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Deciphering the histone code using mass spectrometry

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In honor of Donald F. Hunt on his 65th birthday.

Abstract

During the past decade, studies surrounding chromatin research have grown exponentially. A major focus of chromatin biology is centered on understanding of how histone modifications alter chromatin structure at the molecular and mechanistic levels. Discoveries are being made at a rapid pace due to the advent of new and innovative techniques. Mass spectrometry has emerged as a powerful tool in the field of histone research due to its speed, sensitivity, and ease of use. This has resulted in the identification of a number of novel histone modification sites. In consequence, new roles in biological processes have been discovered and hypothetical models, such as the 'histone code' have been reaffirmed or refined. One significant advantage to using mass spectrometric techniques is that the combinations of modifications on different sites can be determined which is crucial to deciphering the 'histone code'. In this manuscript, the mass spectrometric approaches developed over the past decade for both qualitative and quantitative analysis of histone post-translational modifications (PTMs) are discussed.

Keywords: Chromatin; Histone post-translational modification; Stable isotope labeling; Electron capture dissociation; Electron transfer dissociation

1. Introduction

In eukaryotes, the genetic information is organized into nucleosomes, the building block of a highly conserved structure called chromatin. Each nucleosome contains 146 base pairs of DNA wrapped around a protein core that contains two copies each of the four evolutionarily conserved histone proteins, H2A, H2B, H3, and H4 [1]. The histone N-terminal tails are exposed on the nucleosome surface and are subjected to a variety of enzyme-catalyzed post-translational modifications (PTMs) [2]. The histone variants are synthesized at varying points of the cell cycle and are incorporated into the nucleosomes in a replication dependant and independant manner [3]. It is believed that these modifications and variants provide a control mechanism which dictates the accessibility of DNA by protein machineries and enzymes [4,5]. A repertoire of modifications including phosphorylation of serines and threonines, acetylation of lysines, mono-, di-, and tri-methylation of lysines, mono- and di-methylation of arginines and ubiquitination of lysines [6–9], have been documented to occur on histones. It has been postulated by Allis and co-workers that a single or combinatorial set of these modifications on one or more histones comprise a 'histone code' which determines transcriptional activity at a given gene locus [10–13]. To decipher this code, it is necessary to characterize all possible histone modifications and ultimately associate a particular modification or modification pattern to distinct biological functions such as transcription, replication, chromatin condensation, gene silencing, or apoptosis. This task requires a technology that can identify histone PTMs comprehensively and can track changes in histone modifications due to cellular processes.

2. Classical approaches to histone analysis

Edman microsequencing was one of the classical approaches used for identification of histone PTMs [14–16]. While some of the initial histone modifications were discovered by this technique, the major limitations of protein microsequencing are the requirement of a large quantity of purified samples, the process is time consuming, and the sequence of N-terminally blocked peptides cannot be obtained. Another traditional technique for studying histone PTM is an immunoassay using site-specific

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antibodies [17]. However, antibodies are costly and cumbersome to generate. In addition, the simultaneous presence of multiple modifications lead to epitope occlusion and more importantly, antibodies alone cannot be employed to identify novel modification sites. Conversely, mass spectrometry can determine the exact location of the PTM and acts as a complimentary and improved technique to protein sequencing or immunoassays. Here, we discuss various mass spectrometric approaches to tackle the different aspects of histone modification analysis.

3. Challenges to histone post-translation modification analysis

A major challenge in the study of histones is the presence of a multitude of different modifications that results in a complex mixture of multiply modified isoforms. While these proteins are relatively small, ~11–16 kDa, there may be 100s of isoforms of an individual protein containing different combinations of modifications. For example, a H3 histone peptide of residues 6–20, TARKSTGGKAPRKQL, contains three potential lysine sites which may individually or concurrently be acetylated, mono-, di-, or tri-methylated. There are also two potential arginine sites which may be mono- or di-methylated and three potential phosphorylated sites (two threonines and one serine). Therefore, a total of eight modifications can occur concurrently or in various combinations. In spite of it being only a 15-mer peptide, potentially greater than 100 isoforms of this peptide can be present in a sample. Hence, the ability to chromatographically resolve these isoforms to simplify the mixture is vital. However, the nature of the complexity is further augmented by the presence of a number of isobaric species. The same modification can be localized at different sites of a peptide making them virtually impossible to be resolved by chromatographic means or accurate mass measurements alone. Therefore, high quality MS/MS fragmentation information is essential to provide unambiguous PTM assignments. To further complicate the analysis, the generated peptides of histones are rich in arginine and lysine residues making them extremely hydrophilic and hence challenging to resolve on traditional reverse phase HPLC. In positive electrospray ionization (ESI) mode, the presence of numerous basic amino acids lead to highly charged precursor ions (>+3) and consequently multiply charged fragment ions. Such MS/MS spectra will be impossible to interpret using a low resolution mass spectrometer. Therefore, mass spectrometric methods have to be developed to fit this specific class of proteins and allow comprehensive characterization of variants and modifications of histone proteins.

4. Qualitative analysis: mapping of histone PTMs

Since the exploitation of mass spectrometry for histone analysis by Edmonds et al. [18], peptide mass fingerprinting using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) systems was a common rapid approach to investigate histone modifications [19]. With this method no on-line chromatography was required. The difficulties associated with hydrophilicity and basicity of the histone peptides were therefore not a problem in the early studies. Here, proteins are enzymatically digested and analyzed by the MALDI-TOF system to obtain accurate mass information. For a particular peptide, identification of modifications was made by examining mass shifts between examined mass and theoretical mass. This approach can however lead to misidentification of histone modifications due to the presence of multiple peptide mass degeneracy or genetic variants. Therefore, an alternative peptide mass fingerprinting approach using stable isotope labeling strategy for detecting residue specific mass-tagged peptides was employed by Bradbury and co-workers [20]. This approach allowed labeling of a specific amino acid and monitoring of modifications occurring at that residue. For example, this group utilized incorporation of 50% "heavy" serine-2,3,3-d_3 (Ser-d_3) in HSF (human skin fibroblast) cell cultures for identification and detection of phosphopeptides on histone H2A.X following response of cells to low-dose IR (ionizing radiation). The mass tagged samples were digested and subjected to MALDI-TOF analysis. For the unmodified form of the peptides, each labeled serine present would show a 3 Da mass shift from the unlabeled peptide. The phosphorylated form of the peptide will show an 80 Da increase in mass due to the addition of the phosphate group and will also show the characteristic 3 Da mass split pattern. This approach allowed for extracting the labeled peptides and identifying their modification from a mixture through their characteristic mass-split patterns. Various isotopic/metabolic labeling techniques have also been utilized by other researchers [21–23] for histone analysis such as using stable isotope labeling by amino acids in cell culture (SILAC) in which lysine was replaced by isotopically labeled lysine-D4 to track acetylation patterns of histone H4 [22].

Although peptide mapping using MALDI-TOF instruments provides accurate mass information, it still does not have the resolution to distinguish between histone peptides having nearly identical masses [24]. High resolution and mass accuracy instruments such as the Fourier transform ion cyclotron mass spectrometer (FT-ICR-MS) which has a resolution power of $>10^6$ [25] can differentiate between these masses. The two common histone modifications, acetylation and trimethylation, have a mass difference of only 35 ppm and can be routinely distinguished by FTMS systems. The high accurate mass measurement of this instrument also aids in distinguishing between peptides from different histone variants that have the same nominal mass, but are distinct species. For example, the mass of peptide residues 93–101 from human histone H2A.M, LNKLLGRVT, differs by 15 ppm from the corresponding peptide of variant histone H2A.1, LNKLLGKVT, dimethylated at lysine 99. Only a high resolution mass spectrometer can discriminate between these two peptides.

One of the first groups to apply a high mass resolution mass spectrometer for peptide mass fingerprinting was Freitas and co-workers [24]. An FT-ICR instrument was used to map PTMs on all four core histones. Here, purified proteins were digested with pepsin, V8DE, trypsin and Arg-C. Measured masses were compared to the theoretical masses and mass shift such 42.016 and 42.046 Da were assigned to acetylation and trimethylation, respectively. In order to achieve a high mass accuracy, each spectrum was internally calibrated with four unmodified peptides at a mass range of 400–1200m/z providing an average mass error

of 3 ppm. However, the measured masses are often comprised of peptides containing more than one possible site of modification. Hence, unambiguous assignment of modification sites is not possible using accurate mass alone. To determine the exact site of modification, fragmentation information becomes mandatory.

The two traditional mass spectrometric fragmentation techniques used for peptide analysis are post-source decay (PSD) [26] for matrix assisted laser desorption/ionization (MALDI) and collisional activated dissociation (CAD) [27,28] for electrospray ionization (ESI). While fragmentation can be achieved by PSD, only partial determination of the sequence can be obtained employing this technique [26]. CAD generally produces more comprehensive fragmentation and allows deducing the peptide sequence readily [27,28]. In addition, fragmentation patterns can be exploited to rapidly identify PTMs by examining the MS/MS spectrum for the presence of diagnostic ions characteristic for specific modifications. For example, trimethylated lysine containing peptides show a neutral loss of 59 Da from precursor and fragment ions, and monomethylated lysine residues produce the diagnostic immonium ion at m/z 98 [29,30]. Acetylated peptides show the diagnostic ion of 143 Da [31] and 126 Da [32], corresponding to the immonium ion and loss of NH_3 thereof, respectively, in mass spectrometers that have the capability to detect low mass ions. Serine and threonine phosphorylated peptides exhibit a pronounced neutral loss of phosphoric acid of 98, 48, and 32.6 Da for singly, doubly and triply charged precursors, respectively. These diagnostic ions increase the confidence in PTM identification and can be utilized to rapidly scan MS/MS spectra for the presence of these modifications. Unfortunately, not all modified species are present in high abundance. Phosphorylated peptides are present at low abundance and are rarely selected for MS/MS in the presence of other highly abundant peptides. In this case, instruments with quadrupole selection and fragmentation functionality can be used to selectively analyze phosphorylated peptides using the precursor ion scan mode to monitor the diagnostic mass of 79 Da in the negative ion mode or neutral loss scan mode to monitor losses of 98, 49 or 32.6 Da in the positive ion mode [33]. A different strategy uses immobilized metal affinity chromatography (IMAC) to exclusively enrich for phosphorylated peptides [34–36]. Therefore, increasing the selectivity for specific modifications also increases their chances of being identified.

Taking advantage of both peptide mass fingerprinting and fragmentation techniques, Burlingame and colleagues were one of the first groups to exploit mass spectrometry for determining specific sites of modification [37]. Here, purified histone fractions were enzymatically digested. The peptide mixture was either fractionated or the whole sample analyzed by MALDI-TOF systems to obtain the accurate mass of the peptides. Both static nanoESI-QqTOF and PSD from MALDI-TOF were used to obtain MS/MS information. From the analysis of histone H4 of HeLa cells treated with sodium butyrate, it was found that the tetra-acetylated form was modified at lysine 5, 8, 12 and 16; tri-acetylated form modified at lysine 8, 12, 16; di-acetylated form modified at lysine 12 and 16, and mono-acetylated form modified at lysine 16. Based on the results, the "zip model" was hypothesized describing the direction in which acetylation and deacetylation took place. It was proposed that acetylation first occurred at lysine 16 and then proceeded at lysine 12, lysine 8 and lysine 5, respectively. The deacetylation is accordingly proposed to proceed in the reverse order, first on lysine 5 and last on lysine 16. A similar technique was used for the study of histone H3 PTMs on chicken erythrocytes by this group [38]. To disseminate and simplify the sample complexity, the tryptic peptide mixture was further fractionated by off-line microflow RP-HPLC before being analyzed by MALDI-TOF. In addition, incomplete tryptic digestion of the sample was performed to provide larger histone peptide which may contain multiple sites of modification. Here, the accurate mass measurements with representative immonium ions, 126 for acetylated lysines, 98 for monomethylated lysines, and 84 for di- and trimethylated lysines, respectively, were used for differentiating between methylated and acetylated peptides. From this analysis, lysines 4, 9, 14, 27 and 36 of the N-terminal tail were found to be methylated while lysine 14, 18 and 23 were acetylated. Additionally, lysine 79 from the core domain was found to be mono-, and di-methylated.

To increase speed and sensitivity of histone analysis, it would be beneficial to perform the chromatographic separation on-line with the mass spectrometric analysis. This approach, usually a standard procedure, is challenging for histone analysis, especially for histone H3 and H4. The majority of post-translational modifications on these histones take place on the N-terminal tail, which is very hydrophilic in nature. For histone H3, 15 of the first 50 residues are lysines and arginines. Therefore, most peptides obtained from this region are poorly retained on reverse phase HPLC columns. Even if normal phase chromatography was used, the majority of the peptides will have a charge state of >+3 which will generate fragment ions >+2. Interpretation of histone peptide MS/MS spectra obtained from low resolution mass spectrometers is therefore challenging because these instruments cannot accurately determine multiply charged states. In addition, since most proteases with high specificity cleave after basic or acidic residues and the latter is not abundant in histone proteins, it is not trivial to generate peptides of suitable size for the downstream analysis. Using a protease like trypsin that cleaves after lysine and arginine residues would generate peptides that are too small (4–5 amino acids). Alternatively, proteases like Glu-C that cleave after glutamic acid would generate peptides that are too large for MS/MS interrogation (50 amino acids for the largest peptide). Therefore, a combination of enzymes such as chymotrypsin, pepsin, or incomplete trypsin digests were employed for histone analysis [24]. Furthermore, many of the histone lysines and some arginines are modified which prevents proteolytic cleavage at that residue, thus, making prediction of the cleavage sites of the protein difficult. The latter is especially crippling when a differential analysis on histone proteins is to be performed. Hunt and co-workers were one of the first groups to use a derivatization methodology [39,40] to circumvent the problems associated with histone PTM analysis using RP-HPLC–ESI on ion trap mass spectrometers. The histone proteins are subjected to reaction with propionic anhydride. This reaction will convert amines to propionyl amides and thus reduce the basicity

and add a hydrophobic moiety to every free N-termini, unmodified and monomethylated lysine. This results in increasing the chromatographic retention time on a reverse phase column and facilitating the interpretation of MS/MS spectra as the peptide ions are now mainly doubly and triply charged which generate mostly singly charged fragment ions in ESI. Additionally, since all lysine residues are now either endogenously or exogenously modified, trypsin will no longer cleave at lysine residues. Thus, the proteolytic cleavage from sample to sample is largely unaffected by the modification state of the histone protein. The Hunt group had utilized this methodology [39–41] towards a number of histone studies by nanoESI-LC–MS/MS using ion traps and hybrid ion trap-Fourier transform instruments. A number of novel modifications sites on the N-terminal as well as in the globular core and C-terminal domains have been identified, such as threonine 3 phosphorylation on histone H3 [41], acetylation of the C-terminal lysine 125, 127, and 129 of human histone H2A [30] to name just a few. Probably the most exciting finding of these studies was the existence of the dual modification of serine 10 phosphorylation and lysine 9 methylation on histone H3 from HeLa cells [30]. Previous studies with in vitro HMTase assays demonstrated the inability of synthetic peptides to be methylated at lysine 9 when serine 10 is phosphorylated and vice versa [42,43]. These were among the key data for the histone code hypothesis. Intriguingly, all other 'famous' methyl marks on H3 (lysine 4, 9, and 27) are also direct neighbors of potential phospho marks (threonine 3, serine 10, and 28). This study revealed that the serine 10 and serine 28 phospho marks indeed exist next to a methylated lysine residue. In a subsequent study, the phospho mark on threonine 3 was found next to a methyl mark on lysine 4 as well [41]. These findings led to the refinement of the histone code and gave birth to a new hypothesis, the 'switch hypothesis' [44]. Here, depending on the position of the methyl mark, the phosphorylation is thought to be a regulatory 'on' or 'off' switch that confers another level of regulated control of gene repression and activation by histone PTMs.

The mapping and characterization of histone PTMs have progressed rapidly. A number of breakthroughs in this field have been made possible by using mass spectrometry, such as identification of nuclear receptor coactivator, PRMT1, which mediates methylation of histone H4 arginine 3 [45]. Another example is the identification of a novel acetylation site on yeast histone H3 [46] in the globular domain, lysine 56, which is associated with gene activation by attracting the SWI/SNI chromatin remodeling complex to the location of acetylation. One major recent breakthrough is the identification of a histone demethylase [47], LSD1, which is conserved from S.pombe to human and removes methyl group from histone H3 lysine 4. In the past, it has been unclear whether histone methylation is a permanent modification; this study proves that methylation is dynamic. These are just a few examples mentioned in addition to the ones illustrated in this manuscript. Despite the new and novel findings, importance or function of each individual modification or combination of modifications is still far from being understood. To increase our depth of understanding of the function of these PTMs, it is helpful to obtain quantitative information for each modification.

5. Quantitative analysis: monitoring of histone PTMs

Mapping of global histone modifications by the various strategies led to the confirmation of previously identified PTMs and to the discovery of a multitude of novel modification sites. Once identified, the next questions raised are about the roles of these modifications in chromatin mediated processes. Besides biochemical approaches, mass spectrometry can also aid in answering the biological significances of the modifications by providing quantitative information about the individual histone PTMs and can track changes of modifications based upon the cell cycle or the exposure to different stimuli. The two major factors influencing quantitation studies using mass spectrometry are the signal suppression of peptides in a complex mixture and the differences in ionization efficiencies of peptides. Therefore, for absolute quantitative analysis, synthetic or stable isotopically labeled form of the peptide of interest must be used to normalize its ionization. Nevertheless, in the absence of synthetic or isotopically labeled peptides, semi-quantitative analysis for determination of relative abundances and changes in patterns of histone modifications can be accomplished.

Abnormalities in histone PTM levels have been associated with the development of diseases. For instance, it has been observed that increased levels of histone deacetylases (HDACs) result in inhibition of gene expression. HDACs catalyze the removal of acetyl groups from histones. As acetylation is generally associated with gene activation, it is hypothesized that deacetylation consequently is linked to gene suppression [48]. If the affected gene is a tumor suppressor gene, this could lead to the progression of cancer. Currently, HDAC inhibitors are under investigation as anticancer drugs [49,50] and there is growing interest in monitoring patterns and specific sites of histone acetylation with time course or concentration dependant treatments with HDAC inhibitors.

Freitas and co-workers have performed a number of semi-quantitative studies on monitoring the effects of (HDAC) inhibitors, such as trichostatin A (TSA) and depsipeptide (DDP), on modifications of core histones from diseased cells [51,52]. In the first study [51], changes in histone modifications from leukemic cell lines treated with depsipeptide and valproic acid were characterized. Here, core histones were separated by RP-HPLC and the intact proteins analyzed using a Q-TOF system. The changes in modification levels for each four core histones were quantified by integrating the area of each of the histone population as a percentage of the total histone. Dose dependent changes in the modification levels were observed from this analysis. Also an increase in the acetylated forms of H4 was observed from samples obtained from patients after 4 and 24 h treatment with the HDAC inhibitor, depsipeptide. In the second study [52], acetylation patterns of histone H4 in mouse lymphosarcoma cells were explored. The differently acetylated histone H4 proteins were first separated by gel electrophoresis. Various populations of acetylation on H4 were monitored after treatment with the HDAC inhibitor. The relative abundances of

each of the population were measured to determine the effect of the HDAC inhibitor on changes in levels of acetylation. While the total amount of acetylations can be determined using this methodology, the specific sites of acetylation cannot be identified. To determine the specific sites of modifications, histone H4 was enzymatically cleaved followed by peptide mass fingerprinting using MALDI-TOF and peptide sequence determination from MS/MS information. To distinguish between acetylated and trimethylated species, the immonium ion at m/z 126 and loss of trimethylamine from fragment ions (−59) were monitored, respectively. From this study, the acetylation patterns induced by both trichostatin A and depsipeptide were found to occur initially at lysine 16 followed by lysine 12, and finally lysine 8/5 in agreement with previous studies [37].

A label free semi-quantitation method was also used by Frega et al. [53] to investigate the global modification patterns of human histone H4 in normal and cancer cells. In this study, modification patterns on intact histone H4 isoforms were monitored by LC-ESI/MS using a Q-TOF system and high performance capillary electrophoresis (HPCE). The relative intensities of the various acetylated H4 isoforms were compared to determine the changes in the patterns of acetylations between normal and cancer cells. Site-specific acetylation was assigned through MS/MS fragmentation spectra of tryptic peptides on an ion-trap MS. Results from mass spectrometric and biochemical approaches for this study suggested that cancer cells predominantly show a loss of acetylation on lysine16 and trimethylation on lysine 20. This global loss of monoacetylation and trimethylation of histone H4 is suggested to be a global hallmark of human tumor cancer.

More recently, Ole Jensen and colleagues have also developed a label free mass spectrometric based analytical and computational strategy [54] to obtain relative quantitation of the modifications at the peptide level for all four histones, H2A, H2B, H3 and H4. In this study, human core histones from small cell lung carcinoma cells exposed to six different concentrations of a HDAC inhibitor were extracted followed by separation of the various histones. The tryptic peptide mixture of each histone from the six samples was analyzed in triplicates using a Q-TOF. Peptide alignment was performed using retention times, peptide masses, MS/MS sequence information, and the coefficient of variance (CV) for the ion intensities of the detected peptides across all 18 data sets was calculated. Unmodified tryptic peptides, which had a CV of less than 30% were chosen as 'internal standard peptides' and these peptides showed no changes in intensities upon treatment with the various HDAC inhibitor concentrations. Label free quantitation of peptides based on MS ion intensities can vary significantly from run to run, thus CVs of 25–30% are typical for this type of analysis. The CVs for higher abundance peptides are better than low abundance peptides, an average of 10% versus 40%, respectively [55]. Relative changes in intensity of each modified peptide were then calculated and statistical significant changes determined. As predicted, results showed an increase in the level of acetylation observed with the administration of increased levels of the HDAC inhibitor. On the contrary, levels of dimethylated lysine 57 of H2B decreased and methylated lysine 79 of H3 remained unaffected.

Another strategy employed for quantitation of histones is chemical labeling. Imhof and co-workers [56] have used both propionic anhydride and d_6 acetic anhydride to study histone modification changes during various stages of Drosophila embryonic development. This group used MALDI-TOF for peptide fingerprinting and Q-TOF for MS/MS analysis of Arg-C cleaved peptides. These derivatization strategies will label N-termini, unmodified and monomethylated lysines residues. Initially propionic anhydride was used for derivatization, however, the addition of 56 Da by the reagent also corresponds to four methylations or one acetylation and one methylation. To avoid ambiguity, d_6 acetic anhydride was utilized because the addition of 45 Da instead of 56 Da made it possible to distinguish between modifications for peptide mass fingerprinting. This derivatization analysis showed a frequent miscleavage of Arg-C between lysine 36 and 37 producing the peptide of residue 27–36 which allowed for a comparison of the effect of lysine 36 on the methylation of lysine 27. Lysine 27 is not found to be di-, or tri-methylated when lysine 36 is di-, or tri-methylated and vice versa. This provided a possible explanation of how lysine 27 maintains a repressed state by preventing methylation of lysine 36, and consequently how lysine 36 maintains an active state by repressing methylation of lysine 27. In addition, lysine 36 and 37 were also found to be methylated and an increase in methylation at these residues during development of drosophila was observed which indicated that expansion in chromatin maturation occurs as cells differentiate.

Similarly, Jenuwein and co-workers used both chemical labeling and synthesized histone peptides to study histone H3 methylation states of lysine 9 and 27 [57]. Histone H3 proteins were extracted from wild-type, G9a and Suv39h HTMases (histone methyltransferase) deficient mouse embryonic stem cells. Proteins were treated with propionic anhydride to derivatize unmodified and monomethylated lysine residues and mass spectrometric analysis performed on an ion trap instrument. Relative quantitation was done by determining the ratio of an individual methylated lysine peptide to the sum of all modification states of that lysine in a given peptide fragment. Synthesized peptide standards were used to normalize the relative differences in ionization efficiency of these histone peptides. Results from this study revealed a decrease in trimethylated lysine 9 and an increase in monomethylated lysine 9 for Suv39h HTMase deficient samples. This suggested that the Suv39h HMTases are H3 lysine 9 trimethylating enzyme which can convert monomethylated lysine 9 into the trimethylated state. Sample with the absence of G9a showed a reduction in mono-, and di-methylated lysine levels, but an increased level of the unmodified peptide. This indicated that unmodified lysine 9 is used as a substrate by G9a to induce mono- and di-methylation but not trimethylation. Analogous analysis of lysine 27 did not show any significant changes in levels of its methylated states between the wild-type, the Suv39h and G9a deficient samples.

Utilization of stable isotope labeling techniques has been a prevalent method for quantitation. Hunt and co-workers [40] developed a stable isotope labeling approach by which the carboxylic groups of peptides are converted to their corresponding ethyl esters. Comparative analysis of the modifications obtained

from two cell populations is achieved by converting peptides from the two sample sets to d_0- and d_5-ethyl esters, respectively. The two sets of histone samples were first derivatized by propionic anhydride and enzymatically cleaved using trypsin. Subsequently, the two samples sets were then subjected to derivatization with deutero (d_6) or ethanolic (d_0) HCl to generate deuterated and non-deuterated ethyl esters, respectively. The samples were mixed and analyzed by a hybrid ion trap-Fourier transform mass spectrometer. Identical peptides from the two samples will appear as doublet peaks separated by 5 Da for singly charged species. The relative abundances of peptides are obtained by integrating the area under the curve of the mass chromatogram. Additionally, with the accurate mass information provided by the FTMS, trimethylated peptides can be readily differentiated from acetylated peptides. One crucial factor in obtaining an accurate picture of changes in levels of modification is the means to normalize between the samples. In the case of histone H3, the peptide of residues 41–49 was used for normalization. So far, no modifications have been found on this peptide and it should therefore be present in equal amounts in both samples for the same amount of material. Analysis using this comparative methodology was applied to a number of studies such as monitoring of changes in histone H3 modification levels between wild-type and Kryptonite HTMase mutated form of Arabidopsis Thaliana [58]. Here, a significant change was found in the level of dimethylated lysine 9 which was down regulated in the mutated sample. Mutation of Kryptonite is known to suppress the gene silencing process [59], thus this finding suggests that dimethylated lysine 9 is the major species responsible for gene silencing in Arabidopsis. Similarly, changes in histone modifications from splenocytes of Lupus mice which were treated with histone deacetylase inhibitors were explored using this methodology [60]. Results from this analysis revealed global site-specific hypoacetylation and hyperacetylation on histone H3 and H4 of lupus versus wild-type mice. Also, identified were novel modifications such as histone H3 lysine 18 methylation, and H4 lysine 31 methylation and acetylations that are differentially expressed between the two sample sets. Moreover, in vivo treatment of the HDAC inhibitor, TSA, corrected the site-specific hypoacetylation states on H3 and H4 in lupus mice with improvement of the disease state.

The quantitation of histone modifications based on the MS chromatogram is not always the best method since the peptide in question can contain more than just one possible modification site. The measured peak area in such a case will be a composite of one modification but at different sites. For example, an H4 peptide of residues 4–17 contains four lysine residues that are all known to be acetylated. Peptides with one acetyl group on any of the four sites will have similar elution profile and will co-elute. Hence, determining the abundance of acetylation at each of the four different sites is not possible using the mass chromatogram. To tackle this problem, Smith et al. [61,62] developed an algorithm based on the MS/MS fragment ions of peptides within the N-terminal tail of histone H4 to determine the fraction of acetylation at specific lysine residues in the presence of multiple lysines. This group utilized deuterated acetic anhydride to derivatize unmodified lysines and obtain more uniformly cleaved tryptic peptides. There is a 3 Da mass difference between protiated and deuterated acetyl groups (42 Da versus 45 Da), hence the endogenous level of acetylation can be determined by mass spectrometry. Analysis of derivatized histone H4 peptides from the amino tail was done using an ion trap mass spectrometer and only doubly charged precursor ions were chosen for analysis. The intensities of peaks corresponding to the protiated and deuterated forms of various b and y ions were recorded at their maximum ion current. To determine the fraction of molecules with a specific acetylated lysine (f_{ac}), the measured signal intensity of a particular fragment ion was divided by the sum of the intensities for all protiated and deuterated species for that particular fragment. One key factor for the success of this approach for quantitation is the ability to obtain high yields of the fragment ions. Therefore, peaks with low ion current and those showing interference with other ions were omitted from quantitation analysis. As for any derivatization techniques, the labeling efficiency influences the quantitation; over- or under-derivatization will increase uncertainty in the measurements. Thus, following derivatization, the H4 protein was analyzed by LC–MS to determine the extent of derivatization, and results show no evidence of any under-derivatized lysines. From the analysis of MS/MS spectra of three sets of yeast histone H4, lysine 16 was found to be the most highly acetylated site at 80%, followed by lysine 12 at 54%, lysine 5 at 32% and lysine 8 at 24%.

The quantitative analysis has provided insights into the overall changes of histone PTMs during the developmental stages, from diseased versus healthy, and drug treated versus control cells. These initial quantitative studies highlight mass spectrometry as one of the key players in understanding the roles of the changes in levels of histone modifications towards regulation of chromatin structure and function.

6. Top down analysis: characterization of PTMs on intact histones

With the progress in extensively mapping, quantitation, and differential analysis of histone PTMs, the challenge of histone PTM analysis has shifted from simply identifying sites of modification to identifying combinations of histone modifications and classifying a modification pattern that results in a defined biological read out. As more sites are being discovered, more questions are raised as to the biological function or significance of a single and concurrent modifications identified on histones. The major technical hurdle on the mass spectrometry side for answering the new emerging questions is the task of examining all modification sites present simultaneously on a given histone protein. This is essential for understanding the biological role of the complex modification patterns of histones. The classical method for structural analysis of proteins by mass spectrometry, now referred to as ‘bottom up’, requires that the proteins be treated with proteases prior to analysis by MS/MS to create peptides suitable in size for sequence determination. However, after generating a peptide mixture, it is impossible to conclude which peptides originated from the same molecule. The interdependence of modifications localized on the various generated

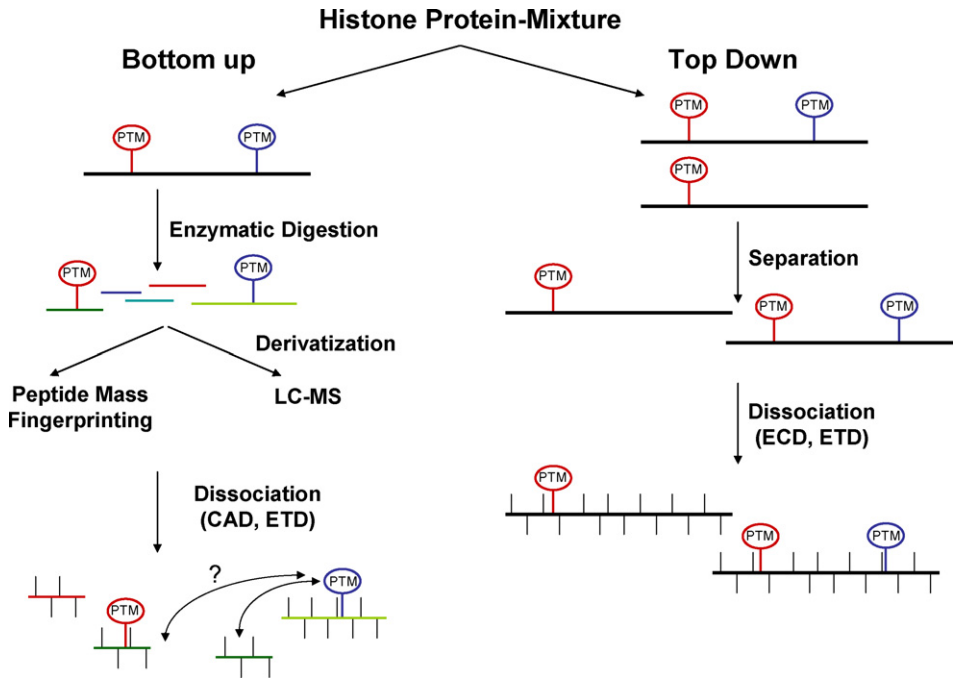

Fig. 1. Schematic showing the 'bottom up' and 'top down' approaches. In the top down analysis, the protein is dissociated and characterized without digestion which allows for detection of all PTMs on a single protein. In the bottom up analysis, the proteins are first digested into peptides and then analyzed which results in the loss of the information regarding the interdependence of modifications.

peptides is lost (Fig. 1). Hence, characterizing the PTM pattern of a given histone molecule is not possible using these MS/MS techniques alone.

A conceptually different approach is the 'top down' method pioneered by McLafferty and co-workers [63]. Here, the entire protein is directly dissociated to provide structural information. This dissociation is termed electron capture dissociation (ECD). ECD cleaves the peptide bond between the amide nitrogen and the α-carbon to form c- and z-type fragment ions. The intact proteins are introduced by ESI into the ion cyclotron resonance (ICR) cell of a Fourier transform mass spectrometer (FTMS). In the cell, they are immersed in a dense cloud of low energy electrons. Upon capture of an electron by the multiple positively charged proteins, an uneven electron species is created that dissociates in pathways different to low energy CAD or infrared multiphoton dissociation (IRMPD). Here, the energy is not deposited in increasing vibrational increments and consequently the weakest bond in a protein or peptide is no longer the preferred cleavage site. The resulting dissociation is therefore less dependent on the amino acid composition or presence of labile post-translational modifications. Hence, this technique provides extensive backbone cleavage even for whole proteins. For the same reason, post-translational modifications (i.e., phosphorylation), labile under low energy CAD conditions, remain intact in ECD. In addition, the intact protein mass is measured prior to dissociation with high resolution and mass accuracy. In the case of histone MS analysis, this provides a direct qualitative determination of the number and characterization of modifications present on a given histone population. The Kelleher research group used this dissociation technique successfully for studies on intact histone H4 from HDAC inhibitor treated (butyrate) and asynchronously grown HeLa cells [64]. The histones were acid extracted and histone H4 isolated with RP-HPLC. The sample was introduced into the custom built 8.5 T quadrupole enhanced Fourier transform mass spectrometer (Q-FTMS) via infusion and 15–40 ESI/FTMS full scans were averaged to obtain accurate mass information for the intact histone H4 proteins. About 100 ECD spectra per mass species were averaged and the complex dissociation data interpreted with help of ProSight PTM [65]. ProSight PTM is a search engine developed in the Kelleher lab tailored to searching *top down* data. To facilitate automated data interpretation, an 'overpopulated' histone H4 database was created. Besides unmodified histone H4, the database includes all potential protein forms based on published modification sites on histone H4. The full scan data showed the presence of five major populations in butyrate-treated and two in the asynchronous cells of histone H4. These major histone H4 species could be characterized and the measured mass shifts from the unmodified mass of histone H4 explained based on the ECD dissociation data, albeit some ambiguity of the exact localization of modifications.

Freitas and co-workers compared results from ECD of intact and tryptic peptides of histone H4 with the information obtained from peptide mass mapping using a Q-TOF system [66]. Here, the ECD results were in good agreement with the data obtained from peptide mapping, but the authors observed fewer sites identified with ECD.

Similarly, Burlingame, Marshall and co-workers used ECD to characterize Tetrahymena histone H2B variants and post-translational populations by ECD FT-ICR mass spectrometry [67]. The authors first acquired high resolution data on a LT-FTMS and subsequently interrogated several components with

CAD. Two major variants were present exhibiting a mass shift of +42 and +84 Da. As predicted, the information obtained from the CAD data was marginal as CAD only cleaved a few peptide bonds in the center of the protein sequence. It did however allow the identification of the two Tetrahymena H2B isoforms based on observed y-type fragment ions. On the contrary, ECD analysis allowed to identify both protein variants and to describe the modifications found concurrently on both variants. Still, some ambiguity existed as to the location of one trimethylation. This could be resolved by mapping the modifications using CAD LC–MS/MS on a tryptic and AspN digest of histone H2B in a subsequent analysis. This study highlights the power of combining bottom up with top down approaches. The top down data gives an overview of the major modification status of a given protein and the bottom up data allows mapping the modification to the exact site. Bottom up approaches alone can overlook modification sites if the peptide of interest carrying the amino acid change or modification was not identified.

In consecutive papers, the Kelleher lab showed ECD studies on histone H2B, H2A, and H3 [21,68,69]. Contrary to histone H4 and especially H3, fewer post-translational modifications on histone family members of H2B (with isoform H2B.A being the most abundant one) and H2A are described. Kelleher and co-workers were able to show the expression of seven different H2B isoforms and 14 H2A isoforms. In addition, using IRMPD, a high molecular weight species that was detected in histone samples isolated at the mitosis-G1 transition, was identified as ubiquitination on the variant H2A.O. A combination of intact mass measurements, a sequence tag matching five H2A family members, and five b-type ions from IRMPD fragmentation of ubiquitin allowed the indirect detection of ubiquitination on H2A.O as the modification of only this family member matched the measured molecular mass. The identification of these isoforms would not have been a trivial task using bottom up strategies. The differences between the individual isoforms are often only a change in a single amino acid and this change often corresponds to the addition or removal of a methyl group, i.e., the change of aspartic acid versus glutamic acid, valine versus alanine, etc. This makes the shift in mass identical to a monomethylated and dimethylated lysine or arginine residue. To tackle this problem, the authors labeled HeLa cells with L-methionine-methyl-^{13}C methyl-d_3 [21]. The metabolic labeling will result in methionines and methyl groups transferred by histone methyltransferases to appear 4 Da higher. This allows differentiating methylated histones from non-methylated but isobaric amino acid variants. To further prevent generation of complex spectra due to isotopic overlap, two methionines present in all histone H2B isoforms were oxidized using performic acid. Without this step, the difference of 16 Da for oxidized methionine would have overlapped with the difference of 14 Da from different isoforms or potential methylations and would complicate the resulting ECD spectra. Histones were acid extracted, purified using RP-HPLC and infused into the custom built 8.5 T Q-FTMS. The top down approach in combination with metabolic labeling allowed identifying histone isoforms differing only by a few amino acids to be described and their abundance quantified over the course of the cell cycle.

Among the four core histones, histone H3 harbors the greatest number of known modifications [10]. The analysis of histone H3 therefore poses the greatest challenge for a 'top down' analysis. The difficulty of histone analysis using 'top down' approaches is the occurrence of isobaric species, species that have the same accurate mass, but show a different distribution and composition of modifications, i.e., trimethylation in one case and mono- and dimethylation in another. A single mass therefore will be a composite of numerous differently modified histone molecules. The resulting ECD spectrum consequently will be a mixed one and render spectrum interpretation even more demanding. Kelleher and co-workers interrogated histone H3 with ECD and tried to limit the amount of isobaric species by separating RP-HPLC purified histone H3 on a cation exchange column [69]. This will separate histone H3 molecules according to their acetylation status. The different fractions were subsequently desalted using RP-HPLC and infused into the Q-FTMS. Acquisition times ranged from 6 to 26 min per spectrum. The resulting spectra are nevertheless so complex that the interpretation was increasingly difficult as the fragment size and the combination of modifications increases. Nevertheless, modifications from the N-terminal tail could be determined up to lysine 36. Even though this analysis could not reveal the modification status of an individual histone protein, it allowed to observe the occupancy of histone modifications based on quantification of c-type fragment ions. For instance, methylation of lysine 4 was found to be present in ~5% and dimethylation of lysine 9 at ~50% occupancy. Lysine 14 and lysine 23 were found to be the major sites of acetylation. Thus, this study provided an overview of the population status of histone H3 modifications not previously described.

Recently, Kelleher and co-workers have presented a quantitative study on human histone H4 [70] in which ion abundance from MS/MS fragment ions were correlated to their intact ion abundances. The protein/peptide relative ratios (PIRRs) and fragment ion ratios (FIRRs) of mixtures of histone H4 peptides and recombinant intact proteins were determined using an FTMS with ECD and IRMPD. The top three most abundant isotopes were integrated to calculate the intact ion abundances for three different charge states which were then averaged to calculate the PIRRs. As for the MS/MS fragment ions, the top three most abundant isotopes from abundant c ions were used to calculate the FIRRs of a mixture of two peptides/proteins or positional PTM isomers. Results show that PIRRs are represented truthful by their FIRRs for both peptide and protein fragmentation data. Most importantly, this study showed that ionization efficiencies for differently modified proteins are largely unaffected in sharp contrast to differently modified peptides which vary significantly with modification status (>20%). This methodology was then applied to the study of intact histone H4 isolated from asynchronously grown HeLa cells with and without the histone deacetylase inhibitor, butyrate. The relative acetylation occupancy determined by this methodology did not change dramatically over the butyrate incubation timecourse. Furthermore, the findings in this study suggest a different progression of acetylation on histone H4 than the previously mentioned study [37]. Acetylation was not found to progress from lysine 16 to 12, 8 and finally 5 because di-acetylated species were identified that are

acetylated on lysine 12 and lysine 8/5. This quantitative analysis using fragment ion ratios from intact protein analysis will be a powerful methodology for obtaining quantitative information of isomeric intact proteins.

An obvious limitation for intact histone analysis using ECD is the sensitivity, acquisition speed, and the limited dynamic range of fragment ions, as the complexity of histone mixtures increases. Imagine a +15 charged precursor ion of a 100 amino acid long protein fragmenting over the entire peptide backbone. The original total ion current is distributed over numerous fragment ions, many of which are present in more than one charge state or different modified states. Not only is the individual signal intensity of fragment ions low, but also the presence of a high density of fragment ions, some of them overlapping, makes spectral interpretation demanding. Furthermore, if the fragmented precursor was a composite of isobaric proteins that differed in their location of PTMs, usually the case for histone analysis, an additional level of complexity and ion signal dilution is introduced. Currently, the efficiency of ECD requires acquisition times of minutes and precludes on-line chromatography. As a result significantly more material is needed for ECD analysis as compared to bottom up strategies.

To overcome these problems, recently Hunt and co-workers developed an ion/ion analogue of ECD, electron transfer dissociation (ETD) [71]. Instead of an FTMS instrument, a Thermo Finnigan linear ion trap is used. Here, the electrons are introduced by means of radical anions, such as fluoranthene, that are generated in a chemical ionization source positioned opposite to the ESI source. The peptide cations are injected as usual by ESI, and after isolation of the precursor ion of interest, radical anions are injected from the back end of the linear ion trap and are allowed to react in the gas phase with peptide cations. As the radical anions transfer electrons to the cations, a rapid fragmentation along the peptide backbone is observed in a similar manner as in ECD leading to c- and z-type ion formation. Intact proteins can be directly dissociated with great efficiency, but lead to fragmentation spectra too complicated for analysis. Recall that the ion trap mass spectrometer, when operated in the usual scan mode, cannot resolve m/z for $z > 2$ and that multiply charged precursor ions give rise to multiply charged fragment ions. Hunt and co-workers therefore combined a second ion/ion reaction-termed proton transfer charge reduction (PTR) [72], employing deprotonated benzoic acid, for simplifying the complex dissociation spectra of intact proteins. Stephenson and McLuckey demonstrated that ion/ion reactions increase proportionally with the square of the charge [73]. Hence, the second reaction rapidly converts multiply charged fragment ions to singly and doubly charge species, resulting in a clean and straightforward interpretable spectra. Using these sequential ion/ion reactions, ETD followed by PTR of the product ions, Hunt and co-workers were able to read off the modifications on residues 1–50 of the N-terminal peptide of histone H3 in a complex mixture of asynchronous histone H3 N-terminal peptides which was introduced directly via chromatography into the mass spectrometer. In a second example, Hunt and co-workers identified an additional member of the H2A gene family not previously described, by analyzing a mixture of H2A.Z proteins employing on-line chromatography and tandem ETD/PTR reactions [72]. The benefits of ETD versus ECD are that the acquisition time is much faster, on the order of milliseconds, and only a few scans need to be averaged to provide high quality data on intact proteins, thus making this technology compatible with chromatography. Since similar chromatography conditions are compatible with ETD/PTR as the one used for classical bottom up approaches, similar amounts of sample are needed. Hence, ETD offers similar sensitivity as the existing CAD based strategies. The current limitations of ETD are the low resolution of the ion trap mass spectrometer and the limited mass range offered by this device. Since only singly and doubly charged fragment ions can be sufficiently resolved without sacrificing scan speed, the N- and C-termini with a mass range of 2000–4000 Da of a protein can be sequenced. That is usually sufficient to identify a protein, but it is not sufficient to characterize the modification status of highly modified histone proteins, such as histone H3. In a recent study, Hunt and co-workers used a new approach [manuscript in preparation] combining chromatographic separations and ETD/PTR for the analysis of histone H3 PTMs from the model organism Tetrahymena thermophilia. The study focused on the 50 amino acid N-terminal peptide that encompasses the majority of histone H3 modification sites. First, the heterogeneous population of histone H3 proteins was successfully resolved based on their acetylation status by cation exchange chromatography. Second, fractionated proteins are digested by the protease Glu-C and the accurate mass of the resulting polypeptides recorded with the LT-FTMS. Third, the polypeptides are fragmented by ETD with each fragmentation spectrum simplified by PTR prior to mass analysis and the data acquired using the zoom scan function of the ion trap. The high quality MS/MS spectra yield sequence coverage of >95% of the N-terminal peptide based on significantly higher S/N from PTR and extensive fragmentation from ETD.

ECD and ETD are techniques that have successfully allowed to assess the population status of histone modifications on the intact protein level and describe modifications present simultaneously on a given histone. These techniques offer new and exciting applications for protein analysis in general and will especially be an indispensable tool for histone research. Certainly, ECD and ETD come with their own set of difficulties, but with further development and wider accessibility of these techniques to more research groups, more progress will be made in this field and more questions to the role of histone modifications will be answered.

7. Conclusions

Histone PTM analysis has proven to be a challenging area and mass spectrometry has been demonstrated to be a very powerful tool in both qualitative and quantitative analysis of histone modifications. Constant improvements in methodologies and new techniques allowed to move histone analysis forward in a rapid pace. ECD and ETD offer an overview and description of all modifications present on an intact histone which cannot be provided by the classical bottom up approaches. Conversely, bottom up approaches offer more detailed information that top down

approaches cannot provide. The application of all approaches described in this review will remain crucial for understanding the role of histone PTMs in regulation of chromatin mediated processes.

Despite the number of histone research increasing at a fast pace, we are still at the early stages in this field. One of the research area that is growing rapidly is the clinical aspect of the effects of HDAC inhibitors on histone modifications. Drugs which act as HDAC inhibitors are able to block angiogenesis, cell cycle progression and promote apoptosis. By targeting these tumor proliferation factors, HDAC inhibitors are playing an important role in cancer therapy. Future research involving development of more screening tools and a better understanding of the roles of HDAC agents in affecting cellular functions will aid in producing therapeutics with the right selectivity and efficacy for a specific illness.

While modification patterns are being identified, the current technologies still limit us to determine these modifications only on either the N- or C-terminal tails of the histones without ambiguity. With more central domain sites with biological relevance being identified, the current challenge is to develop approaches to efficiently determine all sites of modifications on a given histone protein. Despite the identification of these modifications, their physiological roles are still poorly understood. A significant challenge in the future lies in correlating all the catalogued sites and patterns of modifications to specific and meaningful biological readouts. Certainly biological approaches such as chromatin immunoprecipitation (ChIP) and microarray analysis for studies of identified histone modifications on a genome-wide scale will be complimentary to mass spectrometry. Ultimately, it is the combination of both the biological and mass spectrometric techniques that will provide a better understanding of the complexity of the histone code.

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