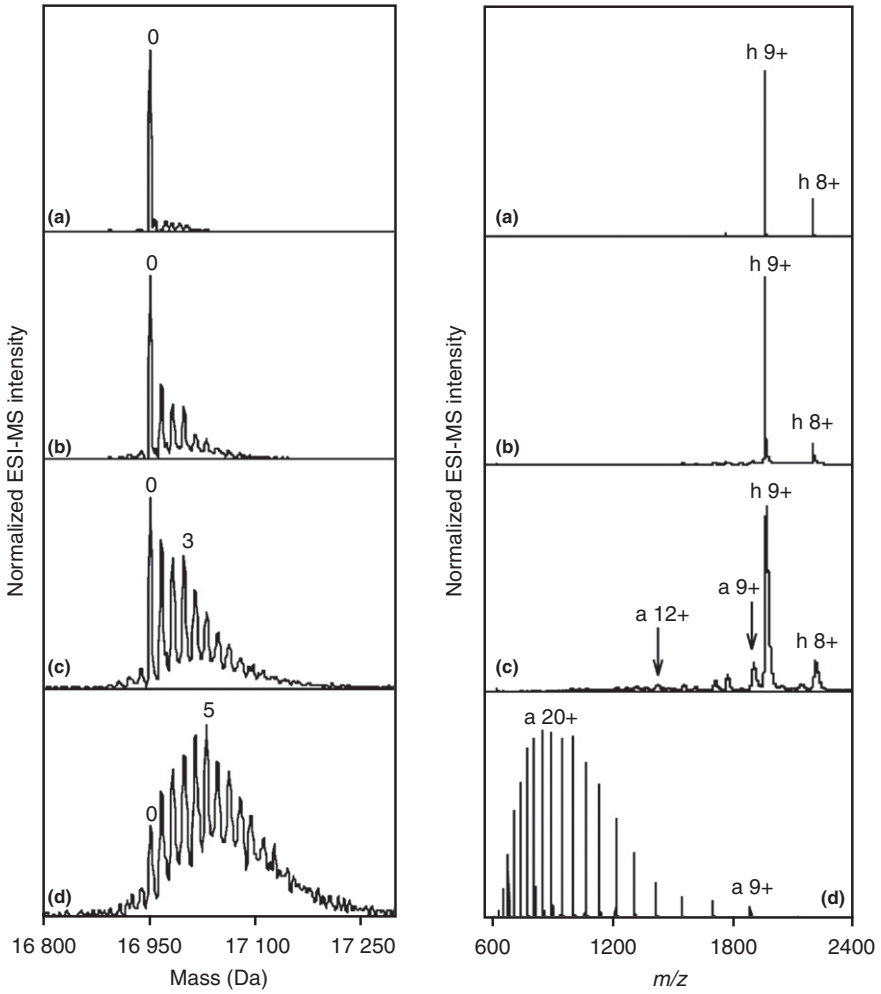

Figure 13 (Right) Deconvoluted mass spectra obtained after γ-ray exposure of 22 μM holomyoglobin at pH 7.0 for (a) 0 s (control), (b) 80 s, (c) 160 s, and (d) 320 s. (Left) ESI mass spectra of 22 μM myoglobin recorded by direct infusion under native solvent conditions (pH 7). Prior to mass analysis, the protein had been exposed to γ-rays for (a) 0 s, (b) 80 s, and (c) 320 s. For comparison, panel d shows the spectrum of acid-denatured myoglobin at pH 2 in dilute formic acid (without γ-irradiation). a, apomyoglobin; h, holomyoglobin. Also indicated are the charge states of selected ions. Reprinted with permission from Tong, X.; Wren, J. C.; Konermann, L. Effects of protein concentration on the extent of gamma-ray-mediated oxidative labeling studied by electrospray mass spectrometry. *Anal. Chem.* **2007**, *79*(16), 6376–6382. Copyright by American Chemical Society.

shift of the average envelope mass is attributed to the increase in the overall number of individual labeled side chains over time. The panel on the right shows structural variants of holomyoglobin from the control to the 320-s labeling and the acid-denatured protein at pH 2 for the purpose of comparison. Both spectra a and b (right) show no significant structural changes between the unlabeled protein and the protein labeled for 80 s. Even after 320 s, the extensive labeling yields predominantly folded $+9$ charge state of the protein with few structurally different forms (aMb $+12$ and aMb $+9$). Comparatively, panel d on the right shows a spectrum of a fully denatured protein. Therefore, based on the panel on the right in **Figure 13**, it is evident that structural changes do occur during the course of oxidative labeling; however, large-scale structural changes due to labeling are relatively minor, as previously reported.[157]

Oxidative labeling can be used to study protein structure, dynamics, folding properties, and protein-protein interactions. Lars Konermann and co-workers used oxidative labeling to study transient acid-induced unfolding intermediates by laser-induced oxidative pulse labeling.[124,158] **Figure 14(a)** shows the rapid mixing device used by the authors to initiate unfolding of holomyoglobin, generate reactive hydroxyl radicals via laser irradiation, and collect discrete time points after the addition of denaturant. The discrete post-denaturation time points were collected, digested with trypsin, chromatographically separated, and analyzed via ESI LC/MS (Q-TOF). The oxidation levels of selected tryptic peptides were determined from full-scan MS data (without using MS/MS fragmentation) by comparing masses of the labeled peptides to the unlabeled reference. **Figure 14(b)** shows the oxidation level of select tryptic peptides in various regions of the protein

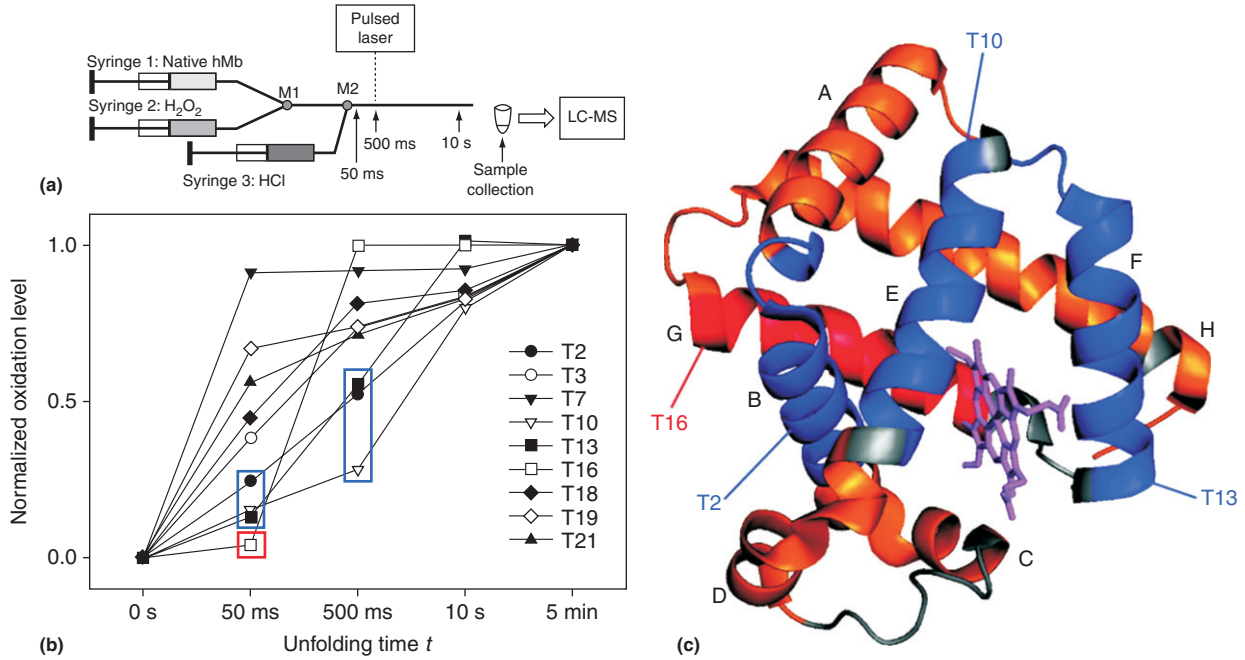

Figure 14 (a) Diagram of the three-syringe, continuous-flow, rapid mixing setup used for all labeling experiments. Details are provided in the text. (b) Normalized oxidation levels of tryptic peptides plotted as a function of unfolding time, *t*. Blue boxes highlight three peptides (T2, T10, and T13) that retain considerable protection from •OH attack at 50 and 500 ms. The red box highlights T16, which is highly protected at 50 ms but not at 500 ms. (c) Crystal structure of native hMb (pdb code 1WLA) with its eight α helices, A–H. Regions that unfold first are shown in orange. Tryptic peptides T2, T10, and T13 represent regions that retain significant protection 500 ms after the onset of unfolding (blue). T16 (red) corresponds to an additional protected region for $t = 50$ ms. Sections not covered by peptide mapping are depicted in gray. The heme group is shown in magenta. Reprinted with permission from Stocks, B. B.; Konermann, L. Structural characterization of short-lived protein unfolding intermediates by laser-induced oxidative labeling and mass spectrometry. *Anal. Chem.* **2009**, *81*(1), 20–27. Copyright by American Chemical Society.

as a function of unfolding time. At 500 ms, peptides T2, T10, and T13 show considerably more protection than the rest of the peptides. At 50 ms, the trend is similar with the exception of the peptide T16, which becomes fully exposed at 500 ms. Figure 14(c) shows a crystal structure of native hMb, with the regions represented by the peptides of interest highlighted. One of the structural insights that can be gained from these structural data is that porphyrin-protein interactions stabilize regions close to heme during unfolding.[101,158] For more in-depth information about the oxidative labeling technique, consult several reviews.[101,144,145,159]

1.17.7 Structural Mass Spectrometry

This section focuses on the structural approaches often used to study protein conformation, dynamics, folding, and non-covalent protein complexes: ESI-MS, native MS, and ion mobility MS. The unifying feature among these methods is that the information about the target protein(s) comes from its physical behavior inside the mass spectrometer. Previously discussed methods, such as PTM characterization, hydrogen exchange, and oxidation labeling approaches, use mass spectrometry for the purpose of signal detection only. Mass spectrometry is used as a high mass accuracy molecular scale to measure the changes originally registered via labeling techniques. For example, in the case of deuterium exchange, other less sensitive detection techniques, such as density gradient column or scintillation counting (tritium exchange), gave way to mass spectrometry once the instrumentation and the methodology allowed for intact protein ionization and detection. Thus, for many applications of structural characterization of macromolecules, mass spectrometry is the latest complimentary approach for making measurements in systems. However, the methods discussed here are the structural biology tools in their own right, similar to X-ray crystallography, NMR spectroscopy, and electron microscopy.

1.17.7.1 ESI Mass Spectrometry

The ESI MS method takes advantage of the multicharging nature of ESI to study protein conformation, dynamics, and shape. As mentioned previously, ESI is one of two ionization methods applicable to large biological molecules.[160] ESI and nano-ESI involve passing a sample through a capillary that is held at a large positive potential (2–5 kV). The liquid sample is drawn toward the source of the mass spectrometer by electrostatic potential, atmospheric pressure differential, and the liquid back pressure by either the HPLC pump or the infusion syringe. One of the properties of ESI is the generation of multiply charged ions – a feature that initially was considered a drawback of the method because multiply charged species complicate spectra. However, in the early 1990s, two research groups reported a relationship between the charge state distribution of protein molecules and the solvent conditions.[161,162] The finding was

explained within the framework of IEM,[16] in which a tightly folded, often globular compact protein has a smaller projected area and therefore a smaller number of charges than an unfolded protein. Because of the compact shape of a folded protein, with its core sequestered from the solution, it has fewer charges due to protonation (and lower charge density) than does an unfolded protein. ESI ionization of folded proteins yields fewer charged states and higher m/z values of the charge distribution compared to a partially unfolded protein, which has more protonation sites available due to its extended conformation.[163] Research by Igor Kaltashov and Lars Konermann[142,164–168] has established ESI-MS as a method to probe the 3-D structure of proteins in solution.

An example of the information that can be learned from ESI-MS experiment is illustrated in **Figure 15**. ESI full MS spectra of 58-kDa antithrombin III was acquired under four different conditions ranging from near-native to acid denatured. There is a transition from the native state (high $m/z + 16$) to a mixture of partially unfolded (medium $m/z + 20$) and fully unfolded states (low $m/z + 32$ and $+38$) as a function of the denaturant amounts.[163] ESI-MS can therefore be used to resolve individual protein states by visualizing unique conformations. However, extracting the quantitative information from the distributions is challenging because the abundances of different conformations do not always correlate with the ion intensity signal recorded by the instrument. Studies show that different conformations of the same protein species have different ionization efficiencies (a propensity for ionization) due to a different hydrophobicity of the residues at the solvent interface. Denatured proteins have higher hydrophobicity and higher ionization efficiency due to their extended conformation in solution. The measure of conformation called solvent-exposed surface area dictates the extent of the multiple charging in gas phase.[164] In addition to characterization of various conformations of the same protein, ESI-MS has also been used to study macromolecular assemblies such as 14-meric molecular chaperonin GroEL. **Figure 16** shows a composite spectrum of various aggregation states of GroEL (14-mer) in gas phase. After a mild CID, the following oligomeric species are observed: intact 14-mers, dimers of 14-mers, and dissociation products such as 13-mers and the monomers. In addition to fragmenting proteins and peptides, CID can be used to dissociate noncovalent assemblies and protein complexes within the mass spectrometer.

Therefore, ESI-MS is a unique method that allows characterization of partially unfolded conformational states and the study of noncovalent assemblies. A particular strength of the method is that the transient gas-phase structures and assemblies can be studied simultaneously, providing information about the dynamics of a particular system. However, this method provides only a rough structural estimate of a particular species. Attempts to quantitatively link the observed charge state distribution to the solvent-exposed surface area and other physical properties are hindered by ionization-related complications.

1.17.7.2 Native Mass Spectrometry

As mentioned previously, the multicharging nature of ESI allows for monitoring of conformational states of the proteins via mass spectrometry. This approach has long been used by Carol Robinson and co-workers to study multiprotein assemblies and high-molecular-weight protein complexes. This methodology has allowed the study of the intact 70S *Escherichia coli* ribosomal assembly and the 30S and the 50S subunits, as well as their stability and dissociation.[169,170] However, some technological hurdles involving the ionization of large biological molecules and transmission of these large ions inside the mass spectrometer had to be overcome first to enable the analysis of large protein assemblies.

Low-flow electrospray ionization and nano-ESI are the preferred methods of analysis of large biological molecules, but they suffer from low efficiency. ESI efficiency has been estimated to be 1–10%, depending on the emitter (needle tip) size and the analyte flow rate, measured as a ratio of the detected ions to the analyte concentration. In addition to the overall low efficiency, large proteins and protein complexes present additional challenges during ionization. The charged residue model of electrospray ionization[171] postulates that the final desolvated droplet forms as the solvent molecules eject via coulombic fission, which eventually leads to the naked charged analyte ion.[172] This process of droplet evaporation has been reported to lead to the formation of nonspecific oligomers, where several protein molecules are present in the final desolvated droplet.[173] In addition to nonspecific oligomerization, large proteins and their assemblies trap buffer and solvent adducts, thus increasing the observed mass of the complex.[174] The oligomerization and the presence of solvent adducts result in broadening and mass shift of the isotopic peaks. One way to reduce the effects of nonspecific oligomerization and solvent adducts is to use mild CAD to increase the internal energy of the ions and facilitate further droplet reduction. However, when analyzing protein assemblies, CAD often leads to disruption of the important noncovalent bonds within protein complexes. Another complication to interpreting spectra of intact large assemblies stems from the multicharging nature of ESI. The number of differently charged species of a protein is proportional to its size; therefore, in the case of a large megadalton protein assembly, the neighboring charge states isotope envelopes overlap. This hinders data interpretation because these overlapping envelopes have to be deconvoluted in order to be identified.

Transmission of the ions inside the mass spectrometer presents another challenge when analyzing large proteins and protein assemblies. Because the kinetic energy of an ion is proportional to its mass, intact protein ions and protein complexes have much greater kinetic energy than do smaller peptide ions (1 eV to 1 keV).[175] Ions with high kinetic energy have trajectories that deviate from the linear path within the guiding optics of a mass spectrometer. Several studies show that transmission efficiency can be improved by increasing the pressure in the source region of the mass spectrometer.[170,176] Increased pressure results in more frequent collision of ions with the gas molecules, which dissipates the high kinetic energy of large protein ions and reduces their velocity. As the velocity of the ions decreases, the oscillation of the ions around the axis of the transfer multipole decreases, resulting in a narrow, more focused ion beam.[172]

A variety of mass spectrometers are used for structural work, ranging from low mass accuracy quadrupole and ion trap

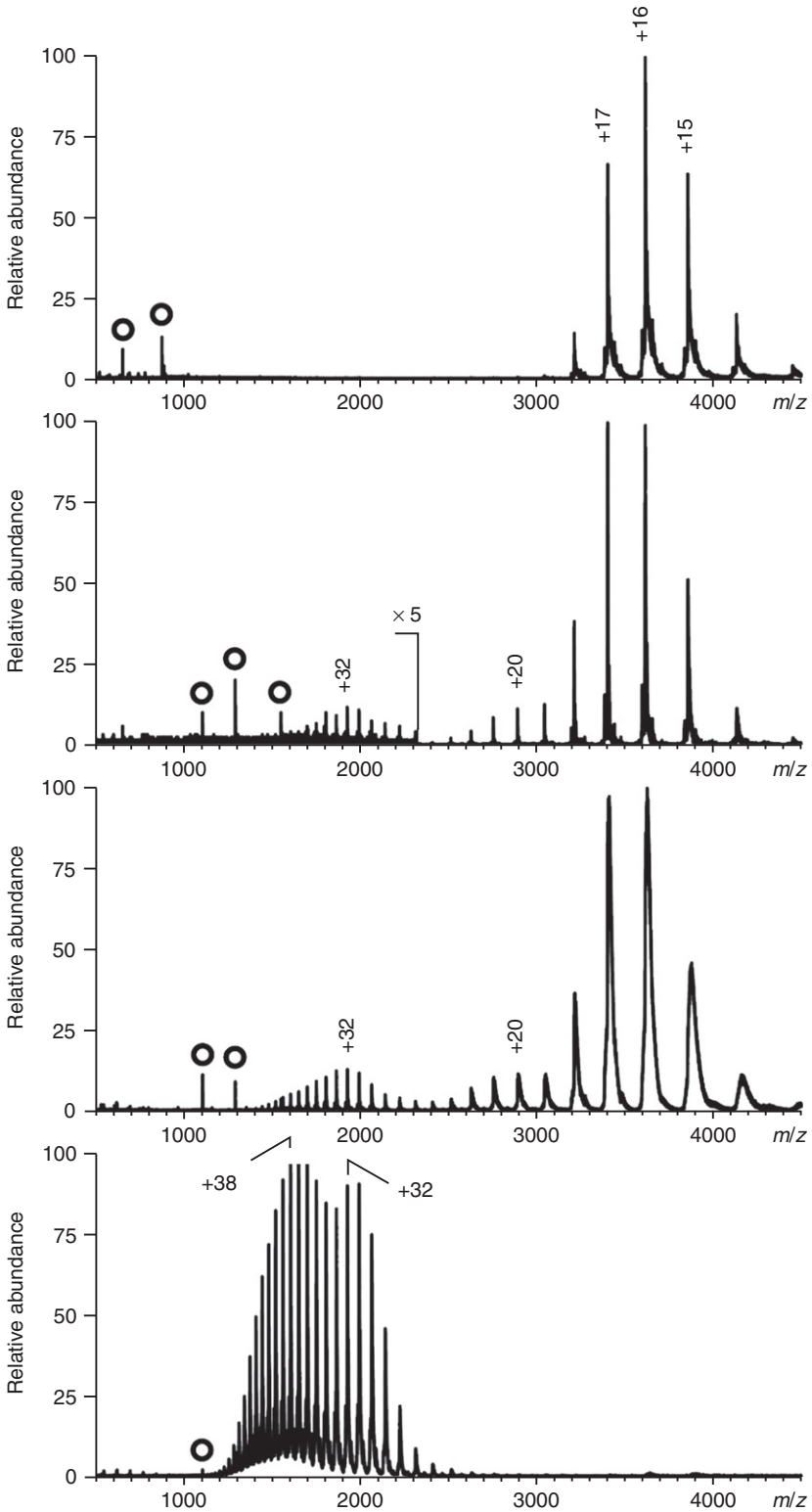

Figure 15 ESI mass spectra of antithrombin III acquired under the following conditions (from top to bottom): 20 mM ammonium acetate, 20 mM ammonium acetate/methanol (50:50), 20 mM ammonium acetate/methanol/acetic acid (49:50:1, vol:vol:vol), and 20 mM ammonium acetate/methanol/formic acid (45:50:5, vol:vol:vol). Ion peaks labeled with circles correspond to low-molecular-weight impurities. Reprinted with permission from Abzalimov, R. R.; Kaplan, D. A.; Easterling, M. L.; Kaltashov, I. A. Protein conformations can be probed in top-down HDX MS experiments utilizing electron transfer dissociation of protein ions without hydrogen scrambling. *J. Am. Soc. Mass Spectrom.* **2009**, *20*(8), 1514–1517.

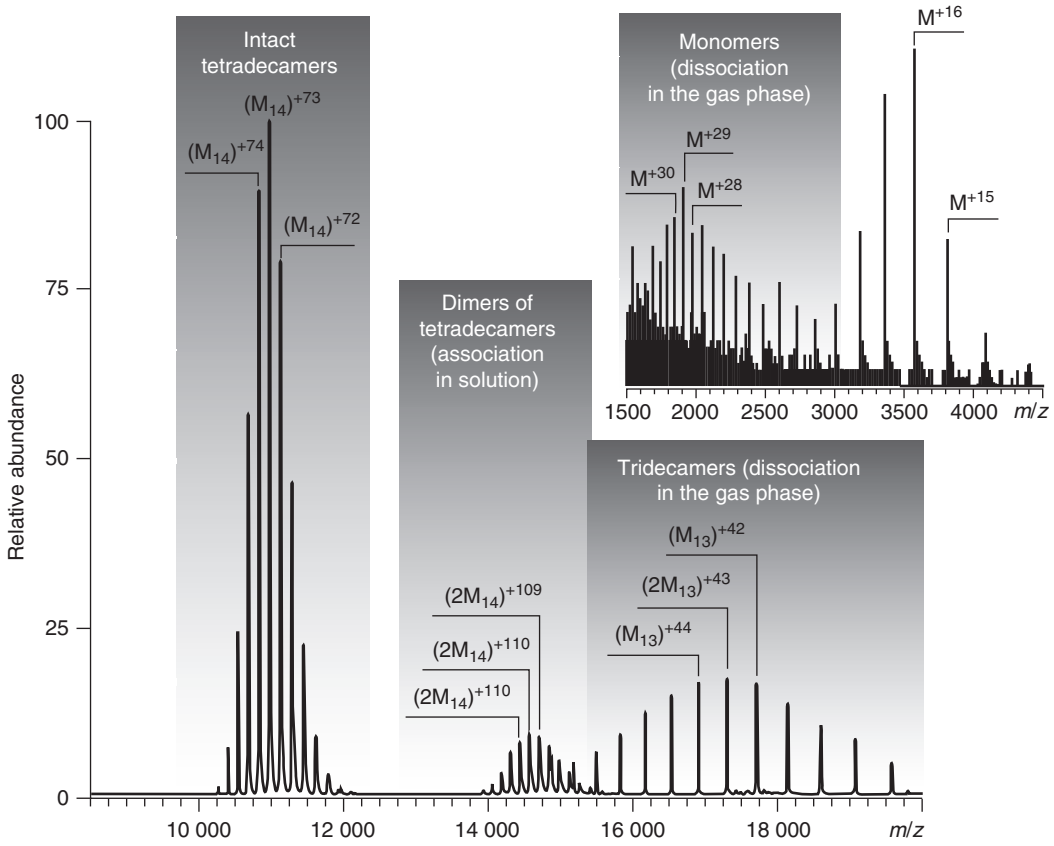

Figure 16 ESI mass spectrum of GroEL acquired under near-native conditions in solution (100 mM ammonium acetate) and mild collisional activation in the ESI interface. The low *m/z* region of the spectrum is shown in the inset. Highly charged monomers and low charge density tridecamers are products of dissociation of tetradecameric structures in the gas phase. Oligomerization of GroEL tetradecamers (formation of 2M14 species) is likely caused by increased protein concentration in ESI droplets as a result of solvent evaporation. Reprinted with permission from Abzalimov, R. R.; Kaplan, D. A.; Easterling, M. L.; Kaltashov, I. A. Protein conformations can be probed in top-down HDX MS experiments utilizing electron transfer dissociation of protein ions without hydrogen scrambling. *J. Am. Soc. Mass Spectrom.* **2009**, *20*(8), 1514–1517.

instruments to high mass accuracy instruments such as FTICR, LTQ-Orbitrap, and TOF. Whereas FTICR and LTQ-Orbitrap instruments were discussed within the context of top-down mass spectrometry, TOF instruments play a special role in the development of structural mass spectrometry. Advantages of TOF instruments compared to the other instruments include a potentially unlimited mass range, high mass accuracy, and lower maintenance costs than FTICR instruments (**Table 1**). As mentioned previously, most TOF analyzers are actually hybrid instruments that feature either a quadrupole (Q-TOF)[30,177] or an ion trap (IT-TOF)[178–180] at the front end of the instrument. These instruments are still referred to as TOF instruments because the mass measurements are most commonly done in the flight tube, whereas the quadrupoles and ion trap are used for ion selection, storage, and accumulation. A Q-TOF instrument usually contains two front-end multipoles – the quadrupole Q and the RF-only hexapole q used from CAD fragmentation only. Thus, a Q-TOF is actually a Qq-TOF. Dissociation of the complexes is usually carried out via CID or electron dissociation (ECD and ETD). When using a Q-TOF instrument, the target ion selection and its fragmentation are carried out in the two front-end multipoles. Because quadrupole mass analyzers have limited mass range (maximum 4 kDa), the front-end multipoles are often modified to isolate and fragment large ions up to 150 kDa.[19]

Some of the pioneering work that defined the field of native mass spectrometry was carried out by Carol Robinson and co-workers. In their 2003 study of the *E. coli* ribosomal structure via native mass spectrometry, the authors were able to not only describe the composition of the ribosome under different solvent conditions but also propose a functional role for the ribosome elongation factor G.[169] **Figure 17** shows annotated mass spectra of the intact *E. coli* ribosome at various pH levels. At pH 7.0 (**Figure 17(a)**), four proteins dominate the spectra – L10, L11, and L7/L12, which form the 50S stalk complex. Thus, it is to be expected that these proteins would dissociate together. However, at pH 4.5, the spectra are populated with proteins L1, L6, L9, and S8 (**Figure 17(b)**). According to the crystal structure (**Figure 17**), these proteins do not have mutual interaction. Therefore, at lower pH, the overall stability of the gas-phase ribosomal assembly is lower than that at physiological pH.

In addition to the task of discovering the stoichiometry and composition of protein assemblies, native mass spectrometry

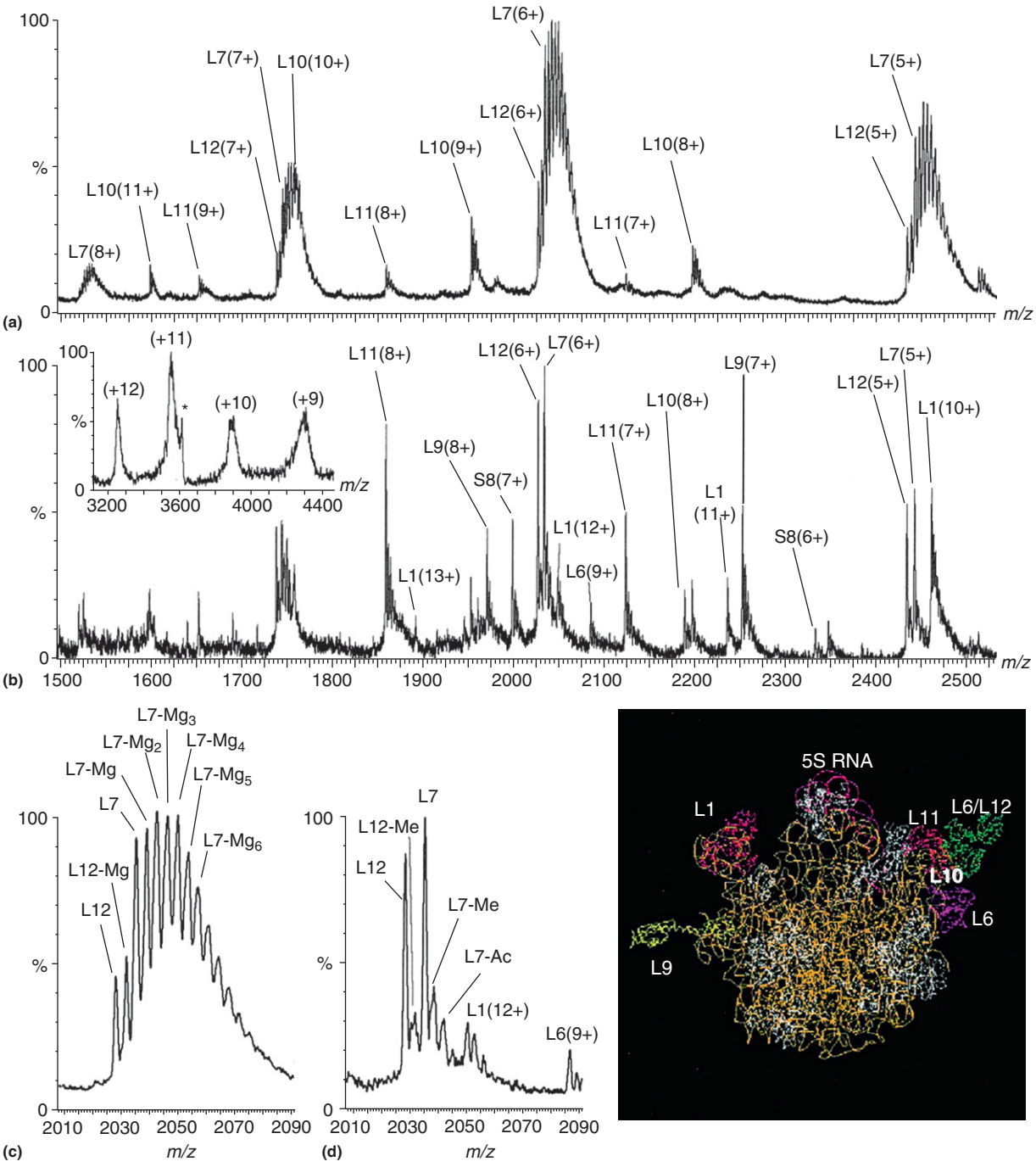

Figure 17 Ribosomes recorded from solution at pH 7.0 (a) and pH 4.5 (b). (c) The +6 charge state shows multiple peaks, each separated by 22 Da, corresponding to the binding of Mg^{2+}. From solution at pH 4.5, Mg^{2+} binding is removed, revealing the extent of methylation and acetylation of L7 and L12 (d). The 50S subunit structure corresponds to the coordinates from *T. thermophilus* at 5.5-Å resolution, Protein Data Bank code 1GIY. Reprinted with permission from Hanson, C. L.; Fucini, P.; Ilag, L. L.; Nierhaus, K. H.; Robinson, C. V. Dissociation of intact *Escherichia coli* ribosomes in a mass spectrometer: Evidence for conformational change in a ribosome elongation factor G complex. *J. Biol. Chem.* **2003**, *278*(2), 1259–1267. Copyright by American Society for Biochemistry and Molecular Biology.

is also used to study the dynamics of protein complexes.[181] Stengel et al. observed more than 300 distinct stoichiometries of interaction between an oligomeric small heat shock protein sHSP and its target. **Figure 18** shows the time course of the complex formation between the heat shock protein HSP18.1 and its target, the unfolding Luc protein. The HSP18.1 dodecamer was incubated with Luc at 42 °C. From the onset of incubation, the amount of free Luc decreases rapidly, as does the abundance of free $HSP18.1_{12}$ (**Figure 18(a)**). As time progresses, the sHSP-target complexes become more

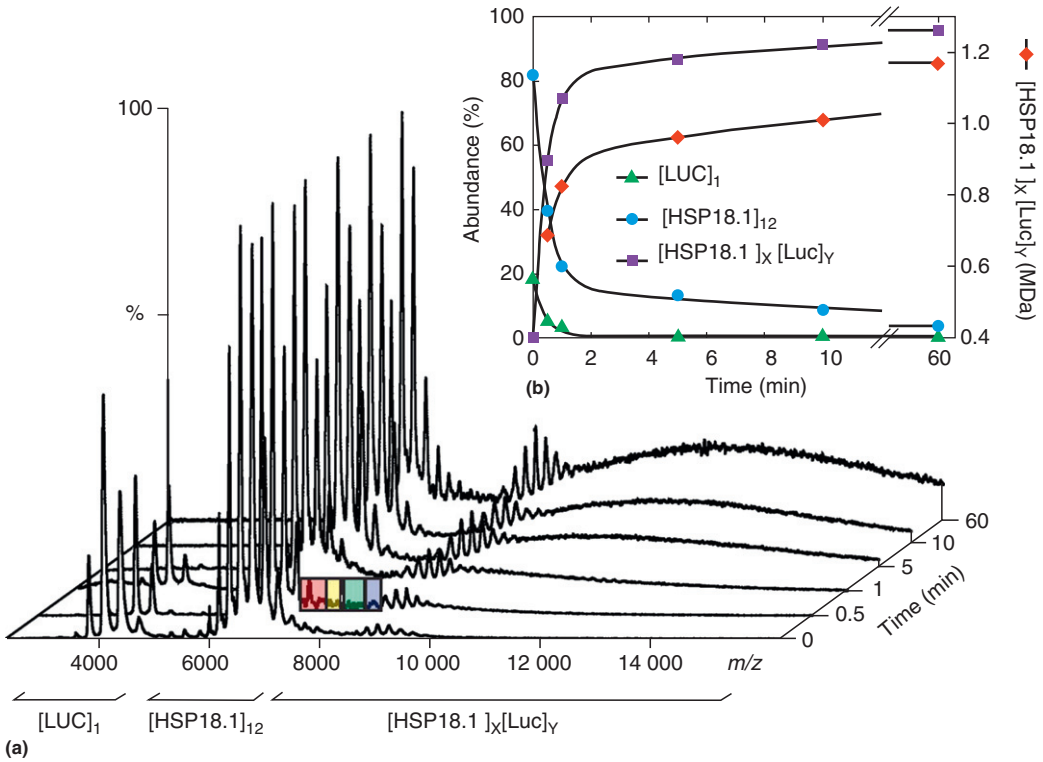

Figure 18 Time course of the formation of complexes between HSP18.1 and unfolding Luc. (a) Incubation with HSP18.1 (≈6350 *m/z*) at 42 °C results in a decrease in the abundance of Luc (≈4100 *m/z*) and the concomitant appearance of signal at high *m/z* (>8000 *m/z*) corresponding to sHSP: client complexes. Data are normalized such that the HSP18.1 is displayed at an intensity of 100%. (b) Quantifying the relative abundances of the different species shows the initial rapid binding of Luc by HSP18.1, followed by further incorporation of HSP18.1 into the resultant complexes. The average mass of these complexes (red) mirrors this behavior, revealing the presence of distinct 'binding' and 'augmentation' steps in the chaperone action of HSP18.1. Reprinted with permission from Stengel, F.; Baldwin, A. J.; Painter, A. J.; Jaya, N.; Basha, E.; Kay, L. E.; Vierling, E.; Robinson, C. V.; Benesch, J. L. Quaternary dynamics and plasticity underlie small heat shock protein chaperone function. *Proc. Natl. Acad. Sci. USA* **2010**, *107*(5), 2007–2012. Copyright by Proceedings of the National Academy of Sciences.

abundant, heterogeneous, and heavier. This means that there is a population of $sHSP_x$-$target_y$ complexes with a propensity for oligomerization. In addition to observing the binding events with native mass spectrometry, the rates of Luc disappearance and heat shock complex formation can be calculated from the time series (**Figure 18(b)**). Several excellent reviews cover the ESI-MS and native MS approaches.[142,182–185]

1.17.7.3 Ion Mobility Mass Spectrometry

Ion mobility (IM) mass spectrometry, also known as gas-phase electrophoretic macromolecule analysis, is a technique that can separate the ions in the gas phase according to their charge and shape. Within the scope of structural proteomics, IM is used for the analysis of individual proteins and large protein complexes. Previously described techniques of chemical labeling, ESI-MS and native MS analysis answer some of the structural questions about the macromolecules and their assemblies. The chemical labeling methods address the topographical features of individual proteins and their complexes, whereas the ESI-MS and the native MS analyses help to determine the composition and the stabilities of the macromolecular assemblies. IM attempts to study the shape of the macromolecules.

Although the absolute values for the thermodynamic stability of protein complexes in solution and in vacuum are significantly different due to the lack of solvation and the hydration contributions in the gas phase, the relative contributions from the individual subunits within the protein complex are similar. In addition, the stoichiometry measurements done in the gas phase usually match those done in solution or obtained from other structural approaches. However, there have been few studies involving a mass spectrometer that compared the geometry and the size of the gas-phase proteins to the X-ray crystallography data. IM mass spectrometry is a method that attempts to describe the macromolecular assemblies not only in terms of their composition but also in terms of their size and geometries.

Ion mobility mass spectrometry is performed by ionizing the protein complex of interest using ESI.[186] The ions travel through the guiding region of the mass spectrometer, where they encounter neutral gas that dampens their kinetic energy. However, in addition to ion guiding potential, the ion mobility chambers (drift tubes) use a weak constant electric field along with the neutral gas to separate the ions based on their mobility. The larger the ion, the larger the number of collisions it encounters with the neutral gas molecules in the IM region. IM is determined by measuring the so-called 'drift

time' of the ion within the IM region. This should not be confused with the drift time inside the TOF mass analyzer because IM drift is achieved in the pre-TOF portion of the instrument. As the ions migrate through the neutral molecules, they separate according to the ratio of their ion-neutral collision cross section (Ω) to charge (z).[186] Within a calibrated IM device, the drift time is converted to Ω. Calibration of the IM drift time is accomplished with a mixture of protein ions, usually small proteins with a cross section smaller than 3500 Å,[186] and the drift time is extrapolated for larger protein complexes. The ensuing collision cross-section data are combined with other structural data, such as X-ray crystallography data, to generate topologies of proteins or protein assemblies.

One of the commercial mass spectrometers that offer IM capability is the SYNAPT HDMS-TOF from Waters. This versatile instrument (**Figure 19**) features a Q, an LIT, and an IM separation module prior to the high mass accuracy analyzer TOF. This mass spectrometer is also capable of interfacing with either ESI or MALDI ionization sources, making it capable of doing both functional and structural proteomics experiments. IM devices are characterized in terms of resolution, which is defined as the centroid (average) of the ion mobility time distribution over the width of that distribution ($t/\Delta t$).[186] Thus, the higher the resolution, the lower the changes in (Ω/z) and the smaller the molecular shape differences that can be separated. The IM data combined with the mass analyzer data provide the drift time (protein shape and geometry), the mass (protein size), and the intensity (protein abundance), adding a third dimension to data.

IM mass spectrometry has been used to differentiate between the two conformational states of the prion protein (PrP) from Syrian hamster.[187] The prion proteins are responsible for the transmission and onset of transmittable spongioform encephalopathies, also known as a bovine spongioform encephalopathy or 'mad cow disease' in cattle. The onset of the disease is triggered by a conformational change of PrP, going from α-helical soluble monomers to β-sheet misfolded aggregates and amyloid plaques. This disease is untreatable, affecting the brain and neural tissue, and is universally fatal. **Figure 20** shows the arrival time distribution, also known as the drift time t, of the α and the β conformations of the PrP for two different charge states and pH conditions. At pH 5.5, the $+9$ charge state (**Figure 20(a)**) of the α-helical ions (red trace) has 60% wider drift time than that of the β-sheet conformation. There is also a 40% increase in the intensity of the secondary peak in α-helical species relative to that of the β-sheet drift time. Similar data are observed for the $+10$ charge state in **Figure 20(b)**. The wide distribution of the α-helical species suggests that there are multiple conformations present compared to the β-sheet conformation. Under physiological conditions, pH 7, both the $+9$ and the $+10$ charge states feature narrower drift time distribution of both the α-helical and the β-sheet species. A review by the research group of Carol Robinson is an excellent source for specific information on setting up native MS experiments.[186]

1.17.8 Conclusion and Future Directions

Mass spectrometry is a rapidly evolving field that has embraced the needs of the biological community in the past two decades. In addition to being an analytical technique used to study small molecules, mass spectrometry is now routinely

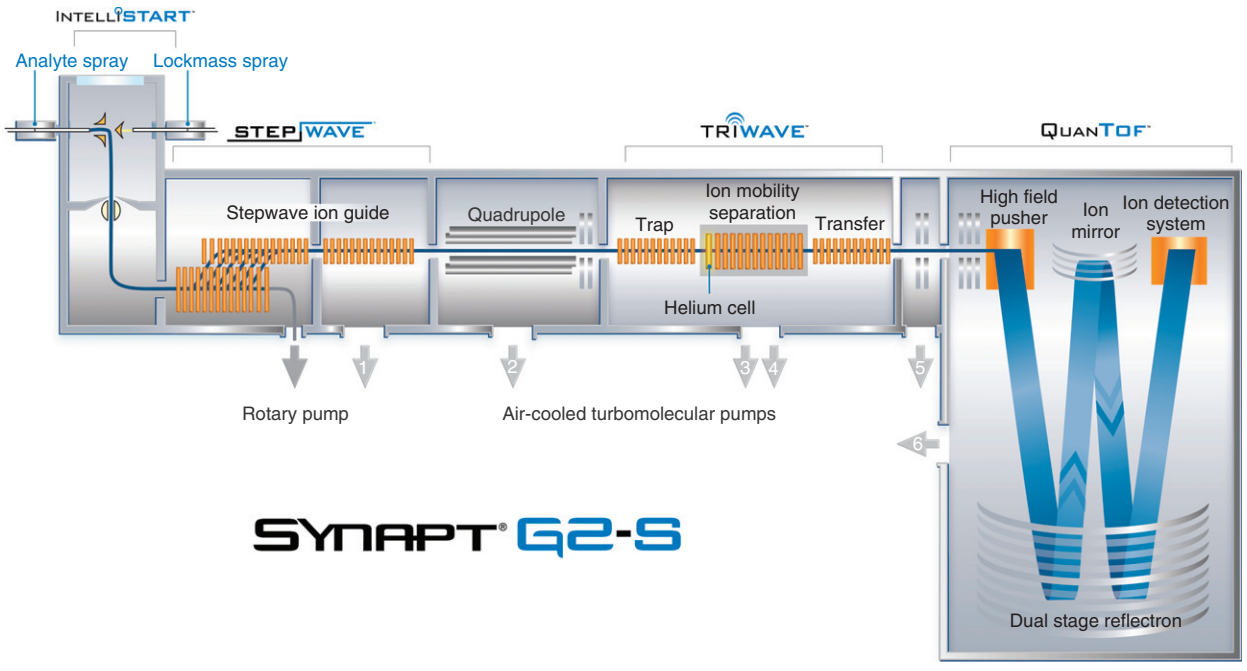

Figure 19 SYNAPT G2 HDMS mass spectrometer. Corporate Literature of Waters, http:www.waters.com. © 2011 Waters Corporation. Used with permission.

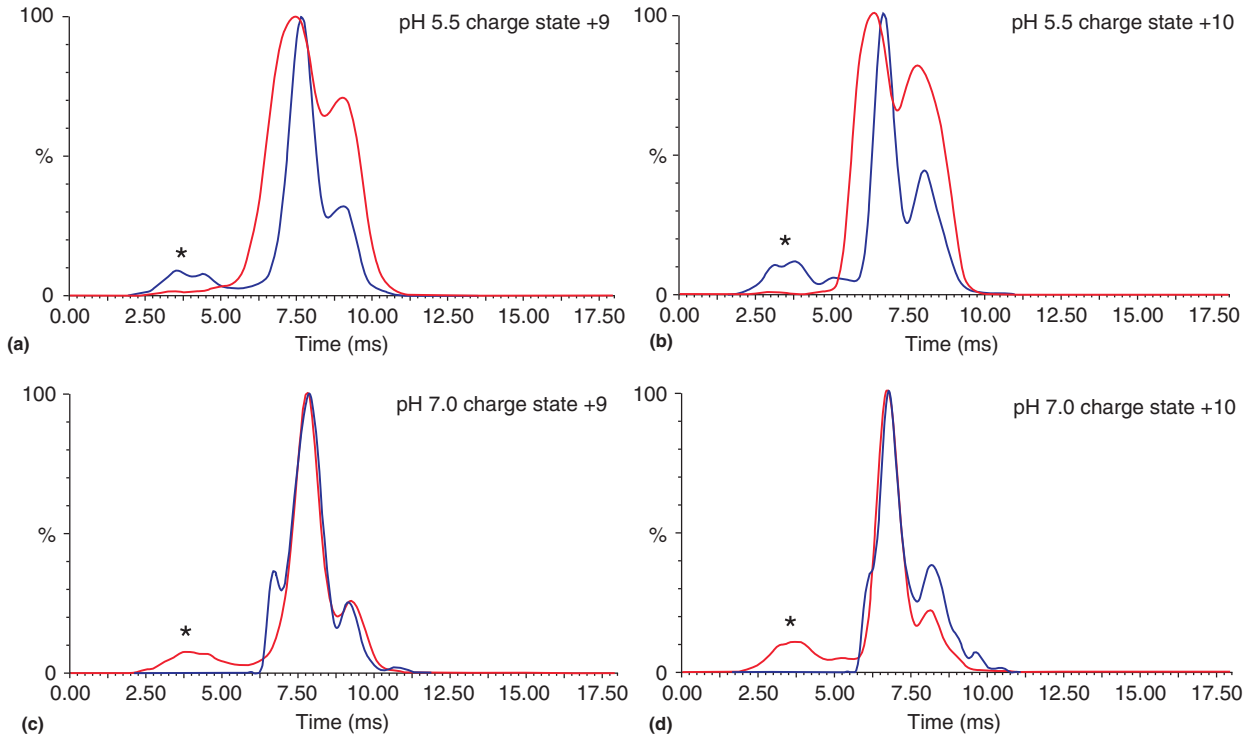

Figure 20 Comparison of α-helical (red) and predominantly β-sheet (blue) SHa PrP(90-231) selected arrival time distributions (ATDs) for charge state +9 and +10. (a) Overlaid ATDs for charge state +9 at pH 5.5. (b) Overlaid ATDs for charge state +10 at pH 5.5. (c) Overlaid ATDs for charge state +9 at pH 7.0. (d) Overlaid ATDs charge state +10 at pH 7.0. Reprinted with permission from Hilton, G. R.; Thalassinos, K.; Grabenauer, M.; Sanghera, N.; Slade, S. E.; Wyttenbach, T.; Robinson, P. J.; Pinheiro, T. J.; Bowers, M. T.; Scrivens, J. H. Structural analysis of prion proteins by means of drift cell and traveling wave ion mobility mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2010**, 21, 845–854.

used to study macromolecules such as proteins. Several technological improvements have propelled mass spectrometry into the realm of biology. Improvements in the area of separations and chromatography allowed for the analysis of high-complexity biological samples such as protein digests and cellular metabolites. A powerful multidimensional approach of orthogonal separation further increased the sensitivity and the resolving power of the front-end HPLC separation. The soft ionization techniques of ESI and MALDI allow for ionization of semivolatile proteins and DNA molecules. The salt-tolerant and sensitive electrospray ionization method, the nanospray ESI, is currently a popular platform to study both individual proteins and large-scale high-complexity samples via mass spectrometry. Complimentary to improvements in front-end separation and ionization, mass spectrometers offer a wide variety of mass analyzers. In addition to the long-established time-of-flight and quadrupole mass analyzers, linear ion trap and Orbitrap mass analyzers offer high scanning rates and high mass accuracy. Several hybrid instrument configurations are tailored to both high throughput and high mass accuracy, including the LTQ-Orbitrap and the SYNAPT HDMS. The latter is especially geared toward structural studies of large proteins because it has an ion mobility cell for IM mass spectrometry of proteins. Finally, there have been many advances in data analysis tools since the early 1990s, spurred by the decrease in computational cost and the increased availability of high-performance computing clusters. The bottom-up computational methods of database search and the top-down methods of *de novo* search have expanded tremendously due to improved understanding of gas-phase chemistry and fragmentation and improved informatics and algorithms.

The field of structural mass spectrometry has especially benefited from the previously mentioned developments. Constant research and improvement of quadrupole analyzers has allowed for transmission and detection of large ions. The soft fragmentation techniques of ECD and ETD allow for improved fragmentation of high charge state ions that tend to be large peptides and small proteins. Electron dissociation techniques also preserve post-translational modifications, allowing for a comprehensive modification analysis of the intact protein. One of the areas of structural top-down proteomics that is underdeveloped compared to the functional bottom-up approaches involves separation techniques. Whereas RP material is excellent for peptide separation, protein RP chromatography is more challenging due to the poorly resolved peaks of large molecules and the large starting material requirement. Therefore, the large quantities and the high purity of the protein sample are also a hurdle for the structural proteomics approach. Although researchers routinely analyze high-complexity samples such as entire proteomes in an automated high-throughput manner via bottom-up proteomics, structural studies require a more targeted approach. Some structural approaches, such as oxidative labeling, are more suitable for high-throughput data collection and analysis than approaches such as deuterium

exchange because in the case of HX, the labile nature of the deuterium label (back-exchange and scrambling effects) imposes a strict set of experimental conditions. The mass spectrometry methods of ESI-MS, native MS, and IM MS face additional challenges with regard to automation because of the poor ionization and transmission of large macromolecular ions. Future developments in the areas of separation, ionization, and ion transmission will go a long way to streamline the structural MS approaches and make these available to a wider group of researchers.

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