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Limiting the Hydrolysis and Oxidation of Maleimide–Peptide Adducts Improves Detection of Protein Thiol Oxidation

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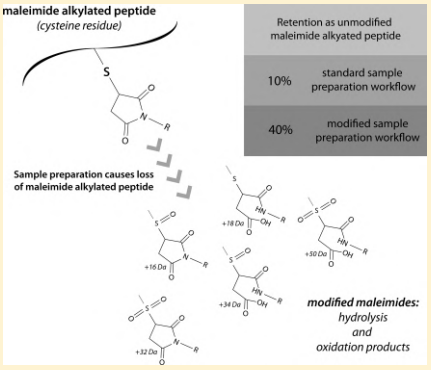
Supporting Information

ABSTRACT: Oxidative stress, caused by reactive oxygen and nitrogen species (RONS), is important in the pathophysiology of many diseases. A key target of RONS is the thiol group of protein cysteine residues. Because thiol oxidation can affect protein function, mechanistic information about how oxidative stress affects tissue function can be ascertained by identifying oxidized proteins. The probes used must be specific and sensitive, such as maleimides for the alkylation of reduced cysteine thiols. However, we find that maleimide-alkylated peptides (MAPs) are oxidized and hydrolyzed under sample preparation conditions common for proteomic studies. This can result in up to 90% of the MAP signal being converted to oxidized or hydrolyzed MAPs, decreasing the sensitivity of the analysis. A substantial portion of these modifications were accounted for by Coomassie "blue silver" staining (~14%) of gels and proteolytic digestion buffers (~20%). More than 40% of the MAP signal can be retained with the use of thioglycolic acid during gel electrophoresis, trichloroethanol–UV protein visualization in gels, and proteolytic digestion buffer of pH 7.0 TRIS. This work demonstrates that it is possible to decrease modifications to MAPs through changes to the sample preparation workflow, enhancing the potential usefulness of maleimide in identifying oxidized peptides.



KEYWORDS: *mass spectrometry, gel electrophoresis, oxidative stress, proteomics, sample preparation*

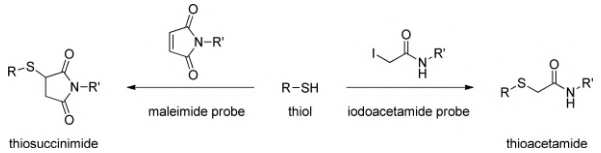
INTRODUCTION

Reactive oxygen and nitrogen species (RONS) can cause oxidation of the thiol group of protein cysteine residues. Oxidation of cysteines can be biologically irreversible through the formation of sulfonic acid derivatives, for example, or biologically reversible such as the formation of sulfenic acid derivatives, intra- and intermolecular disulfides, and S-nitrosylation. A functional consequence of reversible protein thiol oxidation is to transduce the chemical signal of the redox imbalance to a biologically relevant conformational change in the protein, affecting the function of biochemical signaling cascades. Protein thiol oxidation can result in changes to signaling networks in both normal cellular function and pathology.[1,2] It has been proposed to affect signaling relevant to cancer,[3] inflammatory disease,[4] and diabetes.[5] The reversible nature of the modification is especially pertinent when considering its involvement in transient, nonpathological stimuli, such as muscle fatigue.[6]

Protein thiol oxidation is a post-translational modification that has the potential to affect many aspects of cell function because of its diverse protein targets. An understanding of the complexities of protein thiol oxidation-mediated processes, at the proteome level, is therefore relevant to understanding changes in tissue function under oxidative stress. This need for profiling of the redox proteome has necessitated a change toward high-throughput approaches. Established shotgun proteomics techniques center on thiol alkylation chemistry, using either iodoacetamides (including the oxICAT technique based on Sciex's Isotope-Coded Affinity Tag (ICAT) technology),[7−11] or maleimide-based molecular probes (including isotopomeric variants)[12−17] (Scheme 1). Both sets of reagents do not alkylate oxidized thiols, allowing the estimation of the proportion of oxidation of cysteine residues.

There are challenges in detecting the oxidative modifications of protein thiols, and techniques have been constrained by the lack of sufficient specificity, sensitivity, and robustness.[18] For example, the high reactivity of the deprotonated thiol group (the thiolate)[19] can result in artifactual oxidation during sample

Scheme 1. Generic Outline of the Two Main Classes of Molecular Probes Used To Label Thiols



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preparation. Methods to accurately assess protein thiol oxidation state must therefore be rapid, irreversible, and complete.

When probing for thiols, iodoacetamide (IAM) chemistry is most commonly used. Maleimides, although less-used,[20] offer advantages. The rate of reaction of small thiols with *N*-ethylmaleimide (NEM) is 20 times faster than with IAM.[21] In selected proteins from murine skeletal muscle tissue samples, NEM completely alkylated all free thiol groups in 4 min, markedly faster than IAM, which did not reach completion after 4 h of incubation.[22] It has been observed that 6 h is insufficient for complete alkylation of protein cysteine residues with IAM in some settings.[23] From this perspective, maleimides are therefore superior for rapid and complete thiol alkylation.

The difference in reaction rates between thiols and the two classes of probes is reflected in the stoichiometric excess required to effect global thiol tagging. In biological samples, complete alkylation was achieved with a 125 fold excess of NEM, in contrast with a 1000 fold excess required for IAM and iodoacetic acid.[22] The larger excess required with IAMs increases the chance of nonspecific alkylation, that is, of other nucleophilic functional groups.

Alkylation with IAMs is also inherently less selective than with maleimides. When alkylating the cysteine residues of hemoglobin, the rate of the reaction of IAM with cysteine thiols was comparable to its reaction with histidine residues.[24] Furthermore, IAM has been shown to alkylate lysine residues[23] in sufficient proportions to be misidentified as the characteristic diglycine formed by the digestion of ubiquitinated lysine.[25] Nonspecific alkylation with IAM could lead to false reporting of cysteine oxidation if used, for example, to produce a mass shift in protein gel electrophoresis. Maleimides have also been shown to alkylate lysine and histidine residues in addition to cysteines; however, these nonspecific reactions have been shown to be minimal under acidic conditions for reaction times of up to 90 min.[26] Where this nonspecific alkylation has occurred, it has been observed to comprise only 2% of the total product.[27]

The oxidation state of thiols can be preserved through acidification, commonly through preparation in 20% trichloroacetic acid (TCA) solution or irreversibly through alkylation.[21] At low pH, the thiol group is protonated and less electron-rich, so it is less susceptible to artifactual oxidation. Low pH is therefore desirable when alkylating protein thiols.[28] However, the thiol is also less nucleophilic than the thiolate. In this context, IAM has been shown to react incompletely with thiols at low pH, whereas maleimide is reactive at pH 4.3.[22] The pH favoring complete alkylation with IAM, that is pH $\geq$8,[22] has been shown to increase artifactual oxidation.[29] Higher pH values also increase the prevalence of the free base forms of lysine and histidine residues, resulting in higher rates of alkylation of these residues.[30]

As explained, alkylation of cysteines using maleimide chemistry is superior to IAM-based approaches. Maleimides react with thiols more quickly, completely, and more selectively at neutral pH and below. However, most current techniques for interrogating protein cysteine residue oxidation state, such as OxICAT, employ IAM chemistry.[7,15,31−33] We therefore examined whether maleimide-based approaches can be employed in identifying oxidized protein thiols. We find that there is a substantial chemical modification of the maleimide-alkylated peptide (MAP) following the initial alkylation event.[34,35] This causes a corresponding loss in sensitivity of the detection of the tagged peptides by mass spectrometry. Through empirically derived data, we provide a practical set of guidelines for limiting the modification of maleimide-tagged protein thiols during a proteomics sample preparation workflow, which will increase the value of this technique.

EXPERIMENTAL PROCEDURES

Preparation of Standard Protein and Peptide

Solutions of purified lysozyme (Sigma-Aldrich) and L-glutathione (reduced, Sigma-Aldrich) were prepared in 0.25 M tris[hydroxymethyl]aminomethane (TRIS, Biorad), pH 6.0. The addition of tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich) to a final concentration of 1.18 nmol/μg protein and incubation for 1 h at room temperature ensured that the lysozyme and glutathione were fully reduced. Alkylation was carried out with biotin-maleimide (Sigma-Aldrich) (Figure 2) at 1 nmol/μg of protein or peptide (2 μg/μL) for 1 h at room temperature.

SDS-PAGE

TRIS-glycine electrophoresis: Samples were mixed 1:1 v/v with sample buffer (125 mM TRIS, pH 6.8, 4% sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 0.02% bromophenol blue). Standards and samples were applied to a 15% polyacrylamide, 0.1% SDS gel containing either pH 6.8, 250 mM TRIS stacking buffer, or pH 8.8, 375 mM TRIS resolving buffer. All gels were polymerized with ammonium persulfate and tetramethylethylenediamine (TEMED). TRIS gel electrophoresis was performed with 25 mM TRIS, 192 mM glycine, 0.5% w/w SDS buffer (Biorad) using the Bio-Rad Mini Protean III system. Some gels were electrophoresed at 60 V for 2 h in running buffer containing 0.5% thioglycolic acid. Buffer containing thioglycolic acid was discarded and replaced with running buffer prior to protein electrophoresis.

BIS-TRIS electrophoresis: Samples were added to diluted NuPAGE LDS sample buffer (Thermo Fisher Scientific). Gels were cast with 0.3 M bis(2-hydroxyethyl)amino-tris-(hydroxymethyl)methane (BIS-TRIS, Sigma-Aldrich) pH 6.5 and 6 and 12% acrylamide/bis-acrylamide (Bio-Rad) in the stacking and resolving gels, respectively. Gel electrophoresis was carried out in diluted NuPAGE 3-(*N*-morpholino)-propanesulfonic acid (MOPS) SDS running buffer (Thermo Fisher Scientific).

Stain-free gels were constructed through the addition of 0.5% trichloroethanol (TCE) to the resolving gel solution.[36] Proteins were visualized using a Chemidoc MP Imaging system (Bio-Rad). Nonfluorescent gels were labeled with Coomassie "blue silver" stain.[37] Glutathione cannot be visualized with Coomassie staining or with TCE and so was located in gels by comparison with the migration of fluorescent maleimide-alkylated glutathione.

Gel bands were cut into 1 mm cubes and then destained three times with 100 μL of either 25 mM ammonium bicarbonate (Sigma-Aldrich) in 50% acetonitrile (ACN) or 25 mM TRIS in 50% acetonitrile (Sigma-Aldrich) at 37 °C for 45 min. Unstained gel bands were washed three times with ACN. Gel pieces were then dried by vacuum centrifugation. Protein was digested overnight at 37 °C by the addition of 125 ng sequencing grade Trypsin (Roche) in 10 μL of 25 mM ammonium bicarbonate or 25 mM TRIS. Digested protein was extracted by two additions of 20 μL of 1% trifluoroacetic acid (TFA) in ACN and incubation at room temperature for 20

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min. Extracts were pooled and desiccated by vacuum centrifugation.

Streptavidin Purification

Biotin-conjugated peptides were enriched through interaction with a streptavidin-coated 96-well plate (Sigma-Aldrich). Peptides were dissolved in 200 μL of 150 mM ammonium chloride (Sigma-Aldrich), 10 mM ammonium phosphate (Sigma-Aldrich), or 250 mM TRIS pH 7.0, then incubated for 1.5 h. Wells were washed three times with 250 μL of either 50 mM ammonium phosphate or 250 mM TRIS pH 7.0, followed by three washes with 250 μL of double-deionized water. Peptides were extracted by 1 h of incubation with 2.5% formic acid (Sigma-Aldrich) in 70% ACN and dried by vacuum centrifugation.

Cation Exchange Purification and Avidin Purification

Biotin-conjugated peptides were alternatively cleaned up via cation exchange and enriched via an avidin column. Purification using these ICAT cation exchange and ICAT avidin affinity columns (Sciex) was carried out as per protocols provided by the manufacturer (available at http://sciex.com/Documents/Downloads/Literature/mass-spectrometry-4337577C.pdf)

Mass Spectrometry

Extracts were dissolved in 10 μL of 80% ACN/0.1% TFA, and 0.6 μL of this solution was combined with 0.6 μL matrix solution (5 mg/mL α-cyano-4-hydroxysuccinamic acid, 10 mM ammonium citrate, 80% ACN/0.1% TFA) on a MALDI-TOF plate and allowed to air-dry. Analysis was performed with a 5800 MALDI-TOF/TOF mass spectrometer (Sciex, MA).

MS/MS analysis to identify the modified protein residue was carried out with a 5600 TripleTOF system (Sciex, MA).

Analysis Programs

Peaklists were generated with TOF/TOF Series Explorer 4.1.0 (Sciex, MA) with the following parameters: MS (peak filters mass range of 800−4000 Da, peak density maximum 10 peaks for 200 Da, minimum signal/noise 5, minimum area 30, max peaks/spot 40; MS/MS peak filters 60 Da precursor 20 Da, peak density maximum 10 peaks per 200 Da, minimum signal/noise 5, minimum area 30, maximum peak/precursor 100). MS/MS data were imported into the database search engine Mascot (version 2.4.1, www.matrixscience.com) and searched against the Swiss-Prot database with the following search conditions: trypsin digest with allowance for up to one missed cleavage per peptide, no fixed modifications, variable modifications of oxidation on methionine residues and alkylation of cysteines with biotin maleimide, MS tolerance of 0.2 Da, and MS/MS tolerance of 0.6 Da. A significance threshold of $p < 0.05$ was used for identification of peptides. T2d converter was used to convert mass spectra to mzml files (www.pepchem.org). mMass3[38] was used to label peaks and find the area under the curve and carry out baseline correction where necessary.

Experimental Design and Statistical Rationale

A sample size of three technical replicates ($n = 3$) was chosen to detect a difference in means of 5% with $\alpha = 0.05$ and $\beta = 0.2$. Statistical analyses were carried out using GraphPad Prism version 6.0 g for Mac OSX, GraphPad Software, San Diego, CA, www.graphpad.com. Unless otherwise stated, t tests or one-way ANOVA with post t tests and Holm-Šídák correction for multiple analyses were used. Groups were considered significantly different where $p < 0.05$. Raw peak areas and statistical analyses for all data are provided as Supporting Information.

The extent of modification observed in mass spectra is expressed as a proportion of all signals attributed to the peptide, both modified and unmodified. All peak areas of the relevant signals were summed to give a total amount of signal. Each individual signal at the relevant m/z was expressed as a percentage of its area relative to this total signal area for the peptide. Alkylated cysteines are represented as C*.

The data set has been deposited in the MassIVE repository and can be accessed at http://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=6731a45582f14908a5de475edf3a82f2.

RESULTS

Observed Modifications of Maleimide-Alkylated Peptides (Thiosuccinimides)

Purified lysozyme was used to test the efficacy of cysteine alkylation as it forms cysteine-containing tryptic peptides suitable for MALDI-TOF mass spectrometry. Biotin maleimide was used to alkylate the surface cysteine residues of reduced lysozyme, resulting in MAPs. Upon identification of peaks in the mass spectrum corresponding to the expected tryptic peptides, a signal at +16 Da to the MAP was observed (Figure 1a). Other modifications to the expected mass were observed at +32, +34, and +50 Da. We propose that these modifications correspond to oxidation of the thioether sulfur atom to

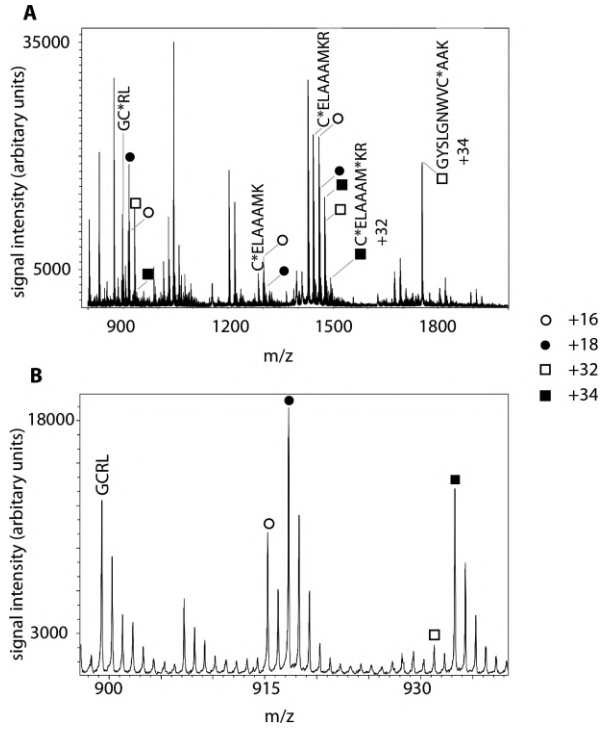

Figure 1. MALDI-TOF mass spectrum of the modified forms of tryptic peptides of lysozyme alkylated with biotin maleimide. Lysozyme was reduced and alkylated with biotin maleimide, purified by SDS-PAGE, and digested in gel. (A) Occurrence of unmodified (denoted by peptide sequence), singly oxidized (+16 Da), hydrolyzed (+18 Da), doubly oxidized (+32 Da), and oxidized + hydrolyzed (+34 Da) forms of the maleimide-alkylated peptides is indicated on the spectrum. (B) Enlarged view of spectrum shown in panel A between 887 m/z and 938 m/z. Four permutations of oxidation and hydrolysis (Figure 2) of the MAP biotin-maleimide-GC*RL are observed.

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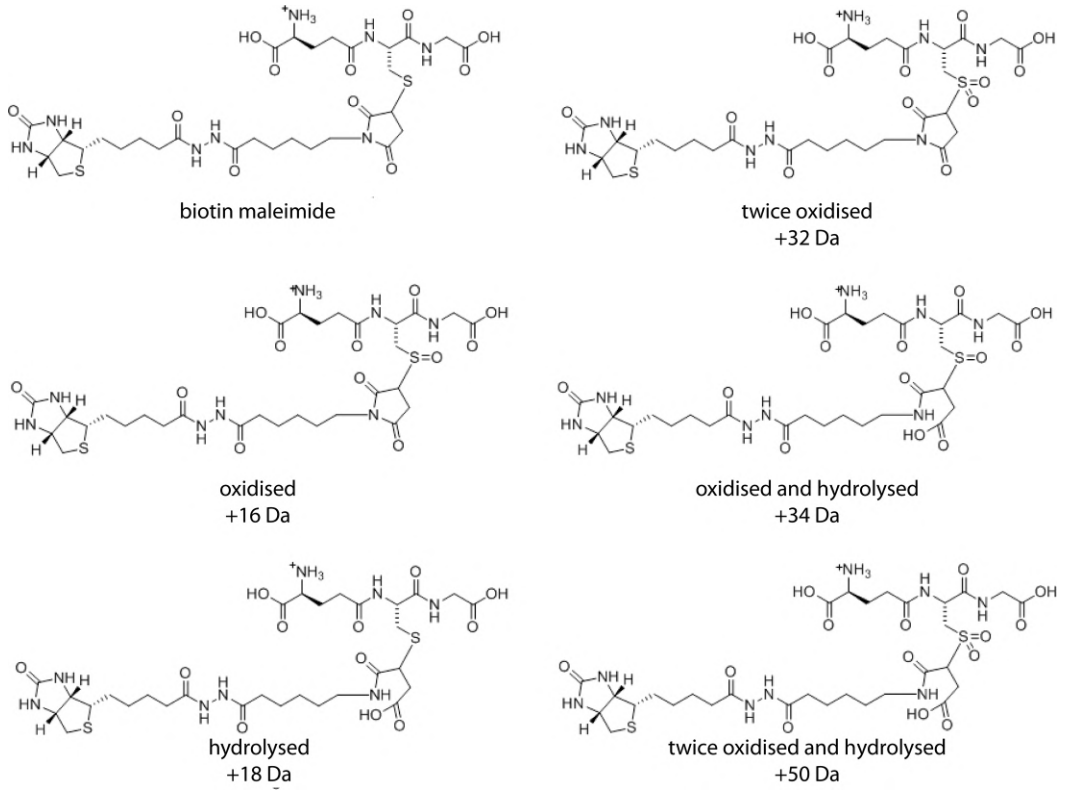

Figure 2. Putative structures for the modification of biotin-maleimide resulting in mass increases of 16, 18, 32, 34, and 50 mass units. Regio- and stereoisomerism are ignored in this Figure. Single oxidation of the MAP at its sulfur atom gives a mass increase of 16 Da and double oxidation a 32 Da increase. Hydrolysis of the succinimide yields a mass 18 units greater than the unmodified MAP. Hydrolysis with single oxidation of the succinimide gives a mass increase of 34 Da, and double oxidation combined with hydrolysis of succinimide gives a mass increase of 50 Da.

sulfoxide and sulfone functional groups (mass increases of 16 and 32 Da, respectively), hydrolytic ring-opening of the succinimide (mass increase of 18 Da), and combinations thereof, giving increases of +32, +34, and +50 Da (Figure 2).

For some peptides the signal at the expected m/z was nearing the limit of detection, while the modified ions were dominant (Figure 1b). The extent of modification for three tryptic peptides was further quantified in three separate experiments. These results show that the degree to which a particular MAP was modified was consistent between experiments, but there was substantial variation in the extent of modification of different peptides (Figure 3).

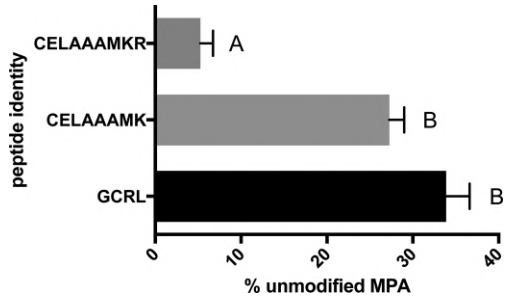

Figure 3. Tryptic peptides of lysozyme alkylated with biotin maleimide have different levels of modification. Signals corresponding to the predicted m/z and modifications at +16, +18, +32, +34, and +50 Da were identified. The areas under each of these peaks were summed, and each peak area was assessed as percentage of this total. Shown here is the percentage of the signal that is retained at the predicted m/z. Annotations with different letters indicate that a significant difference was observed between these two groups (n = 3).

Modification Occurs on the Succinimide Moiety

To establish whether the modification was occurring on the succinimide or the biotin moieties, tryptic lysozyme peptides were alkylated with NEM, which does not contain biotin. As with biotin maleimide, peaks at +16 and +18 Da to the expected mass for the MAPs were observed. These mass shifts were not present for the corresponding nonalkylated peptide. These data indicate that the modification was occurring on the succinimide group of the MAP. Modifications at both +16 and +18 Da suggest both oxidation (incorporation of an oxygen atom) and hydrolysis of the maleimide ring. Possible structures for these modifications to the MAP of glutathione are shown in Figure 2.

Experimental Conditions Causing Modifications of MAPs

To investigate the experimental conditions causing the modification of the MAPs, glutathione was used as a model cysteine-containing peptide. Enrichment of labeled peptides is routinely performed prior to analysis, commonly by taking advantage of the biotin−streptavidin interaction. Therefore, biotin maleimide was used as an alkylating agent. The extent of hydrolysis of the MAP was tracked through a proteomics sample preparation workflow (Figure 4). Signals corresponding to the predicted m/z and the +16, +18, +32, +34, and +50 Da modifications were identified. The steps involved in this workflow were reduction and alkylation, SDS-PAGE, tryptic digest, and streptavidin purification. Samples were processed through stages of the workflow as shown in Figure 4. The dominant modification was +32 Da to the expected mass. Modifications of +16 and +18 Da were also contributors to the significant loss of signal at the expected mass. When processed

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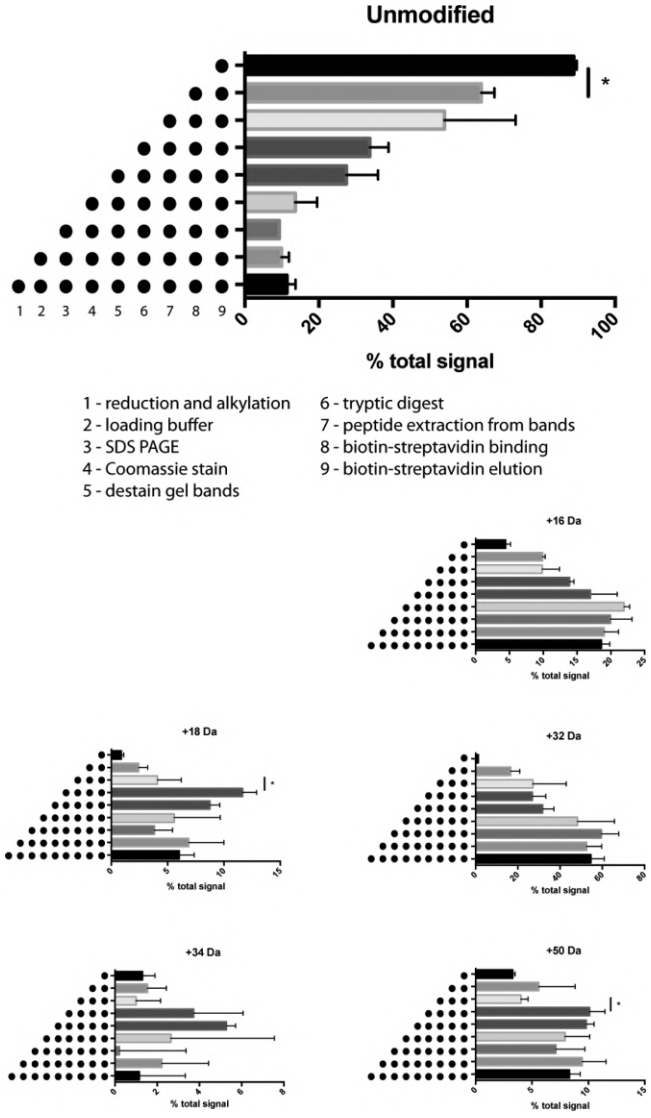

Figure 4. Effect of steps involved in proteomics sample preparation on the modification of glutathione alkylated with biotin maleimide. Biotin maleimide-alkylated glutathione was subjected to subsets of sample preparation workflows to identify steps where modification occurred. Significant differences between successive steps are indicated with asterisks (n = 3). Sample preparation subsets reflect the cumulative modifications through the sample preparation workflow, as indicated in the Figure. Extent of modifications of the MAP, with each permutation identified by the mass added, is shown in separate graphs.

through the whole workflow, 90% of the original signal was lost to modification. Major sources of modification were SDS-PAGE, purification by biotin−streptavidin affinity, and tryptic digest (Table 1).

A range of buffers and alternative buffers to those used in this workflow were tested for their effects on the modification of a MAP (biotin maleimide-alkylated glutathione) (Figure 5). Incubation in 250 mM ammonium bicarbonate resulted in substantial hydrolysis of the MAP. TRIS is a potential alternative to ammonium bicarbonate and was shown to result in substantially fewer modifications to the MAP, notably less hydrolysis. The gel destain buffer (10% v/v acetic acid, 20% v/v methanol) did not cause substantial modification. The use of either ammonium-containing or TRIS buffers in streptavidin

Table 1. Breakdown of the Effect of Steps Involved in Proteomics Sample Preparation on the Modification of Glutathione Alkylated with Biotin Maleimide[a]

experimental step	average % modification	SEM
reduction and alkylation	<1	<1
loading buffer	<1	1
gel electrophoresis	4	2
coomassie "blue silver" stain	14	4
destain gel bands	6	4
tryptic digest	20	8
peptide extraction from gel	10	8
biotin-streptavidin purification	25	1
biotin-streptavidin elution	11	<1
total	90	4

[a]Data shown in Figure 3 have been reworked to show the extent of modification at each experimental step. This shows that steps relating to gel electrophoresis and streptavidin purification are large contributors to the hydrolysis and oxidation of MAPs.

purification (10 mM ammonium phosphate or 250 mM TRIS pH 7.0) resulted in substantial increases in oxidation.

The different steps also changed the prevalence of the types of modification. Streptavidin purification buffers result in substantial conversion of peptides to the putative oxidized forms (+16 and +32 Da), whereas ammonium bicarbonate predominantly resulted in conversion to the putative hydrolyzed forms (+18 Da).

Modifying Tryptic Digestion to Limit Hydrolysis and Oxidation of MAPs

MAP modification occurred in tryptic digest buffer (15 mM ammonium bicarbonate) overnight at 37 °C. Decreasing the time of the tryptic digest did not result in a substantial decrease in modification of the MAP (Figure 6). However, overnight incubation at 37 °C with 25 mM TRIS buffer decreased the relative abundance of the +18 Da modification (Figure 6). Overnight digest of bovine serum albumin in TRIS buffer did not result in any significant change in the functional digestion of the protein when compared with standard ammonium bicarbonate conditions, as judged by the outcome of a Mascot score for the identification of the protein (Figure 7).

Modifying Gel Electrophoresis to Limit Hydrolysis and Oxidation of MAPs

Gel electrophoresis and related procedures were identified as a major source of the modification of MAPs. Coomassie "blue silver" staining resulted in significant increases in hydrolysis (+18 Da) (Figure 8). There was no significant increase in the relative abundance of the oxidized forms of the MAP (+16 and +32 Da) through all forms of electrophoresis.

Because there was a high rate of Coomassie "blue silver"-stain-induced hydrolysis, a "stain-free" gel system was tested where proteins are visualized through the photochemical reaction of TCE with tryptophan residues. Stain-free gel electrophoresis resulted in significantly less modification than when stains were used, but all gel visualization approaches resulted in significantly higher amounts of modifications of MAPs (Figure 8).

Because incubation in a basic buffer was shown to increase hydrolysis (Figure 5), it was hypothesized that basic conditions may result in hydrolysis of MAPs. Gel electrophoresis can be buffered at neutral pH by BIS-TRIS and MOPS buffers as an alternative to the basic TRIS buffers. Coomassie "blue silver"-

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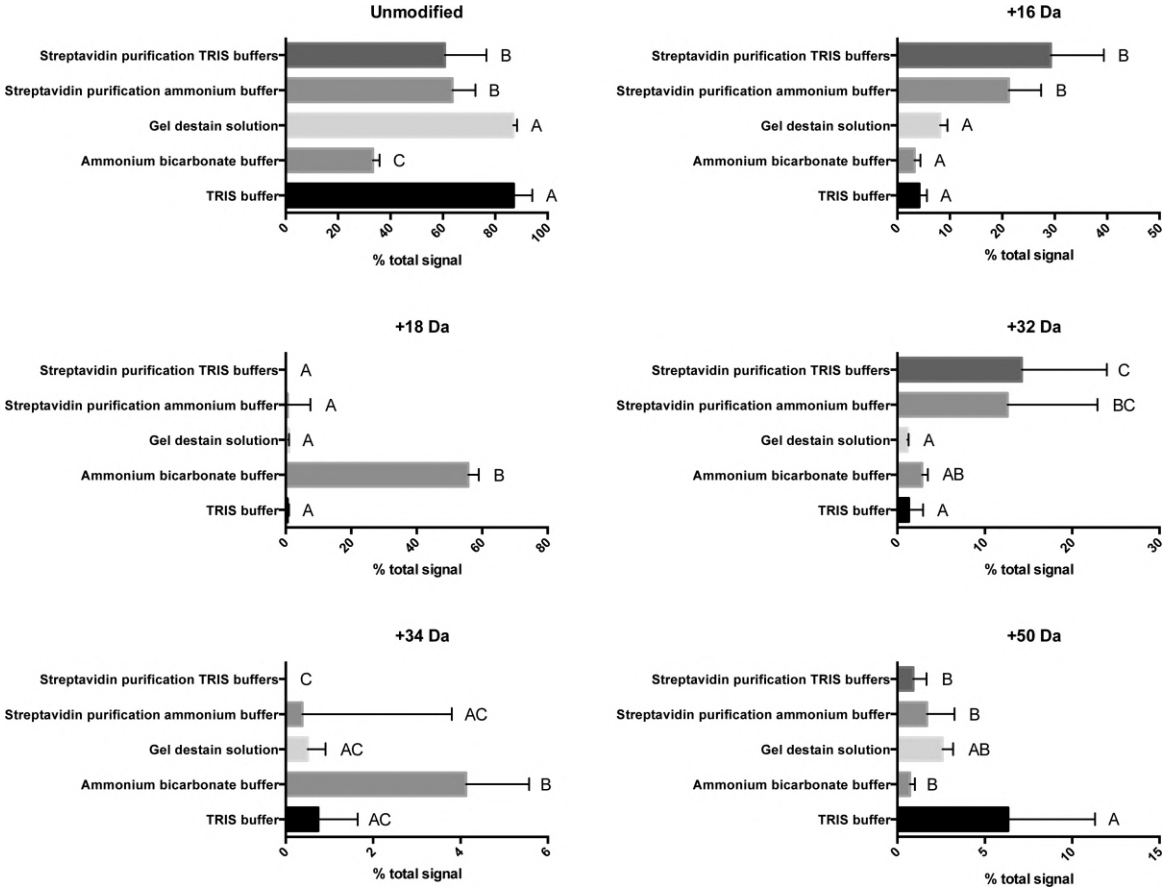

Figure 5. Modification of biotin maleimide-alkylated glutathione in buffers used for proteomic mass spectrometry analysis. TRIS and ammonium bicarbonate (0.25 M, pH 6.8 and 8.0, respectively) were incubated with glutathione alkylated with biotin maleimide for 48 h. MOPS (250 mM, pH 6), TRIS (250 mM, pH 7), and gel destain solution (20% v/v methanol, 10% w/w acetic acid) were incubated with this MAP for 3 h. Buffers containing ammonium (150 mM ammonium chloride, 10 mM ammonium phosphate, pH 7.0) or TRIS (250 mM, pH 7.0) were used when incubating peptides with a streptavidin-coated 96-well plate. Peptides were eluted using 70% acetonitrile 2.5% formic acid after 1 h of incubation. For each graph, annotations with different letters indicate that a difference was observed between those two groups. Annotations with the same letters indicate no significant difference between the two groups (n = 3).

stained BIS-TRIS/MOPS buffered gels overall exhibited no significant difference in the amount of unmodified MAP present when compared with TRIS buffered gels. However, they did show significantly more conversion to hydrolyzed and oxidized (+34 Da) forms than TRIS-buffered SDS-PAGE (Figure 8).

It has been established that oxidation of methionine residues can be limited by pre-electrophoresis of TRIS gels with a buffer containing 0.5% thioglycolic acid.[39,40] Application of this pretreatment in the current work significantly decreased the amount of doubly oxidized MAP (Figure 9). It also significantly decreased the oxidized + hydrolyzed forms and the doubly oxidized + hydrolyzed forms (+32 and +50 Da). As a result, there was an overall significant increase in the amount of unmodified MAP observed (42 ± 5.37 to 57.6 ± 1.51%).

Alterations to Streptavidin Purification to Limit Hydrolysis and Oxidation of Succinimides

Modification adding +16 and +32 Da to the expected mass of MAPs was the major contributor to loss of signal during streptavidin purification. Conducting the streptavidin−biotin binding in the presence of the reducing agent dithiothreitol (DTT) significantly limited the extent of the +32 Da modifications (Figure 10).

Direct Comparison of Standard Workflows and a Workflow Designed to Limit Hydrolysis and Oxidation of Succinimides

The combination of several of the above approaches does result in significant preservation of the signal associated with the unhydrolyzed and unoxidized peptide-maleimide adducts. In a proposed low-modification workflow, samples are processed via SDS-PAGE carried out with gels pre-electrophoresed with TGA, gels are visualized with the stain free TCE/UV system, and tryptic digestion is carried out in TRIS-buffered solution (Figure 11). The GC*RL peptide derived from tryptic digestion of biotin maleimide-alkylated lysozyme shows a significantly greater unmodified signal compared with a standard workflow when processed with the low-modification workflow (p = 0.027). Oxidation (+16 Da) was significantly higher in the low-modification workflow (p = 0.0126), whereas hydrolysis (+18 Da) was significantly higher in the control workflow (p = 0.003). The extent of the preservation of the unmodified signal is significant, from 25 ± 2.32% of the total signal in the control group (n = 3) to 54 ± 8% of the total signal in the low-modification workflow (n = 3).

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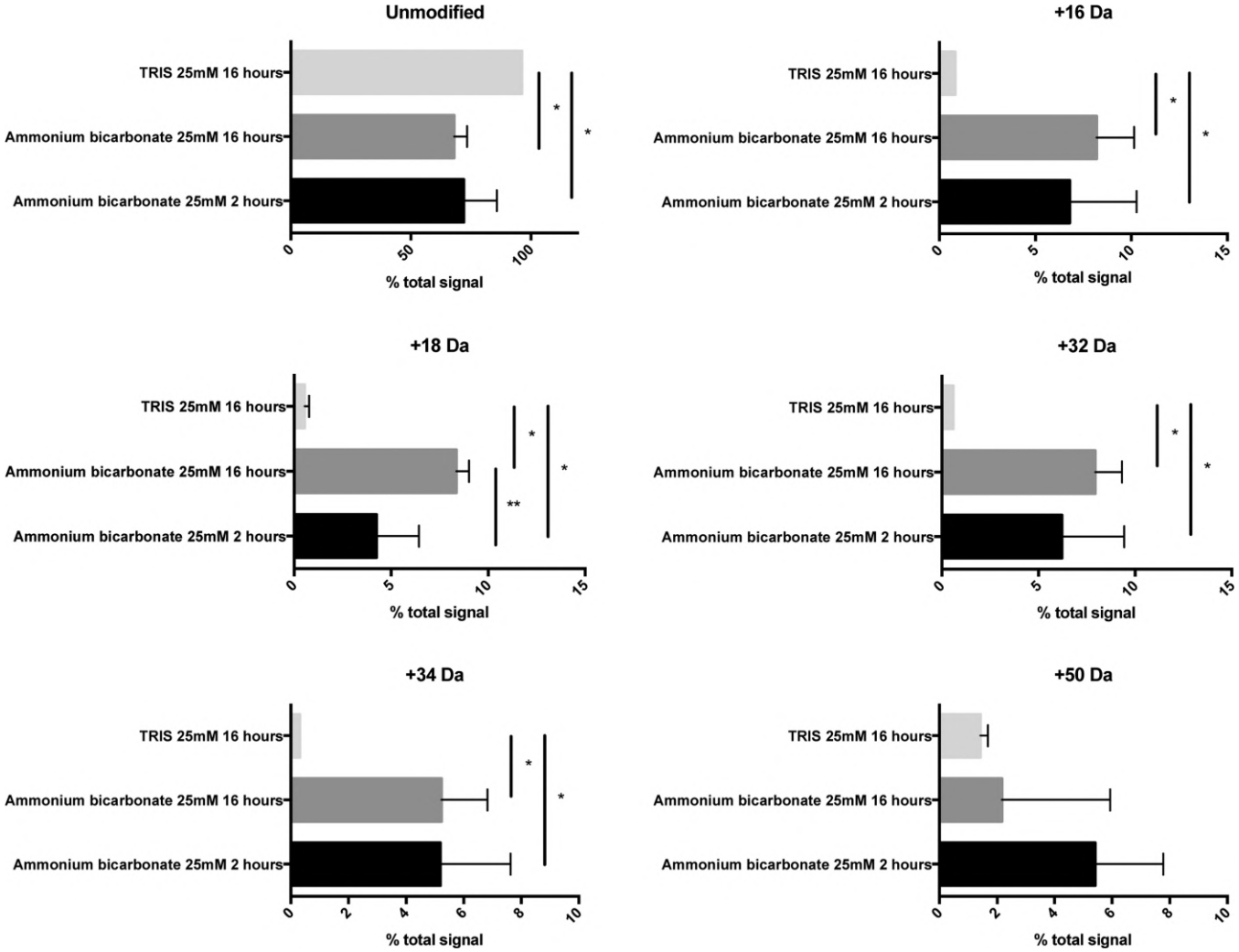

Figure 6. Effect of buffers appropriate for proteolytic digest on the hydrolysis and oxidation of biotin maleimide conjugated with glutathione. The maleimide-conjugated peptide was incubated in either TRIS buffer overnight (16 h) or ammonium bicarbonate (25 mM) for 2 h or overnight (16 h) (n = 3). There was a significant increase in relative levels of all forms of modification bar double oxidation and hydrolysis (+50 Da) with 2 or 16 h of incubation in ammonium bicarbonate when compared with incubation in TRIS buffer for 16 h (n = 3, p < 0.05). Hydrolysis (+18 Da) is significantly higher after 16 h of incubation in ammonium bicarbonate compared with 2 h of incubation (n = 3, p < 0.02).

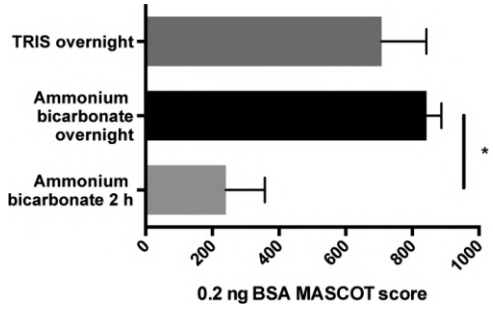

Figure 7. Effect of varying buffers on digestion efficiency of trypsin on bovine serum albumin. BSA (10 μg) was processed via SDS-PAGE and tryptic digest was carried out in gel bands. Digestion was carried out overnight with a trypsin/protein ratio of 1:50; the 2 h digest was carried out at a ratio of 1:10. Significance is indicated by asterisks (n = 3, p < 0.05). Ammonium bicarbonate buffered overnight digest yielded a significantly higher score than that with a 2 h digest. There was no significant difference in the resulting score between the TRIS-buffered and ammonium-bicarbonate-buffered digest of the protein at the overnight time point.

DISCUSSION

Our data indicate that the sample preparation workflow can seriously compromise the detection of MAPs. There is loss of unmodified MAPs throughout the sample preparation workflow, such that after cumulative losses, as little as 10% of the original MAP signal may remain. However, by modifying the workflow, the loss of MAPs can be minimized.

The hydrolysis and oxidation of MAPs could result in changes to ionization efficiency of these peptides. The data relating to the relative proportions of each species in each mass spectra are therefore not indicative of the absolute quantities of each species present but rather how much signal is observed for each in resulting mass spectra. While changes in the proportion of the unmodified and modified signals of MAP are plausibly due to a combination of changes in both the proportion of species present and their differing ability to be ionized, we have not differentiated between this in our investigations, as the focus of this work is to maximize the signal of the unmodified MAP in the mass spectra. As such, this combined effect is what is shown by the charts representing proportion of signal.

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J. Proteome Res. 2017, 16, 2004−2015

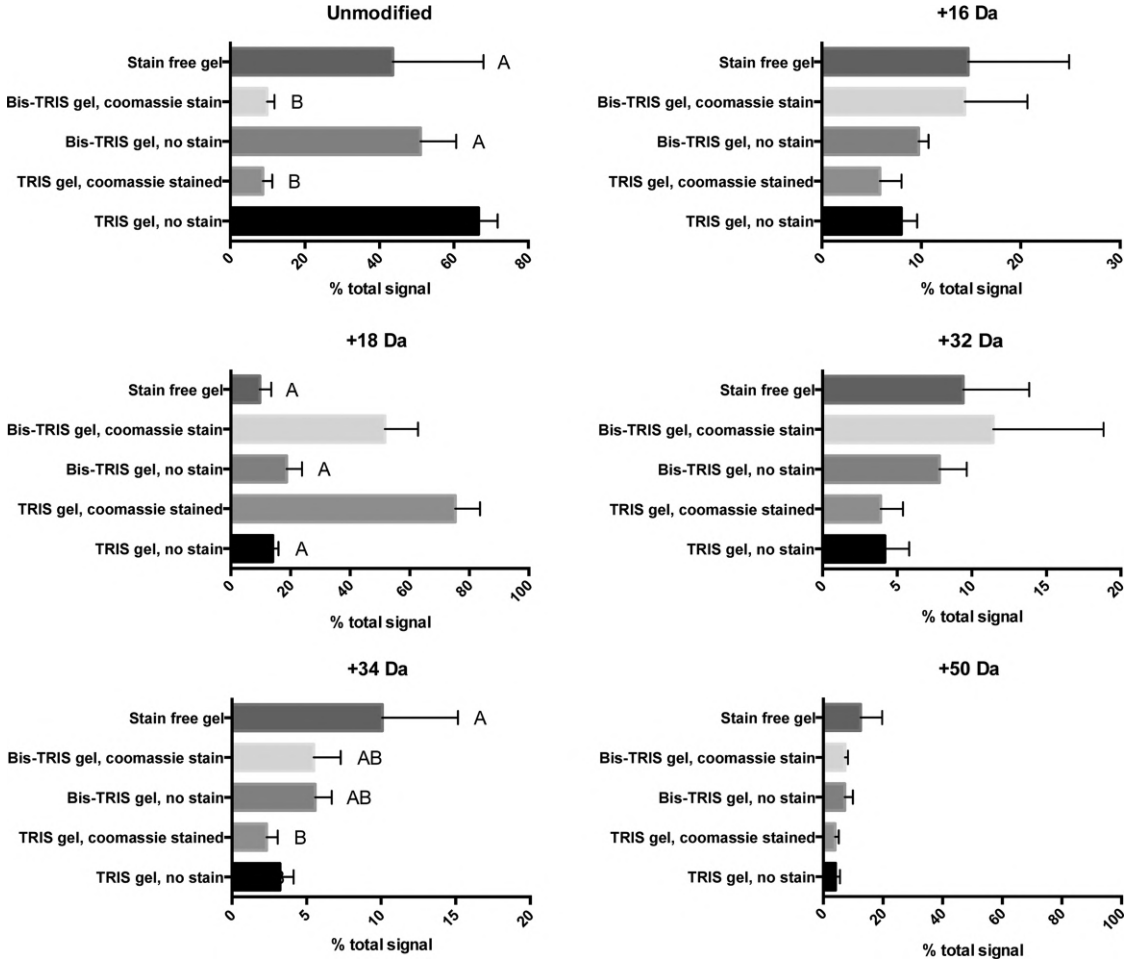

Figure 8. Glutathione alkylated with biotin maleimide was processed through TRIS- or MOPS-buffered SDS-PAGE and visualized using trichloroethanol in conjunction with UV light ("stain free") or Coomassie "blue silver" stain. The proportions of unmodified peptide adduct and modified derivatives were determined by MALDI-TOF mass spectrometry. Annotations with different letters indicate that a difference was observed between those two groups. Annotations with the same letters indicate no significant difference between the two groups. Where there is no annotation in a graph, no significant differences were observed between groups (n = 3).

A review of the literature identified hydrolysis as a cause of the loss of MAPs. Maleimides and the succinimides that form upon reaction of maleimides with thiols, such as cysteine, are known to hydrolyze to the corresponding ring-opened maleamic and succinamic acids.[41−45] Concerns about enzymatic hydrolysis have been described in the literature,[46] but this is not relevant to protein extracts where enzymes have been denatured. The stability of MAP conjugates has also been identified as a potential issue, whereby the maleimide group is hydrolyzed from the peptide with high pH and temperature.[44] However, this is not likely an issue for proteomic workflows given that the related species of the *N*-alkylmaleamic acid (resulting from hydrolysis of NEM) is stable below 50 °C and a pH of 9.26.[47] Hydrolysis of the succinimide, particularly under alkaline conditions,[43,44] is a potential cause of +18 Da increase seen in the mass of MAPs. This is consistent with the observation that the extent of this modification increases with incubation under more basic conditions, such as with ammonium bicarbonate buffers.

Putative hydrolysis (+18 Da) was notably induced where ammonium bicarbonate was used as a buffer during proteolytic digest (Figure 5). Ammonium bicarbonate was initially used because it is widely employed as a mass-spectrometry-compatible salt. It is established that basic conditions increase the rate of succinimide hydrolysis.[48] We observed that the 25 mM ammonium bicarbonate solution used in tryptic digestion protocols reached pH 9.0 after 16 h at 37 °C. Proteolytic digests have been shown to hydrolyze similar chemical structures: The amide functional group in asparaginyl residues undergoes hydrolysis during basic conditions of proteolytic digest.[45] Replacing ammonium bicarbonate with TRIS buffer at pH 7.0 substantially decreased the extent of hydrolysis after a typical overnight proteolytic digest (Figure 6) but did not reduce the efficiency of the trypsin enzyme (Figure 7). Hydrolysis can therefore be ameliorated by the elimination of ammonium-containing buffers in proteomic workflows. While TRIS is not as compatible with mass spectrometry as ammonium bicarbonate, cleanup and enrichment of peptides by chromatography prior to analysis (for example, with sample preparation tips) is increasingly common as a strategy to improve the quality of analysis. The use of TRIS buffers in sample preparation for mass spectrometry is therefore not as problematic as it may have historically been.

In addition to the putative hydrolysis of MAPs, we observed modifications at +16 and +32 Da to the expected ion masses for cysteine-containing peptide-maleimide adducts. This modification of maleimide−thiol adducts has not been previously noted in the literature. However, methionine residues have been

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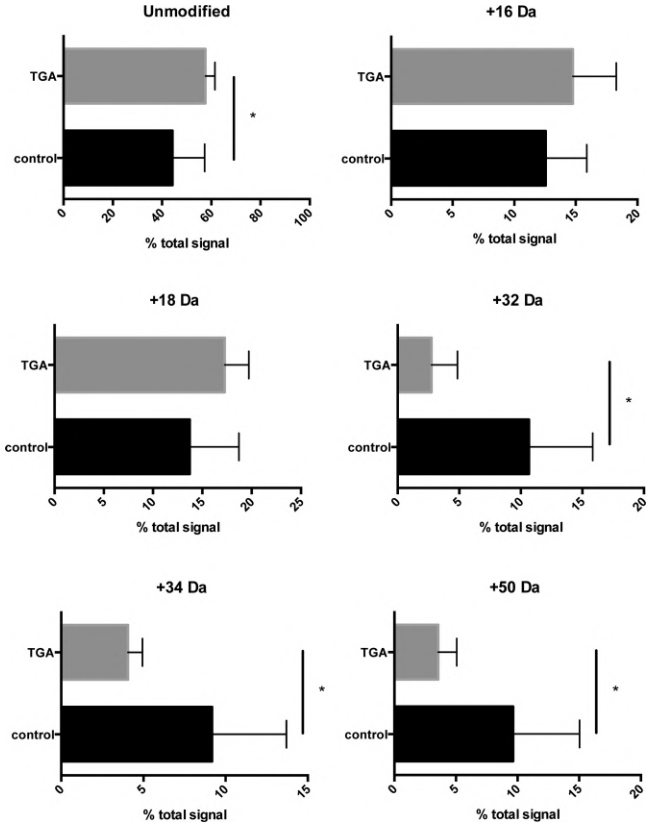

Figure 9. Glutathione alkylated with biotin maleimide processed via TRIS-buffered SDS PAGE with or without the addition of thioglycolic acid (TGA, 0.5%) in running buffer. The proportion of MAP in unmodified and modified forms was determined by MALDI-TOF mass spectrometry. Significance between groups is indicated with asterisks (n = 3, p < 0.05).

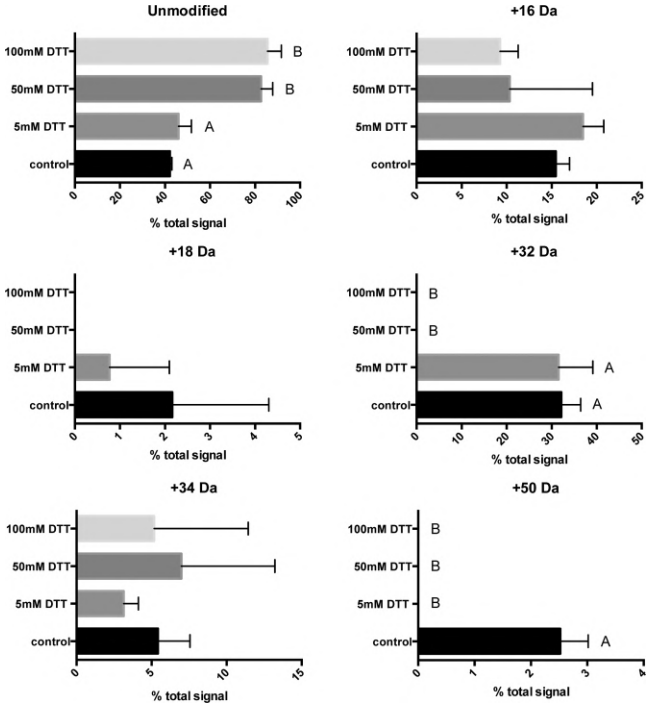

Figure 10. Streptavidin purification with added DTT was tested for the effect on biotin-maleimide-conjugated glutathione. The test MAP was incubated in a streptavidin-coated plate with 250 mM TRIS buffer or 150 mM ammonium chloride with 10 mM ammonium phosphate (pH 7.0). DTT was added to 250 mM TRIS buffer in all other cases. Plates were incubated with the conjugate for 90 min. Elution in 70% ACN/2.5% formic acid occurred over 30 min. The proportion of MAP signal in modified and unmodified forms was determined by MALDI-TOF mass spectrometry. Annotations with different letters indicate that difference was observed between those two groups. Annotations with the same letters indicate no significant difference between the two groups (n = 3).

shown to be susceptible to oxidation, giving rise to sulfoxide +16 and sulfone +32 Da products,[49] so it is likely that oxidation of the sulfur atom of the thioether moiety is causing the formation of these oxidation products (Figure 3). Because of the similarity in proposed mechanism to methionine oxidation, methods that prevent oxidation of methionine were tested.[50] Accordingly, thioglycolic acid preconditioning of gels resulted in a significant decrease in the extent of the +32 Da oxidation form (Figure 9). Oxidation of MAPs was also evident during streptavidin purification. This was limited by the addition of 50 mM DTT during incubation (Figure 10). Taken together these observations show that the putative oxidation of MAPs can be minimized by the use of an appropriate antioxidant.

The nature and extent of the observed modifications of MAPs differs based on the structure of the peptide (Figure 2). This effect has been observed previously with respect to succinimide hydrolysis: the nature of the substituent affects hydrolysis rate due to changes in charge distribution in the molecule[51] and perhaps anchimeric assistance (intramolecular catalysis). If the hydrolysis or oxidation of MAPs is not taken into account and full conversion of the MAP to the modified forms occurs, as was observed for one MAP, then some cysteine residues may not be identified. While it is theoretically possible to account for these modifications in analysis using appropriate software, this would be challenging given the five permutations of hydrolysis or oxidation combined for each MAP (hydrolyzed, oxidized, doubly oxidized, hydrolyzed and oxidized, hydrolyzed and doubly oxidized). For parsimonious detection of MAPs, it is therefore desirable to reduce the extent of the modifications where possible.

We examined the extent to which gel electrophoresis caused modified MAPs because of its utility in mass spectrometry workflows. Gel electrophoresis is established in many laboratories and can effectively remove reagents that interfere with mass spectrometry.[52,53] Gel electrophoresis can also be of analytical advantage when used as a first dimension of protein separation preceding LC−MS: a technique referred to as GeLC−MS. Prefractionation of samples reduces sample complexity, giving greater analytical depth, and can yield superior results to other first dimension separation techniques such as cation exchange.[54,55]

Staining SDS-PAGE gels with Coomassie "blue silver" stain[37] resulted in notable increases in hydrolysis (Figure 8). The use of alternatives to Coomassie "blue silver" staining, such as gels embedded with TCE, was identified as means to minimize the extent of hydrolysis. However, the use of haloalkane photochemistry in visualizing proteins causes covalent modifications to tryptophan, thereby increasing the complexity of the sample. Nevertheless, we have shown that overall this method results in a strong signal corresponding to the unmodified peptide (Figure 11). A second potential limitation of TCE visualization is that there is an increase in MAP oxidation with its use (shown significantly in Figure 11). Exposure to UV light has been shown to oxidize the similarly structured methionine,[56]

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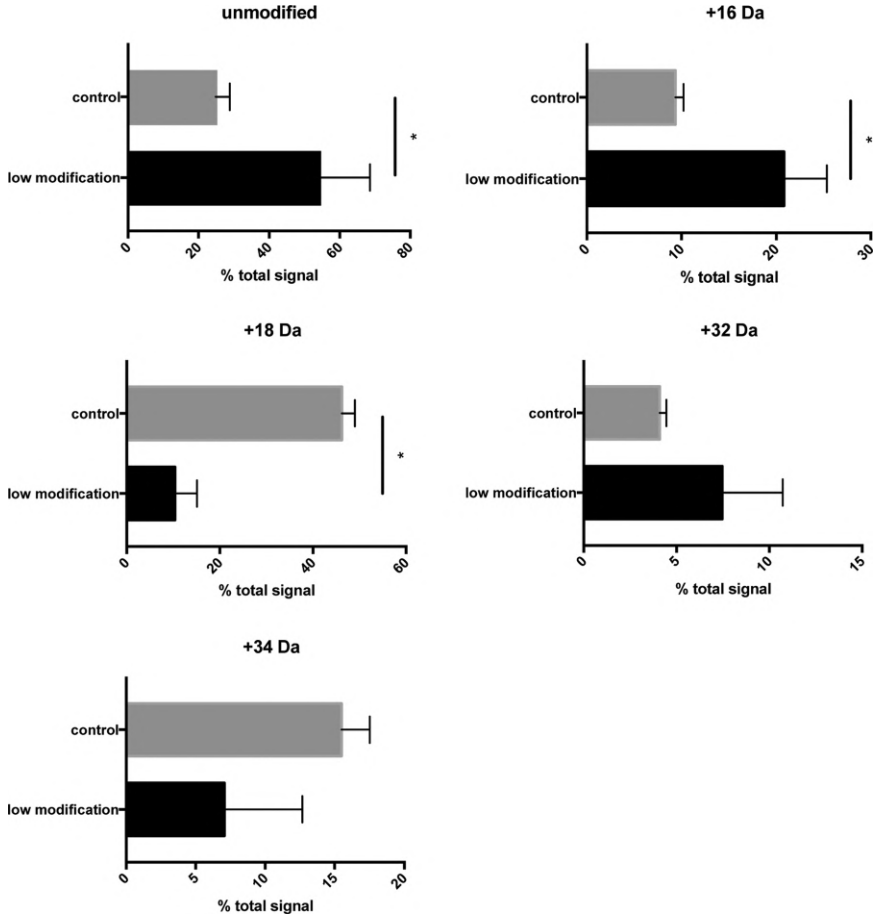

Figure 11. Sample processing with TGA-infused, "stain-free" SDS-PAGE- and TRIS-buffered tryptic digestion decreased the overall loss of signal to maleimide hydrolysis and oxidation. Ten μg samples of biotin maleimide-alkylated lysozyme were subjected to a "low modification" or "control" workflow. The control group was processed via standard SDS-PAGE and stained with Coomassie "blue silver". Tryptic digestion was carried out in 25 mM ammonium bicarbonate solution. The low modification group was processed as follows: TCE-containing SDS PAGE gels were pre-electrophoresed with TGA, and proteins were visualized using TCE in conjunction with UV light. Tryptic digestion was carried out in 25 mM TRIS buffer. Peptide enrichment was achieved by cation exchange and column avidin purification. The proportion of signal from the GC*RL MAP in modified and unmodified forms was determined by MALDI-TOF mass spectrometry. Significance between groups is indicated with asterisks ($n = 3$, $p < 0.05$).

and catalyze the reaction of tryptophan to *N*-formylkynurenine (+16 Da).[36] An alternative that would prevent further oxidation and hydrolysis of MAPs is to exclude gel staining altogether in GeLC−MS workflows.

As we have shown, MAPs are oxidized and hydrolyzed under sample preparation conditions common for proteomic studies. In the context of proteomic studies where isotopically labeled maleimides would be used, oxidation and hydrolysis would cause a proportional loss of isotopically labeled and nonlabeled maleimide. Consequently, the accuracy of the ratio of isotopically labeled to nonlabeled maleimide is preserved, even where the MAP is modified. Nevertheless, the loss of MAPs by modifications has the potential to substantially affect the sensitivity of peptide identification.

As sample preparation workflow complexity increases and so does the proportion of MAPs converted to the hydrolyzed or oxidized forms (Figure 4). The extent of the hydrolysis and oxidation of MAPs is variable across peptides. For the small-peptide glutathione, which was shown to be highly susceptible to these modifications, 90% of the MAP was converted to the oxidized or hydrolyzed forms. For tryptic peptides from lysozyme, modifications ranged from 66 to 95%. By combining the individual approaches to limiting hydrolysis and oxidation, it was possible to limit the extent of these modifications, as shown for a lysozyme-derived MAP in a workflow involving gel electrophoresis, staining, extraction from the gel, and purification with streptavidin (Figure 11). With less complex workflows, where, for example, gel electrophoresis staining is excluded, the oxidation and hydrolysis of MAPs can be limited further. Alternatively, a sample preparation workflow that does not include gel electrophoresis would further decrease modifications to MAPs.

We have not considered the effect of applying endogenous or exogenous oxidants to model systems. Our analytical approaches involve fixing the thiol redox state via quenching by acidification prior to alkylation. This is favored over alkylating nondenatured systems, as it has been shown that up to 20% of thiols in cell culture are inaccessible to alkylation by NEM.[14] Quenching by acidification prior to alkylation with maleimide will remove or denature oxidants that are present in the biological context, and so the effects on MAPs do not need to be considered.

An alternative to limiting the extent of these modifications to MAPs is to account for their presence in analysis by accounting for these modifications in database search algorithms. This would be beneficial for highly abundant, easily ionized peptides.

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J. Proteome Res. 2017, 16, 2004−2015

Where peptides are not abundant and not easily ionized and the MAP is modified substantially in the sample preparation workflow into all five forms, this could result in the peptide signal falling below the limit of detection for the instrument. It is more advantageous for the detection of low abundance peptides to limit the splitting of the signal by these modifications.

In summary, we find that the usefulness of maleimide probes is compromised by modifications to MAPs. Nevertheless, it is possible to decrease modifications to MAPs through changes to the methods employed and by simplifying the sample preparation workflow. An improved ability to detect unmodified MAPs enhances the potential usefulness of maleimide-based approaches in identifying oxidized peptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b01060.

Raw peak areas and statistical analyses for all data. (PDF)

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Notes

The authors declare no competing financial interest.

The data set has been deposited in the MassIVE repository, and can be accessed at http://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=6731a45582f14908a5de475edf3a82f2.

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■ ABBREVIATIONS

MAP, maleimide alkylated peptide; RONS, reactive oxygen and nitrogen species; NEM, *N*-ethylmaleimide; IAM, iodoacetamide; TCA, trichloroacetic acid; TRIS, tris[hydroxymethyl]-aminomethane; TCEP, tris(2-carboxyethyl)phosphine; TEMED, tetramethylethylenediamine; BIS-TRIS, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; TCE, trichloroethanol

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