Mild Performic Acid Oxidation Enhances Chromatographic and Top Down Mass Spectrometric Analyses of Histones

James J. Pesavento‡§, Benjamin A. Garcia¶¶, James A. Streek¶¶, Neil L. Kelleher‡¶¶, and Craig A. Mizzen**‡‡

Recent developments in top down mass spectrometry have enabled closely related histone variants and their modified forms to be identified and quantitated with unprecedented precision, facilitating efforts to better understand how histones contribute to the epigenetic regulation of gene transcription and other nuclear processes. It is therefore crucial that intact MS profiles accurately reflect the levels of variants and modified forms present in a given cell type or cell state for the full benefit of such efforts to be realized. Here we show that partial oxidation of Met and Cys residues in histone samples prepared by conventional methods, together with oxidation that can accrue during storage or during chip-based automated nanoflow electrospray ionization, confounds MS analysis by altering the intact MS profile as well as hindering posttranslational modification localization after MS/MS. We also describe an optimized performic acid oxidation procedure that circumvents these problems without catalyzing additional oxidations or altering the levels of posttranslational modifications common in histones. MS and MS/MS of HeLa cell core histones confirmed that Met and Cys were the only residues oxidized and that complete oxidation restored true intact abundance ratios and significantly enhanced MS/MS data quality. This allowed for the unequivocal detection, at the intact molecule level, of novel combinatorially modified forms of H4 that would have been missed otherwise. Oxidation also enhanced the separation of human core histones by reverse phase chromatography and decreased the levels of salt-added forms observed in ESI-FTMS. This method represents a simple and easily automated means for enhancing the accuracy and sensitivity of top down analyses of combinatorially modified forms of histones that may also benefit for top down or bottom up analyses of other proteins. Molecular & Cellular Proteomics 6:1510–1526, 2007.

1 The abbreviations used are: TD, top down; PTM, posttranslational modification; RP, reverse phase; MPA, mild performic acid; OCAD, octapole collisionally activated dissociation; ECD, electron capture dissociation; SWIFT, stored waveform inverse FT; aLys, α-N-acetyllysine; αSer-1, α-N-acetyllysine 1; 2mLys, dimethyllysine; 3mLys, trimethyllysine; mArg, methylarginine; OMet, oxidized methionine; BME, β-mercaptoethanol (BME) or DTT. However, these reagents are not as effective in the low pH conditions typically used to extract and recover histones and in common methods for their analysis and purification.
cation. Partial oxidation of methionine to methionine sulfoxide has been shown to occur during the purification of histones H2B, H3, and H4 by preparative electrophoresis (11). Similarly partial oxidation of Met-57 was reported to complicate mass spectrometric analyses of Tetrahymena histone H2B prepared by RP-HPLC due to the similarity in mass change with that of methylation (7). Partial oxidation of proteins complicates MS analyses because the signal is spread across multiple oxidized masses, potentially creating artifactual mixtures with biologically important posttranslational modifications or masking their presence. The levels of partially oxidized histones we have detected in samples prepared in our own laboratory and by other laboratories during the course of our work to optimize top down analyses suggest that oxidation is a common occurrence in standard methods of histone preparation. Moreover the existence of methionine sulfoxide reductases capable of reducing partially oxidized methionines argues that some fraction of this oxidation may occur in vivo (12) possibly as a consequence of the activity of DNA repair pathways (13, 14) or due to reaction with H2O2 or other reactive oxygen species under conditions of oxidative stress (15).

Because it is the partial oxidation of proteins that causes problems with mass spectral analysis (see below), an approach capable of completely oxidizing partially oxidized proteins would be valuable. Performic acid oxidation, a method that dates back to the 1960s, completely oxidizes methionine to methionine sulfoxide and cysteine to cysteic acid (16, 17). Recent proteomics research has utilized performic acid oxidation to protect cysteine residues from labeling chemistry (18, 19), to increase the confidence of database searching scores (20), and to enrich cysteine-containing peptides after strong cation exchange liquid chromatography (21). However, other amino acids can be modified in addition to Met and Cys under the conditions originally described (i.e. 9 ml of 88% formic acid + 1 ml of 30% hydrogen peroxide), artifically increasing sample complexity. These include multiple oxidation products of tryptophan, oxidation and chlorination of tyrosine, nonspecific cleavage after asparagine or tryptophan, formylation of lysine, and β-elimination of cysteine (22, 23).

Here we show that incubating proteins with 3% hydrogen peroxide oxidizes methionine and cysteine residues to methionine sulfoxide and cysteic acid, respectively, and can catalyze disulfide bond formation between cysteines that share sufficient proximity. Moreover we show that limited performic acid treatment (3% hydrogen peroxide + 3% formic acid) selectively and quantitatively oxidizes only Met and Cys residues in human core histones to methionine sulfoxide and cysteic acid, respectively. When performed prior to chromatography, oxidation also enhanced the separation of core histones by RP-HPLC. TDMS analysis revealed that the intact mass profiles of oxidized histones are more representative of the true PTM composition due to loss of overlap between differentially oxidized forms and a decrease in the abundance of salt-adducted molecules. Furthermore we found that the decreased spectral complexity of intact histone both enhanced MS/MS spectral clarity and increased the dynamic range for detection of fragment ions after electron capture dissociation (ECD) (24) by approximately 2–5-fold depending on the level of unintentional oxidation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Histone Preparation**—HeLa S3 cells were grown in suspension in Joklik’s modified minimum Eagle’s medium supplemented with 10% (v/v) newborn calf serum and 100 units of penicillin and streptomycin/ml. Samples for biochemical analysis were collected by centrifugation, washed twice with cold TBS, flash frozen in liquid N2, and stored at −80 °C prior to nucleus isolation. HeLa S3 nuclei were prepared by lysing the cells in nucleus isolation buffer (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 250 mM sucrose, 1 mM dithiothreitol, 5 mM microcystin-LR, 500 μM 4-((2-aminoethyl)benzenesulfonyl fluoride, 10 mM sodium butyrate) supplemented with 0.3% Nonidet P-40 followed by low speed centrifugation to prepare nuclei as described previously (25). Crude histones were extracted with 0.4 N H2SO4 and recovered by TCA precipitation. The pellet was washed with acetone + 0.1% (v/v) HCl followed by two washes with 100% acetone, air-dried, and stored at −80 °C. In some cases, 50 mM 2-mercaptoethanol was included throughout the procedure to assess the extent of oxidation attributable to sample preparation.

**Oxidation of Crude Histone and Yeast Mating Factor α**—To determine the time course of core histone oxidation, 320 μg of crude histone was dissolved in a final volume of 323 μl with a final concentration of either 3% (v/v) H2O2 (Fisher), 3% (v/v) formic acid (Fisher), or a freshly prepared mixture of 3% H2O2 and 3% formic acid. 50 μl (50 μg) of samples was collected after 2, 4, 8, 32, and 72 h at room temperature. The time course for oxidation of yeast mating factor α (MF-α; Bachem) was carried out in a similar fashion using 50 μg of peptide dissolved in 50 μl of each reagent with 10 μl (10 μg) taken at the respective time points. For H2O2 oxidation of MF-α, 10 μg of peptide was dissolved in 50 μl of 3% H2O2 and kept at room temperature for 24 h. All reactions were quenched by immediate injection onto a Vydac C18 column and purified by reverse phase liquid chromatography.

**Reverse Phase High Performance Liquid Chromatography**—RP-HPLC-purified histones were prepared by chromatography of approximately 100 μg of crude histone protein, with or without prior oxidation, using a Vydac C18 column (2.0-mm inner diameter × 250 mm) using a multistep gradient from buffer A (0.2% TFA in 5% CH3CN) to buffer B (0.188% TFA in 90% CH3CN). All major peaks were recovered by vacuum drying in a SpeedVac. Recovered fractions were dissolved in distilled H2O, identity and purity were assessed by SDS-PAGE, and fractions were stored frozen at −20 °C prior to further analysis.

**Hydrophilic Interaction Liquid Chromatography (HILIC)** of Reverse Phase-purified H4—Differentially modified forms of H4 in the RP-HPLC peak prepared from asynchronous HeLa cells were resolved by HILIC as described previously (26). Approximately 40 μg of H4 was loaded onto a Poly CAT A column (PolyLC, Columbia, MD) at 25 °C at a constant flow of 0.8 ml/min and resolved using a multistep gradient from 100% solvent A to 100% solvent B. Solvent A contained 65% acetonitrile, 0.015 M triethylamine, H3PO4, pH 3.0, and solvent B contained 60% acetonitrile, 0.015 M triethylamine, H3PO4, pH 3.0, plus 0.68 M NaClO4. The concentration of solvent B was increased linearly from 0 to 10% for 10 min, from 10 to 30% for 40 min, and then to 100% over 5 min. Protein elution was detected at 214 nm. Fractions were collected every minute, and proteins of interest were

**Molecular & Cellular Proteomics** 6.9

**Mild Performic Acid Oxidation of Histones**
Mild Performic Acid Oxidation of Histones

**FIG. 1.** Theoretical isotopic distribution of diacetylated (11,355-Da), diacetylated and monomethylated (11,369-Da, circle), and diacetylated and partially oxidized (11,371-Da, square) forms of histone H4. A, the isotopic distribution of the diacetylated form of H4 is almost completely resolved from the diacetylated, methylated/partially oxidized forms. However, molecules that are diacetylated and methylated overlap significantly with molecules that are diacetylated and partially oxidized (thickened black isotopes signify overlap). B, an example of different histone H4 acetylation profiles on a diacetylated molecule (top; 11,355 Da) versus a diacetylated and monomethylated molecule (middle; 11,369 Da). The diacetylated and methionine sulfoxide (partially oxidized) molecule (11,371 Da) has the same acetylation profile as the parental unoxidized molecule yet has a mass increase of +16 Da. Isolation of a mixture containing the diacetylated and monomethylated form together with the diacetylated and partially oxidized form would result in ambiguous assignment of the acetylation sites to either the monomethyl or methionine sulfoxide form (bottom H4 sequence).

recovered by partial drying to remove acetonitrile followed by precipitation with 20% (w/v) TCA and extensive washing with 20% TCA and subsequently with acetone, 0.1% (v/v) HCl and with acetone.

**Mass Spectral Analysis by ESI-FTMS/MS—** All data were acquired on a custom 8.5-tesla quadrupole-FT ICR MS instrument with an ESI source operated in positive ion mode (27). A quadrupole (ABB Extrel, Houston, TX) was used to select either the +12, +13, +14, +15, or +16 charge state of a histone species; these charge states were then accumulated in a radio frequency-only octupole equipped with a direct current voltage gradient for improved ion extraction to the ICR cell (28). The quadrupole selection window was set at ~40 m/z and centered around the most abundant histone peak. For MS/MS using ECD, stored waveform inverse FT (SWIFT) (29) was used to further filter the selectively enhanced charge states, and the data were collected using the modular ion cyclotron data acquisition system (MiDAS) (29, 30). Typically 30- and 5-μl samples were enough for more than 150 min of stable nanospray using a NanoMate 100 (Advion) with ~200 nL/min and low (~50 nL/min) flow nanospray chips, respectively, providing ample time to acquire high quality MS and MS/MS scans of four to eight intact protein forms per sample.

**Octupole Collisionally Activated Dissociation (OCAD)—** External to the magnet bore, ions were selected using the quadrupole and fragmented using electrostatic acceleration (10–45 V) into an octupole pressurized to ~10 millitorrs with nitrogen gas. All relative molecular weight (M_r) values and fragment masses are reported as neutral, monoisotopic species.

**Electron Capture Dissociation—** ECD was performed by applying 5 A through a dispenser cathode filament (Heatwave Technologies, Crescent Valley, British Columbia, Canada). During the ECD event, ~10 V was applied on the grid potential while ~9 V were sent through the filament for optimal ECD. Typically 15 cycles of ECD were performed with individual irradiation times of 500 ms and a 10-ms relaxation time between cycles. All relative molecular weight (M_r) values and fragment masses are reported as neutral, monoisotopic species.

**RESULTS**

**Partial Oxidation Increases TDMS Complexity—** Combinatorial modification of histones has been suggested to represent a “code” that mediates the regulation of critical DNA-templated processes in chromatin including transcription and replication and may also play a direct role in determining chromatin condensation during the cell cycle (32–34). Thus, the ability of TDMS to resolve combinations of modifications is a primary determinant of the efficacy of this approach for investigating histone biology. One obstacle to unambiguous identification of modifications on intact histones is the presence of mixtures of oxidation products of methionine (methionine sulfoxide and methionine sulfone) and of cysteine (sulfenic, sulfonic, and sulfonic acids of cysteine). These oxidations create mass increases, respectively, of 16 and 32 Da per methionine and 16, 32, and 48 Da per cysteine residue that can overlap with posttranslational modifications such as a single methylation (+14 Da), two methylations (+28 Da), or three methylations or one acetylation (+42 Da), which are relatively abundant in histones. When these modifications occur simultaneously with partial oxidation in proteins, the overlapping isotopes observed on an 8.5-tesla FTMS instrument (~10^6 resolving power) make mass spectral isolation of an oxidized protein from a modified protein extremely difficult (Fig. 1A). If the sites and extent of oxidation are known, it may

ProSight PTM, a Web-based software and database suite, was used to accelerate the characterization of histone protein forms (3, 31).

1512 Molecular & Cellular Proteomics 6.9
be possible to account for the effects of oxidation when interpreting spectra (see "Discussion"). Nonetheless partial oxidation in complex mixtures can be expected to sharply reduce the clarity of top down analyses because they rely on intact masses as a constraint on the mass differences of fragment ions. For example, if a diacetylated H4 (mass of 11,355 Da) has an acetylation profile (aLys-5 + aLys-8) that is different from a diacetylated, monomethylated H4 (mass of 11,369 Da; aLys-12 + aLys-16), oxidation of the former (mass of 11,371 Da) creates a form whose isotopic distribution significantly overlaps the latter (Fig. 1A). These two PTM profiles are merged during subsequent isolation and fragmentation, obscuring the connection between methylation state and the corresponding acetylation profile (Fig. 1B). It should be noted that instruments with lower resolving power will not give isotopic resolution on larger peptides and proteins, making oxidation even more difficult to discern from methylation or other PTMs (e.g. \( \sim 10^8 \) on TOF-based detectors).

Rapid Partial Oxidation of Histone H4 Directly after Sample Preparation Is Readily Detected by Top Down MS—Histone H4 was prepared from butyrate-treated HeLa cells and analyzed directly by FTMS using a NanoMate 100 (Advion) equipped with a low flow rate chip (\( \sim 50 \, \text{nl/min} \)) as the ESI source. The mass spectrum showed several abundant dimethylated forms of H4 (most H4 in asynchronous HeLa cells is dimethylated at Lys-20 (3)) bearing 1–4 \( \epsilon \)-N-acetylions (denoted by \( 2m \) and \( 1–4ac \) in Fig. 2A) and a similar distribution of related forms whose respective masses were all 14–16 Da greater. Thorough examination of the isotopic distribution of these latter species revealed that the mass difference in each case was closer to +16 Da rather than +14 Da, suggesting that it corresponded to an oxidation (Fig. 2A, inset). This oxidation was localized by MS/MS to residue 84, the sole methionine present in each of these abundant acetylated forms of H4 (denoted as [O] in Fig. 2A). The same sample was then subjected to complete, intentional methionine oxidation (see below), and a mass spectrum was obtained using the same instrumental parameters as for the untreated sample. As shown in Fig. 2B, this treatment made partially oxidized forms undetectable at the isoforme level (Fig. 2B, inset) and at the MS/MS level (data not shown). The mass of each of the abundant acetylated, dimethylated forms was increased by 32 Da consistent with conversion of methionine and methionine sulfoxide residues to methionine sulfone (denoted as \( 2[O] \) in Fig. 2B). It should be noted that discriminating a +16-Da (oxidation) from a +14-Da (methylation) mass shift is not trivial because an unknown mixture of the two distributions makes unequivocal mass determination difficult. Furthermore on lower resolution mass spectrometers, especially those lacking top down capabilities, the presence of both methylation and oxidation may lead researchers to incorrect conclusions based on contaminated mass spectra.

We then examined the relative contributions of sample preparation and MS analytical conditions to the levels of partial oxidation detected. Our laboratory and others have experienced increased oxidation at lower ESI flow rates, so we proceeded to analyze the same exact sample of H4 from butyrate-treated cells using a regular flow chip (\( \sim 200 \, \text{nl/min} \)) (Supplemental Fig. 1). Remarkably the MS from the regular flow chip showed very little oxidation even after \( \sim 180 \, \text{s} \) of continual spray. Conversely switching back to a low flow chip again augmented the levels of the +16-Da series of shoulder peaks detected after 15, 60, and 180 s of continual spray time. These findings suggested that the use of a low flow rate chip artifically increased the levels of partially oxidized species over time. Unfortunately the simple remedy of using regular flow chips exclusively is not always possible given the fact that it may take 4 h or longer to obtain high quality ECD spectra, making low flow rate chips the preferred option for minute samples. Furthermore as mentioned below, prolonged storage of protein samples at \( \sim 80 \, \text{°C} \), even in the presence of antioxidants, can increase the levels of protein oxidation. Together these factors illustrate the need for a method capable of reducing or oxidizing partially oxidized protein forms in a quantitative fashion.

Gradual Oxidation of Samples during Storage: Human Histone H4—Despite measures such as including 50 mM BME throughout nucleus isolation, histone extraction, and recovery and during recovery and storage of histones following purification by RP-HPLC, we have been unable to identify conditions that completely prevent partial oxidation of Met residues during histone preparation. The varying abundance of partially oxidized forms most noticeably complicates top down analyses of histones H4 and H3, generally the most extensively acetylated and methylated species in samples from asynchronous cell cultures and animal tissues. The tendency for oxidation to occur during routine storage of samples is particularly problematic. This was first observed in histone H4 prepared from a sample enriched in cells in M phase obtained from a synchronized culture of HeLa cells that was dried following reverse phase purification and then resuspended in water with 50 mM BME. This sample was immediately analyzed by FTMS and then stored at \( \sim 80 \, \text{°C} \) prior to being reanalyzed 1 and 3 months later on a regular flow Advion chip. The relative abundances for the three lower mass forms (11,271, 11,285, and 11,299 Da) did not vary significantly when analyzed at different times (Fig. 3A). However, a dramatic increase in the relative abundance of the mass \( \sim 11,314 \) Da occurred over time. Closer examination of the isotopic distribution revealed that, in addition to an increasing abundance, the molecular mass value observed for this form had increased by \( \sim 1 \) and \( \sim 2 \) Da after 1 and 3 months, respectively, at \( \sim 80 \, \text{°C} \) (Fig. 3A, middle and bottom mass spectra). This gradual increase by 2 Da suggested that the 11,299-Da form was being oxidized (11,299 + 16 = 11,315 Da), creating an overlapping distribution with the 11,313-Da form. Isolation and ECD fragmentation of the 11,315-Da form shown in the bottom mass spectrum of Fig. 3A (asterisk) confirmed the
presence of Met-84 sulfoxide as the predominant form (see Fig. 7 for a thorough investigation of the ECD spectra from this partially oxidized form). We also found that adventitious oxidation occurred during HILIC, which we use as a second dimension following RP-HPLC to fractionate histones H1, H3, and H4 according to PTM content. Analysis of a diacetylated H4 fraction from asynchronous HeLa cells immediately following recovery from HILIC showed an intact MS profile with many peaks that were determined to be a mixture of PTMs and oxidation (Fig. 3B, top mass spectrum). ECD of each intact form above 5% yielded small \(z \pm H1\) ions and large \(c \pm H1\) ions that confirmed the presence of partial Met-84 oxidation (data not shown). To test whether Met-84 could be completely converted to the sulfoxide, hydrogen peroxide (3% final concentration) was added to a 1 \(\mu M\) H4 sample already in an ESI solution (49:49:2, H2O:MeOH:formic acid). This sample was

**Fig. 2.** MS of H4 isolated from butyrate-treated HeLa cells reveals a high degree of partial oxidation. A, histone H4 was resuspended in ESI solvent and immediately analyzed by FTMS using a low flow rate chip as the ESI source. Oxidation of each major acetyl, dimethyl form was determined by comparing the observed isotopic distribution with a theoretical distribution of a triacetyl (3ac) + dimethyl (2m) (●), triacetyl + dimethyl + oxygen ([O]) (□), and triacetyl + trimethyl (3m) (■) (or tetraacetyl (4ac) because they have overlapping isotopic distributions; see inset). B, intentional and complete oxidation of H4 restores the original abundance profile by removing the overlap of partial oxidation with H4 PTM forms. NaBu, sodium butyrate; 1ac, monoacetyl; 2ac, diacetyl.
then analyzed by FTMS after 10 min at room temperature and again after 2 weeks at −80 °C. The samples analyzed immediately showed minimal oxidation (top mass spectrum) with the most abundant peak (asterisks) identifying that cluster of isotopes as having a monoisotopic mass of 11,313 Da (Δm of +84 Da relative to unmodified H4). After storage at −80 °C, noticeable oxidation of H4 was observed because the most abundant peak shifted to higher mass over time. After 3 months (mo), the most abundant peak identified that cluster of isotopes as having a mass of 11,315 Da (Δm of +86 Da), which is the same mass of an oxidized 11,299-Da form immediately to the left of this peak (70 + 16 = 86 Da). B, the MS profile of a representative diacetylated H4 HILIC fraction. The fraction was immediately resuspended in ESI solvent (49% water, 49% methanol, 2% formic acid) following recovery, and the resulting MS profile showed significant levels of oxidized H4 (top mass spectrum). The addition of 3% H2O2 to the ESI solvent shifts the peaks by +16 Da within 10 min at room temperature (middle mass spectrum) and by +32 Da after storage at −80 °C for 2 weeks (bottom mass spectrum). The post-Lys-20 fragment ion relative ratios (i.e., ions that contain all PTMs) match up closely to the protein ion relative ratios after oxidation is complete (bottom mass spectrum).

Fig. 3. Partial oxidation during storage of histone H4 skews the intact MS modification profile. A, histone H4 from cells collected during M phase of the cell cycle was purified by RP-HPLC and resuspended in water supplemented with 50 mM BME. An aliquot was analyzed immediately, and the remainder was stored at −80 °C. The samples analyzed immediately showed minimal oxidation (top mass spectrum) with the most abundant peak (asterisks) identifying that cluster of isotopes as having a monoisotopic mass of 11,313 Da (Δm of +84 Da relative to unmodified H4). After storage at −80 °C, noticeable oxidation of H4 was observed because the most abundant peak shifted to higher mass over time. After 3 months (mo), the most abundant peak identified that cluster of isotopes as having a mass of 11,315 Da (Δm of +86 Da), which is the same mass of an oxidized 11,299-Da form immediately to the left of this peak (70 + 16 = 86 Da). B, the MS profile of a representative diacetylated H4 HILIC fraction. The fraction was immediately resuspended in ESI solvent (49% water, 49% methanol, 2% formic acid) following recovery, and the resulting MS profile showed significant levels of oxidized H4 (top mass spectrum). The addition of 3% H2O2 to the ESI solvent shifts the peaks by +16 Da within 10 min at room temperature (middle mass spectrum) and by +32 Da after storage at −80 °C for 2 weeks (bottom mass spectrum). The post-Lys-20 fragment ion relative ratios (i.e., ions that contain all PTMs) match up closely to the protein ion relative ratios after oxidation is complete (bottom mass spectrum).

Met-84 sulfoxide and Met-84 sulfone (data not shown). After 2 weeks at −80 °C, Met-84 was completely converted to methionine sulfone (Fig. 3B, bottom mass spectrum). Complete oxidation of methionine created intact protein ion intensity ratios that matched closely with the post-Lys-20 c ion intensity ratios (35). These observations led us to speculate that the relatively low concentrations of hydrogen peroxide and formic acid in this mixture were capable of catalyzing the complete conversion of methionine to methionine sulfone.
Because hydrogen peroxide alone or in combination with formic acid has been used previously to oxidize Met and Cys residues, we investigated the use of these reagents to develop a protocol for complete oxidation of Met and Cys residues that would be compatible with TDMS.

Optimization of Mild Performic Acid Oxidation for Histones—Recombinant histone H4 was used as a model substrate to examine the affects of H2O2, formic acid, and both H2O2 and formic acid in a time- and dosage-dependent fashion. Early protocols using performic acid to oxidize proteins used a mixture of 9 ml of 88% formic acid to 1 ml of 30% H2O2 to create performic acid (17). After using this protocol for 4 h, MS of performic acid-oxidized recombinant histone H4 showed formylation of lysine residues as well as oxidation of methionine (data not shown). Adjusting the performic acid formulation to 3% H2O2 + 3% formic acid (v/v, final concentration for each) and treating for 4 h resulted in oxidation of only methionine to methionine sulfone and did not produce any side reactions as determined by MS and MS/MS (data not shown). We then tested the effect of treating acid-extracted whole histone (AEWH) from asynchronous HeLa S3 cell nuclei with 3% formic acid only, 3% H2O2 only, or 3% H2O2 + 3% formic acid for 2, 4, 8, 32, and 72 h prior to purification of individual histones by RP-HPLC. As expected based on past literature, the core histones in untreated AEWH eluted in the following order: H2B, H2A-1, H4, H2A-2, H3.2, H3.3, and H3.1. B, hydrogen peroxide (left side) converts all methionines to the sulfone; however, the H3 chromatographic regions remain dynamic throughout the time course presumably due to differences in cysteine oxidation state. Mild performic acid treatment (right side) results in seven well resolved peaks whose retention remains unaltered after 2, 4, and 8 h of treatment.

![Fig. 4. RP-HPLC chromatographs of AEWH after treatment with 3% formic acid, 3% hydrogen peroxide, or mild performic acid (3% H2O2 + 3% formic acid). A, a typical chromatogram of untreated (left) and 3% formic acid-treated (right) AEWH. Formic acid treatment does not alter the typical histone RP-HPLC elution profile. The order of elution is H2B, H2A-1, H4, H2A-2, H3.2, H3.3, and H3.1. B, hydrogen peroxide (left side) converts all methionines to the sulfone; however, the H3 chromatographic regions remain dynamic throughout the time course presumably due to differences in cysteine oxidation state. Mild performic acid treatment (right side) results in seven well resolved peaks whose retention remains unaltered after 2, 4, and 8 h of treatment.](image)
methionine residues in histones H2A, H2B, and H4 to the sulfoxide very rapidly because the elution position of each of these proteins was quantitatively shifted following the 2-h treatment and was not further altered by longer incubation with H2O2 (Fig. 4B, left). In the case of H2A, the Met-containing variants expressed in HeLa cells all elute in peak H2A-1 (1), and thus the retention of peak H2A-2 is unaffected. The elution of the H3 variants was also altered following incubation of AEWH with H2O2, but in this case, multiple peaks with novel retention times whose relative abundance varied with the duration of H2O2 treatment were obtained. Because previous work suggested that methionine residues are rapidly converted to the sulfoxide by H2O2 alone (11), we suspect that at least some of these “transient” H3 peaks contain partially oxidized forms of cysteine (36) (i.e. sulfenic, sulfonic, and sulfonic acids) but did not characterize them in detail. Treatment of AEWH with the 3% H2O2 + 3% formic acid generated seven major peaks that are well resolved in the chromatogram (Fig. 4, right). Complete conversion of methionine to methionine sulfone and cysteine to cysteic acid appears to occur relatively fast under these conditions because intermediate peaks were not observed during the time course. SDS-PAGE of the major peaks for the 4-h time point revealed that a single band was resolved for each species whose relative mobility was equivalent to that observed for untreated AEWH (data not shown), demonstrating that this duration of treatment did not damage the proteins aside from oxidizing Met and Cys residues (see below). However, as the length of incubation with 3% H2O2 + 3% formic acid was increased beyond 4 h, additional peaks appeared throughout the chromatogram. These extra peaks suggest that it is likely that additional reactions (e.g. formylation, tyrosine chlorination, β-elimination of cysteic acid, etc.) (22) occur during extended treatment, but we have not attempted to characterize these species.

**Intact Mass Analysis of Oxidized Histones**—Several repetitions of the experiment described above demonstrated that the results obtained with 3% H2O2 + 3% formic acid for 4 h at room temperature appeared to give quantitative oxidation of histone Met and Cys residues in highly reproducible fashion. We refer to these conditions as mild performic acid (MPA) treatment below. Because previous work has shown that exposure of proteins to greater concentrations of performic acid can cause chemical modifications besides oxidation, we next used TDMS to investigate whether MPA treatment resulted in any such undesired changes. AEWH from asynchronous HeLa S3 cells was incubated for 4 h at room temperature with either water, 3% H2O2 only, or 3% H2O2 + 3% formic acid prior to RP-HPLC. The major peaks recovered were then analyzed by MS (Fig. 5) and MS/MS (Fig. 6) and are listed in Table I. For the sake of clarity, if multiple family members of a given histone (e.g. H2B.Q, H2B.A, H2B.K/T, H2B.J, H2B.E, H2B.B, and H2B.F) are present in a fraction, a generic representative name is assigned to that fraction (e.g. H2B). The histones in fractions H2B, H2A-1, H4, H2A-2, and H3.2 (see Fig. 4) each have two, one, one, zero, and two methionines, respectively. Histone H3.1 has two cysteines, whereas H3.2 and H3.3 both contain one cysteine. For untreated AEWH, MS analysis confirmed the identity of each major histone species (Fig. 5, B–G, top mass spectra, and Table I). The MS profiles, including both posttranslational modifications and protein family members, are similar to those previously reported by our laboratory (1, 3–5). For histones that are heavily modified, the extent of oxidation in intact MS profiles is very difficult to ascertain. This problem is exemplified by the untreated H3.1 MS profile, which has ~14 masses that are spaced every +14 to +16 Da away from each other (Fig. 5G). Typically the most abundant H3.1 form has a mass increase of +70 Da from the unmodified mass (+28 Da from two methylations and +42 Da from one acetylation) (5). In this study, the intact mass of the most abundant H3.1 species from the untreated sample was +86 Da greater than the unmodified mass, suggesting that partial oxidation had shifted the +70-Da form by +16 Da. Theoretically oxidation of AEWH with 3% H2O2 will shift the mass of each histone in units of +16 Da for each oxygen incorporated depending on how many methionines and cysteines are present in the protein. As shown by the middle mass spectra in Fig. 5, B–G, after treatment with 3% H2O2, each histone (with the exception of H2A-2; Fig. 5E) increased in mass relative to the corresponding untreated mass (Fig. 5, B–G, top mass spectra). Table I lists the theoretical mass and that observed for the most abundant forms of the untreated, 3% H2O2-, and 3% H2O2 + 3% formic acid-treated histones. Considering the mass difference of the oxidized histone H3.2 with the number of methionines/cysteines in that protein, it appeared that methionine was only oxidized to the sulfoxide, whereas cysteine was completely converted to cysteic acid during treatment with 3% H2O2 alone (two Met (+32) and one Cys (+48) = +80 Da). This observation was confirmed by MS/MS (see below). In contrast, after H2O2 treatment the observed mass increase of histone H3.1, which has two cysteines and two methionines, was only 30 Da instead of the expected 128 Da (two Met (+32) and two Cys (+48) = +128 Da). This suggests that both methionines are converted to the sulfoxide (+32 Da), whereas the two cysteines appear to be in a disulfide bond (~2 Da), making them insensitive to H2O2 oxidation. After treatment with 3% H2O2 + 3% formic acid, an additional mass shift was observed for each histone except H2A-2 (Fig. 5, B–G, bottom mass spectra). This mass shift was also a multiple of 16 Da and was proportional to the number of methionines in each histone (except H3.1), indicating that the methionine in these histones was converted to methionine sulfone. The mass increase of histone H3.1 following MPA treatment was +160 Da, suggesting that all cysteines were converted to cysteic acid and that all methionines were converted to the sulfone. Previous work has shown that conventional performic acid oxidation completely reduces disulfide bonds and subsequently oxidizes cysteine to cysteic acid (16, 17). It is inter-
FIG. 5. Top down MS profiles of acid-extracted whole histone after a 4-h treatment with water, 3% H\textsubscript{2}O\textsubscript{2}, or mild performic acid (3% H\textsubscript{2}O\textsubscript{2} + 3% formic). A, RP-HPLC after no treatment (top), H\textsubscript{2}O\textsubscript{2} treatment (middle), and mild performic acid treatment (bottom). B–G, mass spectra of the histone fractions H2B, H2A-1, H4, H2A-2, H3.2, and H3.1 from the corresponding chromatograms in A. The monoisotopic masses from the most abundant form in each MS are listed. The mass difference (\(\Delta m\)) of the observed mass to the theoretical mass (calculated in Table I) is listed below the monoisotopic mass.
FIG. 6. MS/MS of each core histone localizes the oxidation to regions containing methionine or cysteine residues. The GCAD MS/MS spectra corresponding to histone H2B (A), H2A-1 (B), H4 (C), H3.2 (D), and H3.1 (E) after no treatment (top), 3% H2O2 treatment (middle), and mild performic acid treatment (bottom). Ions shaded light or dark gray indicate a mass shift relative to the ions from an untreated sample. For H2B (A) and H2A-1 (B), the family member corresponding to the highlighted fragment ions is identified.
Mild Performic Acid Oxidation of Histones

**TABLE I**

<table>
<thead>
<tr>
<th>Histone</th>
<th>Untreated</th>
<th>H$_2$O$_2$</th>
<th>Mild performic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theor.</td>
<td>Obs.</td>
<td>Δm</td>
</tr>
<tr>
<td>H2B.A</td>
<td>13,766.5</td>
<td>13,766.6</td>
<td>0.1</td>
</tr>
<tr>
<td>H2A.O</td>
<td>13,997.8</td>
<td>13,997.9</td>
<td>0.1</td>
</tr>
<tr>
<td>H2A.C$^b$</td>
<td>13,993.9</td>
<td>13,994.0</td>
<td>0.1</td>
</tr>
<tr>
<td>H4</td>
<td>11,299.3</td>
<td>11,299.5</td>
<td>0.2</td>
</tr>
<tr>
<td>H3.1</td>
<td>15,333.4</td>
<td>15,348.5</td>
<td>15.1</td>
</tr>
<tr>
<td>H3.2</td>
<td>15,317.5</td>
<td>15,318.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^a$ If multiple masses are present, the most abundant mass is listed. These masses include the PTMs (if any) associated with the most abundant form as identified by TDMS in our laboratory (1, 3–5).

$^b$ Four other gene family members also share this sequence (H2A.D, H2A.I, H2A.N, and H2A.P) (1).

**Localization of Oxidized Histone Residues**—To verify that methionine and cysteine were the only amino acid residues being oxidized, we performed MS/MS using OCAD. Because OCAD fragmentation is somewhat dependent on amino acid sequence, localizing the oxidation to a single amino acid residue was not possible in all cases. However, OCAD of H2A-1 generated a fragment ion series ($b_{49}$-$b_{53}$) that covered the only methionine, Met-51 (Fig. 6B). The mass of the b ions pre-Met-51 ($b_{49}$ and $b_{53}$) matched the unmodified sequence of H2A.O in the untreated, H$_2$O$_2$-treated, and MPA-treated samples. Conversely the masses of the b ions containing Met-51 ($b_{51}$, $b_{52}$, and $b_{53}$) all had different values in each of the untreated, H$_2$O$_2$-treated, and MPA-treated samples. The untreated sample contained mostly unmodified $b_{51}$-$b_{53}$ ions; however, satellite peaks of +16 Da, indicative of partial oxidation in vivo and during sample preparation, were also observed at ~10% relative abundance (Fig. 6B). The only $b_{51}$-$b_{53}$ ions observed for the H$_2$O$_2$-treated samples were +16 Da heavier than the unmodified theoretical mass, indicating that only methionine sulfoxide was present. Likewise the $b_{51}$-$b_{53}$ ions from the MPA-treated samples were +32 Da heavier than the unmodified theoretical mass, indicating that only methionine sulfone was present. These results show unequivocally that methionine was the sole residue in H2A.O that was oxidized by MPA treatment and were further corroborated by analyses of histone H2B and H4. The fragmentation sequence coverage for H2B and H4 did not cover the methionines in these proteins as well as was achieved for H2A.O, but fragment ions that spanned the methionines in each of these histones confirmed quantitative oxidation of methionine to the sulfoxide upon treatment with H$_2$O$_2$ alone and to the sulfone following MPA treatment (Fig. 6, A and C). Histone H3.2 contains two methionines and one cysteine. One Met and the sole Cys are covered by the $y_{34}$ fragment ion in Fig. 6D. As expected, the untreated $y_{34}$ matched the theoretical, unmodified mass of H3.2. After treatment with H$_2$O$_2$ alone, the $y_{34}$ appeared +64 Da greater in mass. Because we have shown that methionine becomes oxidized only to the sulfoxide after treatment with H$_2$O$_2$ (mass shift of +16 Da per Met), the remaining +48 Da (i.e. 64 – 16 = 48 Da) is consistent with that cysteine being fully converted to cysteic acid in this case. After treatment with MPA, $y_{34}$ was +80 Da heavier than the theoretical ion, indicating that the methionine in this fragment was converted to the sulfone. Other fragment ions for H3.2 showed similar oxidation states on the other methionine in the un-, H$_2$O$_2$-, and MPA-treated samples (data not shown). Unlike H3.2, histone H3.1 contains two cysteines. After OCAD fragmentation of untreated H3.1, several fragment ions showed partial oxidation (see $y_{34}$ in Fig. 6E) and the presence of an intramolecular disulfide bond (see $y_{43}$ in Fig. 6E). The $y_{43}$ ion contains two cysteines and one methionine. Interestingly the observed mass was –2 Da below the theoretical unmodified mass and had a satellite ion with +14 Da. Therefore, some of the untreated H3.1 population contained an intramolecular disulfide bond (Δm of –2 Da) in addition to methionine oxidation (Δm of –2 + 16 = 14 Da). However, the presence of a $y_{34}$ ion, which lies between the two cysteine residues, demonstrated that this disulfide bond was not present in all of the molecules because this ion would not be observed if that were the case. Other ions larger than $y_{43}$ and in between the two cysteines showed similar mass shifts (data not shown). The masses of small z' ions and large c ions that did not contain any cysteine or methionine matched very closely with the theoretical masses. MS/MS of H3.1 after H$_2$O$_2$ treatment generated a $y_{43}$ ion with a +14-Da mass difference, indicating that methionine was oxidized to the sulfoxide and that both cysteines were in a disulfide bond. Furthermore no fragment ions between the two cysteines were detected, showing unequivocally that all molecules have an intramolecular disulfide bond. OCAD fragmentation of MPA-treated H3.1 showed the $y_{34}$ ion with a Δm of 80 Da and a $y_{43}$ ion with a Δm of 128 Da, corroborating the intact molecular mass values and the complete oxidation of all cysteines and methionines. When the
histones in the H2A-2 fraction were fragmented, every fragment ion mass remained the same regardless of oxidation treatment (data not shown). Taken together, the intact mass shifts and the localization of these mass shifts exclusively to fragments containing methionine and cysteine show that oxidation is the predominant reaction during our mild performic acid treatment. Evidence of any other chemical modifications or oxidation at other residues was not detected.

**Mild Performic Acid Oxidation Leads to Increased Dynamic Range and Identification of Novel PTMs of Intact H4**—We next investigated whether MPA treatment could be used to “rescue” the PTM abundance information of the M phase H4 sample described above that had accrued significant amounts of partially oxidized forms during storage. An aliquot of the same M phase H4 sample was treated with MPA and then analyzed by FTMS (Fig. 7C). The PTM profile following MPA treatment was strikingly similar to that obtained when the sample had been analyzed immediately without treatment following preparation (compare top panel of Fig. 3A with Fig. 7C). We then isolated and fragmented the fourth molecular species from the stored, untreated sample (11,315 Da in Fig. 7A) and the MPA-treated sample (11,345 Da in Fig 6C). ECD of the 11,315-Da form in the stored, untreated sample generated multiple fragment ions covering residues known to frequently be modified (Fig. 7B, ECD mass spectrum). Most fragment ions before Lys-20 showed a mass shift (Δm) of +42 Da consistent with the only modification being an N-terminal acetylation (αacSer-1). A c162+ ion with a Δm of +84 Da was observed at levels too close to noise to be confidently confirmed (also no c172+ +84-Da ion was detected). The two c223+ ions observed showed an abundant ion with a Δm of +70 Da and a minor ion with +84 Da. Because all pre-Lys-20 ions showed an N-terminal acetylation, the +70- and +84-Da forms must represent a +28 Da (dimethylation, 2mLys-20) and a +42 Da (trimethylation, 3mLys-20) on Lys-20, respectively. The c99+ and all fragment ions post-Met-84 have a slightly broadened distribution with an approximate mass shift of approximately +85 Da. This broadened distribution is created by the overlap of the more abundant ααSer-1 + 2mLys-20 + OMet-84 form (Δm = 86 Da) with the less abundant ααSer-1 + 3mLys-20 (Δm = 84 Da). Surprisingly when the same fourth molecular mass from the MPA-treated sample was analyzed, many more fragment ions were observed (Fig. 7D, ECD mass spectrum). The ECD spectrum shows three c152+ ions having a Δm of +42, +56, and +84 Da revealing the presence of an ααSer-1 (+42 Da), ααSer-1 + mArg-3 (+56 Da), and ααSer-1 + aLys-12 (+84 Da). The same set of masses were observed for the c162+ ion, but the fragment ion relative ratios (35) showed that the +84-Da form was now more abundant, indicating that in addition to aLys-12 other molecules contain aLys-16. As expected, all ions between Lys-20 and Met-84 have a Δm of +84 Da, and all ions after Met-84 have a Δm of approximately +115 Da consistent with a Met-84 sulfone (84 + 32 = 116 Da). These results show that full conversion of partially oxidized Met-84 allows a more complete modification profile to be obtained (see Supplemental Fig. 2 for a general illustration of how a complex MS/MS spectrum arises from the presence of multiple isobaric PTMs). Furthermore this is the first time mArg-3 + 2mLys-20 and 3mLys-20 have been described at the intact level, thus allowing a more quantitative assessment of their global abundance over bottom up MS and antibody-based detection methods. We reason that, because ECD fragmentation rarely generates ions that are above a signal to noise ratio of ~20 for histones on our instrument, the presence of the oxidized +70-Da form reduces our dynamic range and thus limits the detection of rare PTMs.

**Mild Performic Acid Oxidation Does Not Affect Methylation, Acetylation, or Phosphorylation**—The true PTM abundance of “real world” samples such as those described above has been defined only partially and can vary between preparations. Thus, we used synthetic histone peptides with known levels of methylated and acetylated lysines and phosphorylated serine to make a critical test of whether MPA treatment leads to any loss of these common histone modifications. Synthetic H4 peptides containing acetyl-Lys at the third position in combination with either mono-, di-, or trimethyl-Lys at the seventh position and a synthetic H1 peptide with phospho-Ser at the fifth position were oxidized by MPA treatment. An N-terminal cysteine incorporated into each peptide provided a positive control for thiol oxidation, but the peptides contained no other readily oxidizable residues. The mass of each unoxidized peptide matched closely with the theoretical mass (data not shown). Upon MPA oxidation, all peptides exhibited a mass shift of +48 Da. ECD fragmentation of each peptide confirmed the presence of the respective PTM in addition to a cysteic acid (Table II). These results show that the MPA oxidation does not alter the absolute levels of acetylation, methylation, or phosphorylation in these model peptides and consequently is not expected to alter the PTM abundances of natural samples.

**MPA Treatment Results in Partial Oxidation of Tryptophan Residues**—Histones from most eukaryotes do not contain tryptophan. Given the evidence that extensive performic acid treatment alters Trp residues, we tested whether mild performic acid treatment has any benefit for analysis of Trp-containing proteins. The small peptide yeast MF-α, which has the amino acid sequence WHWQLKPGQPMY (molecular mass = 1682.9 Da), was selected as a model substrate. MF-α eluted as a single peak with an altered retention time, relative to that of untreated peptide, following oxidation with 3% H2O2 alone for as long as 24 h, suggesting quantitative oxidation of the sole methionine to the sulfoxide (Fig. 8A). This was confirmed by MS and MS/MS (data not shown). Oxidation or other changes to Trp were not detected. In contrast, MPA treatment of MF-α for 2–19 h resulted in numerous peaks in RP-HPLC (Fig. 8B). These multiple peaks appear to correspond to partial oxidation products of tryptophan. MS of the first major peak (~47 min) from the 19-h time point revealed...
Mild Performic Acid Oxidation of Histones

TABLE II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Theor.</th>
<th>Mild performic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Da</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δm&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAAKRHRmKVL</td>
<td>1278.77</td>
<td>1326.78</td>
</tr>
<tr>
<td>CAAKRHR2mKVL</td>
<td>1179.70</td>
<td>1226.72</td>
</tr>
<tr>
<td>CAAKRHR3mKVL</td>
<td>1193.72</td>
<td>1240.74</td>
</tr>
<tr>
<td>CKAPKpSPAKA</td>
<td>1079.52</td>
<td>1127.51</td>
</tr>
</tbody>
</table>

<sup>a</sup> aK, acetyllsine; mL, methyllsine; 2mK, dimethyllysine; 3mK, trimethyllysine; pS, phosphoserine.

<sup>b</sup> The mass shift was localized to the N-terminal cysteic acid.

<sup>c</sup> MS/MS localized the −1-Da mass difference to the C-terminal Leu. The c ions covering the modified residues matched the theoretical mass and were not affected by MPA oxidation.

many different masses ranging from ~1400 to 1800 Da (data not shown). In an attempt to identify conditions facilitating complete oxidation of tryptophan, we examined the effect of increasing concentrations of H₂O₂ + formic acid at room temperature and the effect of performing the MPA treatment at elevated temperature (Fig. 8C). Because multiple peaks were still apparent in RP-HPLC (Fig. 8C), these measures were either unable to completely oxidize Trp or caused additional changes in the MF-α peptide. Finally we carried out the MPA oxidation of MF-α at 4 °C to see whether it was possible to oxidize methionine but not tryptophan at reduced temperature (Fig. 8C). Unfortunately we found that this was not the case. The reaction generated fewer chromatographic peaks in samples taken early in the time course, enabling characterization of methionine sulfone and the initial tryptophan oxidation products by MS and MS/MS (data not shown). However, the time points beyond 4 h showed multiple chromatographic peaks suggesting that the reduced temperature slows the reaction rate but still generates many oxidized tryptophan products. Although we did not characterize many of the chromatographic peaks obtained under these conditions with respect to the oxidation states of Trp, it is apparent that further work, possibly using alternate chemical approaches, is required to assess whether quantitative oxidation of Trp is feasible under conditions that minimize reactions with residues other than Met and Cys.

**DISCUSSION**

We have shown that partial oxidation of histones, either in vivo or unintentionally during sample preparation and FTMS analysis, leads to increased sample complexity and difficulty interpreting MS and MS/MS data for histone proteins. Intact MS profiles of partially oxidized histones decrease the meaningfulness of relative ratios of protein forms. Furthermore partial oxidation, especially in the case of H3 and H4, creates a nearly completely overlapping distribution with neighboring modified forms. Subsequent isolation and fragmentation of these modified forms makes PTM characterization and quantitation much more difficult. It should be noted that, in some cases, it may be possible to account for partial oxidation. By using fragment ion intensity relative ratios (35), contamination can be “subtracted” out if and only if the PTM composition of the unoxidized species is known and the relative amount of the oxidized species contaminating the mass of interest is also known. If ECD is used for MS/MS, care should be taken when using this approach because y ions, which can be generated by ECD, are the same mass (+16 Da) as an oxidized z’ ion and might erroneously increase the calculated amount of oxidation from a lower mass form. An alternative solution to this problem involves complete conversion of partially oxidized residues to completely oxidized residues. We found that treatment of histone extracts with hydrogen peroxide converted methionine to methionine sulfone. This level of oxidation reduced the relative retention of each of the Met-containing histones during reverse phase chromatography and increased the overall separation of the histones. Similarly using H₂O₂ to characterize methionine oxidation in the protein calmodulin (which has no cysteine or tryptophan), Galeva et al. (15) have recently reported that hydrogen peroxide converts nearly 100% of the nine methionines in calmodulin to methionine sulfone. In addition, the use of a milder oxidant, peroxynitrite, converted methionine to the sulfoxide in a dose-dependent manner. In both cases, each methionine sulfone form of calmodulin eluted at different times with the more highly oxidized species eluting earlier. Hydrogen peroxide was also found to convert cysteine to cysteic acid for histones such as H3.2 that contain a single cysteine, whereas the same treatment catalyzed the formation of an intramolecular disulfide bond for histone H3.1, which contains two cysteines. It is known that H₂O₂ oxidation of free cysteine first leads to a reactive cysteine sulfenic intermediate. This intermediate can then react with one or two more molecules of H₂O₂ to form the sulfinic and sulfonic (cysteic acid) forms, respectively. Additionally cysteine sulfenic acid...
can also react with another thiolate anion (\(\text{CS}^-\)) to form a cystine or another cysteine sulfenic acid to form a thiol sulfinate (36). The presence of only the cysteic acid form of H3.2 and not H3.2 dimers might be explained by the great molar excess of \(\text{H}_2\text{O}_2\) to total histone (\(\sim 10^5\)). Conversely the close proximity of the two cysteines in H3.1 may have been the determining factor in the formation of a disulfide bond. This novel reactivity may provide chromatin researchers further
insight into possible mechanisms underlying the functional differences between H3.1 versus the H3.2 and H3.3 variants.

Using performic acid as the oxidant (∼95% formic acid, 5% H2O2), Dai et al. (22) demonstrated that, in addition to complete oxidation of methionine and cysteine, multiple side reactions, such as tyrosine chlorination and lysine formylation, were observed at substoichiometric levels. Using our mild performic acid procedure to oxidize histone proteins, methionine sulfoxide and cysteic acid were the only chemical modifications identified. However, the generation of multiple chromatographic peaks after prolonged exposure to MPA suggests that additional residues can be modified with continued treatment. Similarly treatment of a Trp- and Met-containing peptide, MF-α, generated multiple chromatographic peaks, presumably representing multiple oxidation states of tryptophan (37, 38), after MPA oxidation. Therefore, MPA oxidation should be avoided for Trp-containing proteins when using a strictly top-down MS approach. In these cases, a 24-h treatment with H2O2 avoided for Trp-containing proteins when using a strictly top-down approach. Therefore, MPA oxidation should be avoided for Trp-containing proteins when using a strictly top-down MS approach. In these cases, a 24-h treatment with H2O2 is a possible alternative because methionine will be converted to the sulfoxide and cysteine will be converted to cysteic acid or cystine without modifying any other residue. Alternatively Trp-containing proteins can be digested prior to MPA oxidation and then analyzed by bottom up MS. Considering that Trp occurs at the lowest frequency among amino acids in proteins (comprising only 1.13% of the amino acid composition of all sequences entered in the Swiss-Prot database as of April 2007), the MPA procedure is potentially compatible with bottom up proteomics. The effects of partial oxidation on the chromatographic separation or MS portion of bottom up analysis can be readily discerned by comparing the results for samples that are digested and then intentionally oxidized or not prior to LC-MS. Although MPA treatment is expected to increase both spectral and chromatographic complexity of Trp-containing peptides, this will affect only a small number of peptides in many cases. Furthermore this problem may be offset by reductions in specificity and chromatographic complexity achieved through the complete oxidation of Cys and Met residues, which occur at higher frequencies than Trp in proteins. Thus, it seems likely that both mass spectral and chromatographic complexity and computational demand could be reduced when our method is used in conjunction with bottom up analyses for proteins with limited numbers of tryptophan residues.

To completely oxidize methionine to the sulfone, we used mild performic acid treatment consisting of 3% H2O2 and 3% formic acid for 4 h at room temperature. MS and MS/MS analysis of MPA-oxidized histones revealed that methionine was completely oxidized to the sulfone and cysteine was completely oxidized to cysteic acid. Eliminating partially oxidized species formed during storage restored the true abundance ratios of a mitotic histone H4 sample. Also reduction in MS/MS complexity and an increase in dynamic range were observed after the isolation and ECD fragmentation of a low abundance mass. This single mass contained four isobaric species, none of which were observed when partial methionine oxidation contaminated the corresponding mass in the untreated sample. Two of these isobaric species, mArg-3 + 2mLys-20 and 3mLys-20, have not been observed previously in MS analyses of intact H4 presumably because background levels of oxidation have precluded their detection. Importantly, the detection of these forms via top down MS allows a more quantitative estimate of their global levels. For instance, we estimate that 3mLys-20 occurs on ∼1.5% of total H4 when the confounding effects of partial met oxidation are reduced using MPA treatment. This estimate agrees with the low level of trimethyllysine detected in H4 by conventional amino acid analysis (39) and the low relative abundance of 3mLys-20 forms of H4 resolved by hydrophilic interaction chromatography (26). In contrast, a recent report claiming that a reduction in the level of Lys-20 trimethylation was a hallmark of cancer estimated that the abundance of 3mLys-20 ranging from 20 to 30% and 5 to 15% of total H4 in different types of normal and cancer cells, respectively (40). Because Lys-20 trimethylation was not confirmed directly by MS/MS and the abundances estimated of different H4 forms were based on intact protein mass measurements alone, it is possible that the large discrepancy in the apparent extent of Lys-20 trimethylation is related to the effects of partial oxidation in the latter study compared with our work and that of others (26, 39). The method described here should facilitate further investigation of this critical issue. Furthermore although not elaborated upon here, this technique is expected to be of value in discriminating between highly conserved members of multigene families. We recently used MPA treatment to discern two histone H2B gene family members differing by only a single alanine to serine substitution (Ala to Ser = +16 Da), a feature that was difficult to establish with partially oxidized samples because this is the same mass difference as that for formation of a methionine sulfoxide (4).