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Enrichment of Carbonylated Peptides Using Girard P Reagent and Strong Cation Exchange Chromatography

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It has been shown that oxidatively modified forms of proteins accumulate during oxidative stress, aging, and in some age-related diseases. One of the unique features of protein oxidation by a wide variety of routes is the generation of carbonyl groups. Of major interest in the study of oxidative stress diseases is which proteins in a proteome are being oxidized and the site(s) of oxidation. Based on the fact that proteins are generally characterized through tryptic peptide fragments, this paper reports a method for the isolation of oxidized peptides, which involves (1) derivatization of oxidized proteins with Girard P reagent (GRP; 1-(2-hydrazino-2-oxoethyl)pyridinium chloride), (2) following proteolysis enrichment of the derivatized peptide using strong cation exchange (SCX) chromatography, and (3) identification of oxidation sites using tandem mass spectrometry. Derivatization of aldehydes and ketones in oxidized proteins was accomplished by reacting protein carbonyls with the hydrazide of GRP. The resulting hydrazone bond was reduced by sodium cyanoborohydride to further stabilize the labeling. Derivatization time and concentrations of the derivatizing agent were optimized with model peptides. Oxidized transferrin was used as model protein to study derivatization efficiency at the protein level. Following metal-catalyzed oxidation of transferrin, the protein was derivatized with GRP and trypsin digested. Positively charged peptides were then selected from the digest with SCX chromatography at pH 6.0. Seven GRP-derivatized peptides were found to be selected from transferrin by MALDI-TOF-TOF analysis. Fourteen underivatized native peptides were also captured by the SCX column at pH 6.0. Mapping of the derivatized peptides onto the primary structure of transferrin indicated that the oxidation sites were all on solvent-accessible regions at the protein surface. Efficiency of the method was further demonstrated in the identification of oxidized proteins from yeast.

It is common in biological systems that the redox potential in cells occasionally reaches a level where protein oxidation begins to occur.[1] At extreme levels of oxidative stress, there can be substantial oxidation of proteins accompanied by cell necrosis and death. In fact, protein oxidation plays an important role in a wide variety of diseases ranging from atherosclerosis, arthritis, diabetes, and muscular dystrophy to the formation of cataracts.[2,3] Oxidative stress is also a component of aging.[4] Of major interest is how and where oxidation occurs in cells. It has long been known that protein oxidation is triggered by reactive oxygen species (ROS) and that ROS-mediated modifications of proteins involves either oxidation of amino acid side chains or cleavage of the polypeptide backbone. Carbonyl group formation accompanies both of these processes and is responsible for the associated cross-linking of proteins that is a hallmark of oxidative stress.[5] The number of ways proteins can be oxidized and cross-linked is very complex, adding to the diversity of the phenomenon.[6]

Oxidative chain cleavage occurs either through an α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a modified polypeptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative.[7,8] On the other hand, direct oxidation of lysine, arginine, proline, and threonine residues also produces carbonyl derivatives. In addition, carbonyl groups may be introduced into proteins by reactions with aldehydes such as 4-hydroxy-2-nonenal or malondialdehyde produced during lipid peroxidation.[8,9] Still another route of protein carbonylation is through the oxidation of sugars that nonenzymatically derivatize lysine in the glycation process.[10,11] This is a major issue in diabetes.

Protein carbonyl groups have been quantified in several ways. One is by derivatization with 2,4-dinitrophenylhydrazine followed by quantification with UV spectroscopy.[12] Another is by reduction with tritiated borohydride and subsequent radiography.[13,14] Protein carbonyl groups also react with hydrazine to form a Schiff base,

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which can be reduced to stable secondary amines that are easily quantified. Another way is through derivatization with a fluorophore such as fluorescamine. The resulting secondary amine is fluorescent and has high molar absorptivity at 489 nm.[15] Immunological detection and quantification of protein carbonyl groups is another route. Protein carbonyl groups have also been labeled with digoxigenin−hydrazide and detected by dot-blotting with an anti-digoxigenin antibody.[16] Use of anti-2,4-dinitrophenol antibodies and poly(vinylidene difluoride) membrane slot-blotting to capture 2,4-dinitrophenyhydrazine-derivatized proteins that are detected with a peroxidase-labeled second antibody is yet another method.[17]

The most recent method developed for identification of oxidized proteins utilizes biotin hydrazide as a labeling reagent to react with protein carbonyls and provide an affinity tag for the chromatographic isolation of oxidized proteins.[18] After avidin affinity capture of oxidized proteins, they were further fractionated by reversed-phase chromatography. Fractions from the reversed-phase column were then tryptic digested and the proteolytic digests either analyzed directly by ESI-MS or after further fractionation with reversed-phase chromatography. Even though this method allows comprehensive analysis of oxidized proteins by identification of all oxidized and nonoxidized peptides pooled in the same fraction, it has its own drawbacks. Identification of all peptides of oxidized proteins to identify the site of carbonylation is time-consuming and labor-intensive. The biotin hydrazide tag can also fragment and interfere with the fragmentation pattern of the peptide.[19] As a result, biotinylated peptides are either missed or search engines can have difficulty assigning the sequence.

A new strategy is reported here for isolating peptides from oxidized proteins based on labeling protein carbonyls using Girard P reagent (GRP).[20] Although GRP was originally developed to derivatize and solublize insoluble steroids,[21] it readily derivatizes oxidized proteins. An attractive feature of GRP is that it carries both a hydrazide and quaternary amine group. The hydrazide group reacts readily with carbonyls whereas the quaternary amine adds positive charge to oxidized proteins and peptides. It will be shown that addition of GRP to peptides permits their enrichment by strong cation exchange (SCX) chromatography at neutral pH and enhances their ionization efficiency in MALDI mass spectrometry.

EXPERIMENTAL SECTION

Materials. Synthetic peptides Ac-N-Me-Tyr-Val-Ala-Asp-aldehyde, Ac-Ile-Glu-Pro-Asp-aldehyde, and Ac-Leu-Leu-Met-aldehyde were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Sodium cyanoborohydride, trifluoroacetic acid (TFA), and Coomassie blue (Bradford) protein assay kits were purchased from Pierce Co. (Rockford, IL). 1-(2-Hydrazino-2-oxoethyl)pyridinium chloride (Girard P reagent), dithiothreitol (DTT), trypsin, *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), and apotransferrin (human) were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile, iron(III) chloride, potassium chloride, magnesium chloride, ascorbic acid, urea, and calcium chloride were purchased from Mallinckrodt. (St. Louis, MO). The 218TP54 reversed-phase C_{18} column was purchased from Vydac (W. R. Grace & Co.). The DE44H10426 Zorbax reversed-phase C_{18} column (0.5 × 150 mm) was purchased from Agilent Technologies, Inc. (Palo Alto, CA). The TSK Chelate-5PW column (7.5 × 7.5 mm) was purchased from TosoHaas (Montgomeryville, PA). The strong cation exchange and reversed-phase chromatography (RPC) analyses were done on a BioCAD 20 Microanalytical Workstation (PE Biosystems, Framingham, MA). The LC system used in conjunction with mass spectrometer was an Agilent 1100 series instrument. LC/MS mass spectral analyses were done using a Sciex QSTAR hybrid LC/MS/MS quadrupole TOF mass spectrometer and MALDI-TOF-TOF mass spectra were obtained on a 4700 Proteomics Discovery System (Applied Biosystems). All spectra were obtained in the positive ion mode.

Methods. *Derivatization of Carbonyl-Containing Peptides with GPR.* The carbonyl groups were derivatized with GPR. This reaction is analogous to the well-known reaction of carbonyl groups with hydrazine and hydrazides.[22,23] Reaction with GPR introduces a quaternary amine into carbonyl-containing peptides. Model peptides were prepared separately in PBS buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4). A 2000-fold excess of derivatization reagent was added, and the final concentrations of peptides were adjusted to 250 μg/mL. The reaction was carried out for 8 h. Hydrazone bonds of derivative were reduced at 0 °C with addition of an equal volume of 30 mM sodium cyanoborohydride in PBS followed by incubation for an additional 40 min at the same temperature. Excess reactants were removed by dialysis against PBS.

Optimization of Derivatization Reaction on Synthetic Peptides. Stock solutions (2.0 mg/mL) of the synthetic peptides Ac-N-Me-Tyr-Val-Ala-Asp-aldehyde, Ac-Ile-Glu-Pro-Asp-aldehyde, and Ac-Leu-Leu-Met-aldehyde were prepared separately in PBS buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4). A 2000-fold excess of derivatization reagent was added, and the final concentrations of peptides were adjusted to 250 μg/mL. Reactions were carried out for time periods of 10 and 30 min and 1 , 2, 4, 8, and 12 h, respectively. Aliquots (100 μL) of each peptide solution at different reaction times were withdrawn, and the reaction was stopped by freezing at −80 °C. Three peptide solutions were mixed immediately before injection for LC/MS analysis.

To optimize the derivatization agent-to-peptide molar ratio, aliquots of each peptide were separately mixed with the GPR in different molar excess and the peptide final concentrations adjusted to 250 μg/mL. The peptides-to-GPR ratios examined were 1:1, 1:10, 1:100, 1:250, 1:500, 1:1000, 1:2000, and 1:4000, respectively. Again the reaction was carried out for 8 h in the dark and stopped by freezing at −80 °C.

Model Protein Oxidation. Metal-catalyzed oxidation of apotransferrin (human) was accomplished according to Stadtman et al.[3]

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Apotransferrin was dissolved at a concentration of 10 mg/mL in oxidation buffer (50 mM Hepes buffer, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$) and dialyzed against the same buffer at 4 °C to remove any impurities that might be present in the commercial transferrin and could interfere with oxidation reaction. Oxidation was accomplished by incubation with a freshly prepared mixture of neutral ascorbic acid (25 mM) and $FeCl_3$ (1 mM) at 37 °C overnight in a shaking bath. The reaction was stopped by addition of EDTA (1 mM). This sample was then dialyzed against PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) at 4 °C. A control sample was prepared in oxidation buffer supplemented with 1 mM EDTA.

Protein Oxidation and Derivatization. The metal-catalyzed oxidation of apotransferrin was accomplished as described in the previous section. GPR was added (5 mM final concentration) prior to addition of a freshly prepared mixture of neutral ascorbic acid (25 mM) and $FeCl_3$ (100 μM). The mixture was incubated overnight in a shaking bath at 37 °C. The reaction was stopped by addition of EDTA (1 mM), and the sample was dialyzed against PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) at 4 °C and diluted to 2 mg/mL with the same buffer. Then, the protein concentration was measured using the Coomassie blue protein assay. Hydrazone bonds in the derivatives were reduced at 0 °C with addition of an equal volume of 30 mM sodium cyanoborohydride in PBS followed by incubation for an additional 40 min at the same temperature. Excess reactants were removed by dialysis against PBS. Control samples were prepared in oxidation buffer supplemented with 1 mM EDTA.

Yeast Strain and Culture Conditions. The method of Yoo was followed in the growth[24] of wild-type *Saccharomyces cerevisiae* strain SM1058.[25] Yeast cells were grown at 37 °C in yeast extract−peptone−dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) with a shaking incubator operated at 200 rpm. Cell growth was determined by optical density (OD) measurements at 600 nm. For processing a mid-log-phase culture, the overnight cultured cells were inoculated into fresh medium to produce a cell density of 0.2−0.3 OD_{600}.

Preparation of Total Protein from Yeast. Cells from the 50- or 500-mL cultures were harvested and then washed twice with cold water by centrifugation at 3000 rpm for 10 min at 47 °C. The pellet was resuspended in lysis buffer (pH 7.4) containing 3.8 mM $NaH_2PO_4 \cdot 6H_2O$, 49.4 mM $Na_2HPO_4 \cdot 6H_2O$, 48.4 mM NaCl, 5 mM KCl, 20% glycerol, 1% 2-mercaptoethanol, 0.3% IGEPAL CA-630 (Sigma) as a nonionic detergent Complete-Mini protease inhibitor, and 5 mM GRP. After 8 h, an equal volume of 30 mM sodium cyanoborohydride in lysis buffer was added to reduce C=N bonds. Cells were broken by repeated vortexing in cold conditions for 10 min with an equivalent volume of glass beads (0.6-mm diameter; Sigma, G-8772). Supernatant was collected by centrifugation at 14 000 rpm for 10 min at 4 °C. Protein concentration was measured by the Bradford method using a Coomassie protein assay kit (Pierce). Trichloroacetic acid was then added to maintain a concentration of 10% and the mixture incubated on an ice bath for 30 min, after which the solution was centrifuged at 11000*g* for 3 min and the supernatant discarded. The pellet was washed three times with ethanol−ethyl acetate (1:1) and stored at −80 °C for further analysis.

Proteolysis. To denature, reduce, and alkylate protein samples, urea and DTT were added to a final concentration of 6 M and 10 mM, respectively. Mixtures were incubated for 1 h at 37 °C, iodoacetamide was then added to a final concentration of 20 mM, and the reaction was allowed to proceed for an additional 30 min at 4 °C. Cysteine was then added to a final concentration of 10 mM to quench extra iodoacetamide. Samples were diluted 6-fold with 50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Sequence grade trypsin was added (2%) and the reaction mixture incubated at 37 °C for at least 8 h. Proteolysis was quenched by adding TLCK (trypsin/TLCK ratio of 1:1 (w/w)).

Strong Cation Exchange Selection. A TSK-GEL SP-5PW column (7.5 × 7.5 mm) was used for selection of carbonyl-containing peptides. All chromatographic steps were performed with a BioCAD 20 Micro-Analytical Workstation (Applied Biosystems, Framingham, MA). The flow rate was 1 mL/min with dual detection at 215 and 280 nm. To prepare the column for selection, the column was first cleaned with seven bed volumes of a 50 mM MES buffer (pH 6.0, 1.0 M NaCl) and then equilibrated with 50 mM MES buffer at pH 6.0. Before being injected onto the SCX column, transferrin tryptic peptides were adjusted to pH 6.0. Carbonyl-containing peptides were eluted from the SCX column with 50 mM MES buffer (pH 6.0, 0.5 M NaCl). The same procedure was applied in pH optimization studies where peptide selection was tested at pH 4.5 and 7.5 with the exception that 50 mM sodium acetate buffer was used at pH 4.5 and 50 mM HEPES buffer was used at pH 7.5).

LC/MS Analysis. Derivatized and nonderivatized model peptides were separated on a Agilent Zorbax C_{18} column (0.5 × 150 mm) using an Agilent 1100 series instrument at 4 μL/min. In all LC/MS analyses, 40 μL of derivatized and nonderivatized peptides at a concentration of 250 μg/mL was injected. Solvent A was 0.01% TFA in deionized H_2O (dI H_2O) and solvent B was 95% CH_3CN/0.01% TFA in dI H_2O. The flow from the column was directed to the QSTAR workstation (Applied Biosystems, Framingham, MA) equipped with an ESI source. Flow from the HPLC was diverted to waste for 10 min after sample injection at 100% solvent A to remove salts, remaining derivatizing reagent GRP, and weakly adsorbed peptides. The QSTAR was then reconnected, and peptides were separated in a 15-min linear gradient (from 0 to 60% B). MS spectra were obtained in the positive ion mode at a sampling rate of 1 spectrum/s.

LC Separation of Tryptic Peptides. Peptides from the transferrin and yeast lysate tryptic digest were separated on a Vydac C18 column (4.6 × 250 mm) using an BioCAD 20 Micro-Analytical Workstation (Applied Biosystems) at 1 mL/min. A volume of 250 μL of the oxidized−biotinylated transferrin digest at 1 mg/mL (protein equivalent) was chromatographed. In the case of yeast lysate, the injection volume was adjusted to 200 μL at 2 mg/mL (protein equivalent). Solvent A was 0.01% TFA in deionized H_2O (dI H_2O) and solvent B was 95% CH_3CN/0.01% TFA in dI H_2O. Peptides were separated in a 60-min linear gradient (from 0 to 60% B). Fractions from the LC were collected, dried, and stored for later analysis

MALDI TOF-TOF MS Analysis. MALDI-TOF-TOF mass spectra were acquired on a 4700 Proteomics Discovery System (Applied

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Biosystems, Foster City, CA). All samples were collected in the positive linear mode. A sinapinic acid (SA) matrix was prepared as a saturated, aqueous solution that contained 49.9% acetonitrile and 0.1% TFA. The sample was prepared by spotting 1 μL of transferrin peptide solution (described in the previous section and redissolved in aqueous solution that contained 49.9% acetonitrile and 0.1% TFA) first, followed by 1 μL of SA matrix immediately. The sample was allowed to air-dry before loading into the mass spectrometer. Spectra were obtained by averaging at least 200 laser shots. The MS/MS spectra were obtained using the system's automated acquisition mode and a range of peptide mass from 800 to 4000 amu. MS\MS spectra were analyzed using the 4700 GPS data analysis software.

RESULTS

Analytical Strategy. The charge of a peptide is determined by amino acid composition and pH. Since tryptic peptides normally have a basic amino acid at their C-terminus and a primary amine at the N-terminus, they will be positively charged at neutral or slightly basic pH unless they contain other charged amino acids. Peptides that have one or more acidic amino acids would be either neutral or negatively charged at this pH. Histidine does not contribute charge at slightly basic pH. This means that few tryptic peptides will bind to a SCX column at slightly basic pH and low ionic strength.[26] (The exception would be miss-cleaved tryptic peptides.) This fact will be exploited in this work to select tryptic peptides that have been derivatized with one or more quaternary amine groups.

Aldehydes and ketones are readily derivatized by Schiff base formation. Since the object in this work was to isolate and characterize peptides derived from proteins that have been oxidized to the point that they have one or more aldehydes or ketones, Schiff base formation was used to introduce additional positive charge into these proteins at sites of oxidation. This would mean that after trypsin digestion, peptides thus derivatized will be of increased positive charge and be selectable with a SCX chromatography column. (The exception would be peptides that contain multiple acidic amino acids.)

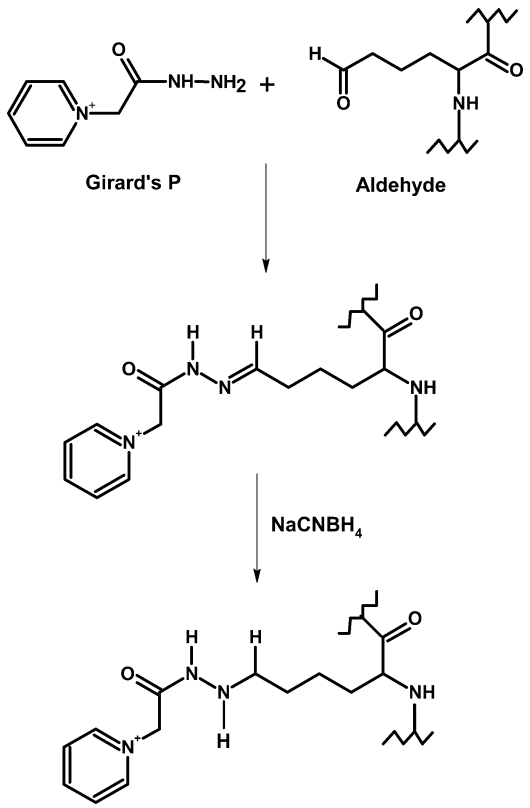

Figure 1. Structure and manner in which GRP was used to label oxidized proteins.

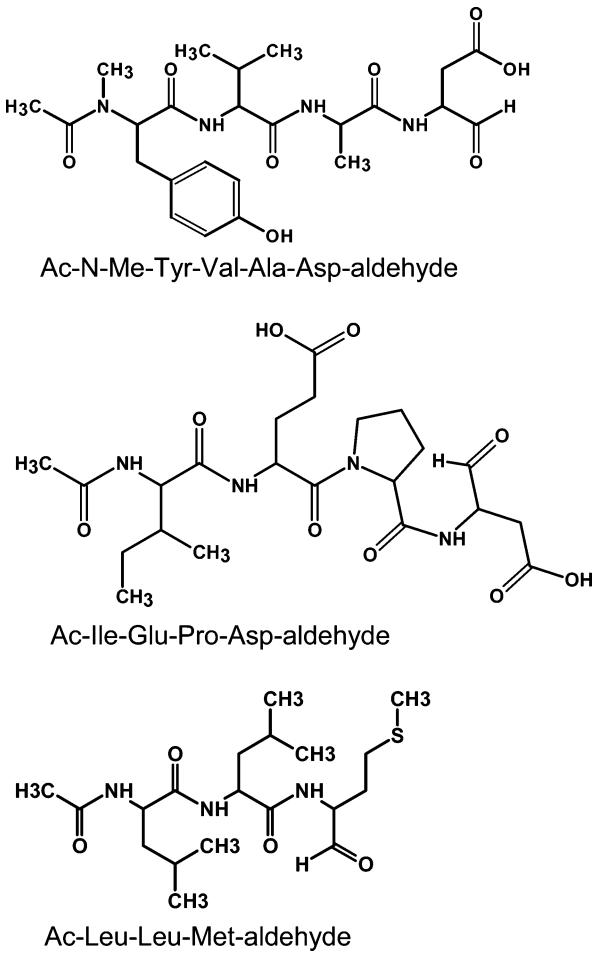

Figure 2. Structures of the three model peptides used for optimization of the labeling.

GPR (Figure 1) is part of a family of reagents originally developed to derivatize and solubilize steroids.[27] Their utility in this work comes from the fact that they have (i) a hydrazide moiety that reacts with aldehydes and ketones in proteins to produce a Schiff base, which can be selectively reduced using sodium cyanoborohydride, and (ii) a quaternary amine moiety for increasing the interaction of tagged peptides with a SCX column. An additional asset is that the quaternary amine on GRP-derivatized peptides will increase ionization efficiency and thus detection sensitivity in MALDI and electrospray ionization mass spectrometry.[26]

The propensity of carbonyl groups to form a Schiff base at neutral pH suggests that they will have a limited lifetime in the amine-rich environment of cells.[8] Moreover, after cell lysis, it is reasonable to expect that this reaction will continue in vitro and

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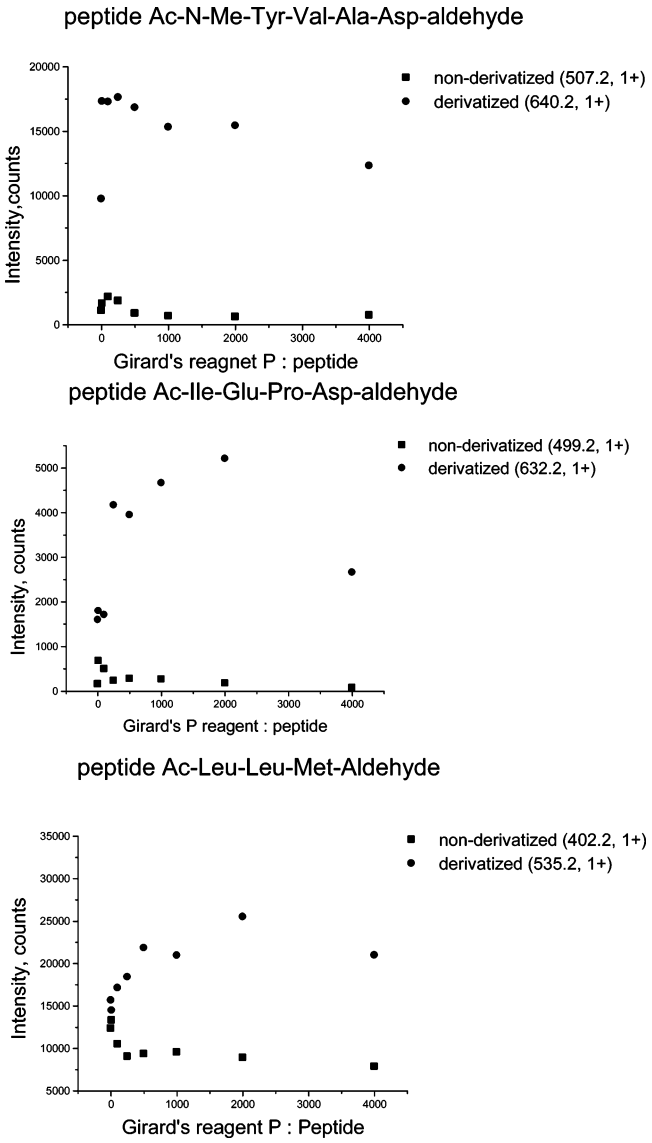

Figure 3. Molar ratio optimization of derivatization reaction. Model peptides were mixed with GPR in 1, 10, 100, 250, 500, 1000, 2000, and 4000 molar ratios. 2000:1 molar ratio shows optimized derivatization efficiency.

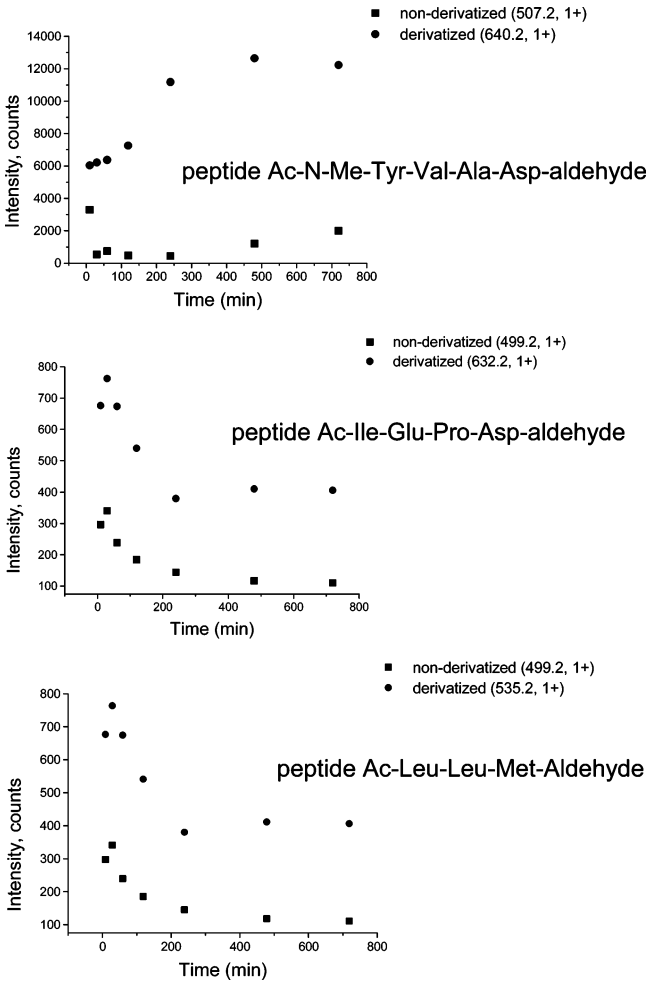

Figure 4. Time optimization of derivatization reaction. Reaction mixtures (molar ratio 1:2000 of peptide/GRP) were stirred in room temperature for 10, 30, 60, 120, 240, 480, and 720 min. At 480 min, the maximum derivatization was accomplished for two of the peptides. Peptide Ac-Ile-Glu-Pro-Asp-aldehyde is unstable even after derivatization. Both derivatized and nonderivatized peptides were decreased. This means in some cases long derivatization time can cause loss of oxidized sites.

the concentration of carbonyl groups will decline further. For this reason, GRP was added to the cell lysing buffer to immediately derivatize oxidized proteins upon cell lysis. The GRP−protein Schiff base thus formed was reduced with sodium cyanoborohydride to preclude hydrolysis.[28] Protein mixtures could then be stored for later analysis. Unreacted GRP was removed by dialysis. Low molecular weight species arising from the oxidation of peptides and lipids were eliminated in this process.

Optimization of the Derivatization Reaction. Derivatization reactions were carried out in the presence of sodium cyanoborohydride so that Schiff base products would be immediately reduced to a more stable product. Reaction conditions were optimized using three synthetic model peptides, Ac-N-Me-Tyr-Val-Ala-Asp-aldehyde, Ac-Ile-Glu-Pro-Asp-aldehyde, and Ac-Leu-Leu-Met-aldehyde (Figure 2). Reactions were carried out at pH 7.4, near-physiological pH where trypsin digestion can subsequently be executed. Fortuitously, Schiff base formation also occurs readily at this pH.[29] The optimum molar ratio of GRP to peptide was examined from 1:1 through 4000:1 in reaction times varying from 10 to 720 min. A high level of derivatization was found through ESI-MS analysis of product formation with the three model peptides to require at least a 2000:1 ratio (Figure 3).

Based on the fact that there was little increase in product formation beyond 480 min, it was concluded that reaction times beyond 8 h would be nonproductive in future studies (Figure 4). In fact, increasing the reaction time to 18 h resulted in a net loss of both derivatized and underivatized peptides due to side reactions (data not shown). This was especially true in the case of Ac-Ile-Glu-Pro-Asp-aldehyde.

Optimization of GRP−Peptide Enrichment by SCX chromatography. The objective here was to find the pH that maximizes both enrichment and selectivity of an SCX column for

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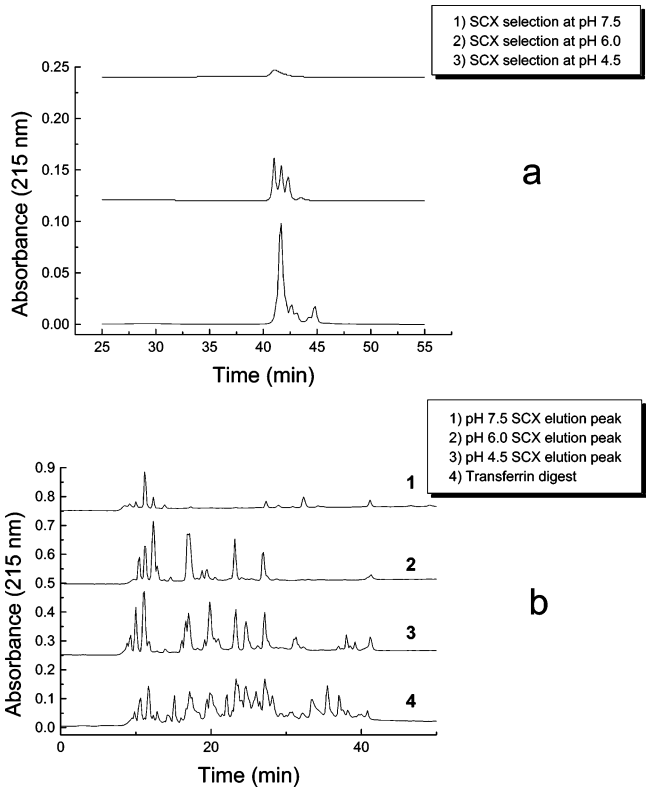

Figure 5. (a) SCX chromatogram of transferrin tryptic digest at pH 4.5, 6.0, and 7.5. (b) Reversed-phase chromatogram of transferrin tryptic peptides collected from SCX column at pH 7.5 (trace 1), pH 6.0 (trace 2), and pH 4.5 (trace 3). Decrease in pH will increase the number of peptides binding to SCX column. Trace 4 in stack of reversed-phase chromatograms shows the RPC profile of the transferrin digest before SCX selection.

GRP-derivatized peptides. Theoretically, the binding of GRP-derivatized peptides on an SCX column will be greatest near neutral pH and low ionic strength. At this pH, the imidazole group in histidine residues is only partially ionized whereas primary amines and guanidine residues will be fully ionized. At slightly basic pH, ionization of primary amines and guanidine will begin to be suppressed as well. Peptide binding at pH 4.5, 6.0, and 7.5 on a TSK-GEL SP-5PW SCX column was examined using tryptic digests of transferrin and oxidized transferrin (Figure 5a). Peptides captured and released from the SCX column were further fractionated with a RPC column (Figure 5b) and identified by MALDI mass spectrometry.

Transferrin is a 77-kDa mass protein that produces 54 peptides when completely tryptic digested, assuming no miss-cleavages. When fractionated by RPC on a C_{18} column and analyzed by automated MALDI-MS/MS using the top 10 peaks in each MALDI spot, a total of 35 (65%) of the 54 native peptides were identified. Analysis of the oxidized and GRP-tagged transferrin sample (Figure 5b, trace 4) resulted in the identification of 23 native peptides, and 6 GRP-derivatized peptides were identified. There were additional GRP-derivatized peptides that could not be identified by MASCOT. Clearly, protein oxidation eliminates some native peptides from the mixture and produces new species that are too complex to be identified with current search engines.

Binding of native tryptic peptides from transferrin to an SCX column at pH 4.5, 6.0, and 7.5 was assessed in a previous study.[26] At pH 4.5, roughly 67% of the native peptides from trypsin were seen, whereas at pH 6.0, ~10% were identified. Of the peptides captured at pH 6.0, all contained histidine residues. Only miss-cleaved peptides were captured at pH 7.5.

Table 1. List of All Derivatized Peptides Identified from MALDI-TOF-TOF MSMS Spectra Search by Mascot[a]

obsd mass	Sequence	Mascot score	peptide sequence
2691.48	547−569	31	GDVAFVKHQTVPQNTGG-**K**N**P**D*P**W**
1282.70	531−540	23	E̲GY̲YGY̲**T**GAF
2548.30	252−273	46	K**P**VDEY̲K̲DC*-HL̲AQVPSHTVVAR
1204.58	363−373	21	WCALSHHE**R**L
1365.70	121−132	50	**K**DSGFQMN̲Q̲LR
1628.83	588−600	24	**K**PVEEYANCHLAR
1659.74	683−695	72	K̲CSTSSLLEAC**T**F

[a] All the peptides have only one derivatized amino acid except GDVAFVKHQTVPQNTGGKNPD*PW, which has three. The derivatized amino acids are boldfaced and underlined. Star (*) indicates other modifications such as alkylation of cysteine or sodiation of aspartic acid. All the oxidation sites were found to be on protein's solvent accessible surface.

Oxidized and tagged transferrin digests were selected with the SCX column (Figure 5a) using the same mobile phases and then further fractionated with the C_{18} RPC column (Figure 5b). RPC fractions were analyzed using a MALDI TOF-TOF 4700 Proteomics Discovery System and the top 10 peaks in each fraction identified as before by selective MS/MS analysis. As expected from the literature,[26] the largest number of peptides were selected at pH 4.5. SCX selection of the oxidized, derivatized transferrin digest at pH 4.5 allowed the identification of 7 GRP derivatized and 19 underivatized native peptides.

Increasing the pH at which peptides were captured on the SCX column to 6.0 resulted in the identification of 7 GRP-derivatized peptides (Table 1) and 14 native peptides. Of the 14 native peptides identified, only 4 were histidine containing without miss-cleavage (SCHTGLGR, APNHAVVTR, WCALSHHER, INHCR), one is a peptide without a miss-cleavage or histidine (ASYLDCIR), and the rest are peptides with at least one miss-cleavage. Clearly, multiple basic amino acids in a peptide increase retention on an SCX column. This problem can be solved by increasing the digestion time or by use of immobilized trypsin columns. Many other GRP-derivatized peptides were observed based on their molecular weight, but they could not be identified by the search engine. As noted before, the GRP-derivatized peptide in oxidized protein mixtures can be oxidized at multiple amino acid and be very difficult to identify.

At pH 7.5, the capture of GRP peptides was poor. Only 1 GRP-derivatized peptide was captured along with 10 underivatized peptides. Although not observed in this work, it has been noted that miss-cleaved peptides with additional lysine and arginine residues will also be captured at pH 6.0 and 7.5.

Identification of Oxidation Sites Using GRP Tagging of MCO Oxidized Transferrin. GRP-derivatized peptides were used to determine the oxidation sites on transferrin (Figure 6). Although arginine, lysine, proline, and threonine were all oxidized to the carbonyl level, lysine and proline were found to be oxidized more frequently than the other two (three different lysine and proline residues were found oxidized in transferrin while only one

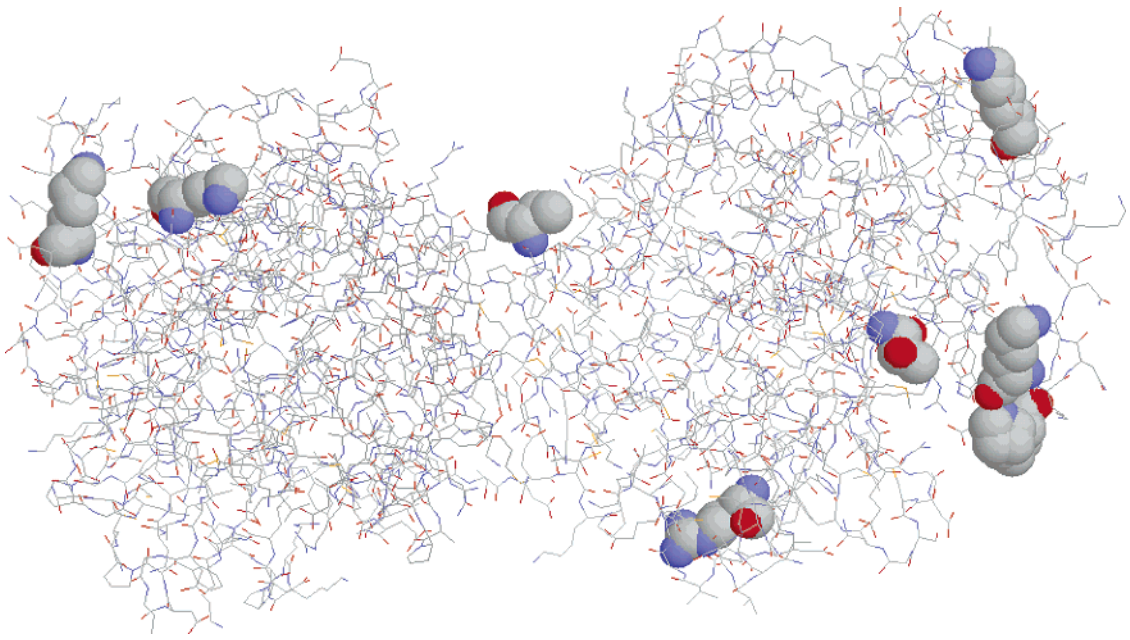

Figure 6. Map of carbonylation sites on oxidized transferrin. There are three lysines and three prolines as well as two thereonine and one arginine found oxidized. All the identified peptides are in the surface of the protein easily accessible by ROS. Two of the peptides carry an iron binding site, which may have contributed to oxidation. As was expected, the in vitro oxidation of the proteins is random at more accessible sites.

arginine and two threonine residues were found to be oxidized). All of these oxidation sites are on the surface of the protein and accessible to solvent. It should also be noted that two of the five oxidized peptides identified have a metal binding site in their sequence. In the peptide K**P**VDEYKDC*HLAQVPS**H**TVVAR, proline 253 is oxidized and histidine 268 is an iron binding site. Similarly, in the peptide EGYYG**YT**GAF, theronine 537 is oxidized while tyrosine 536 is an iron binding site. Since the oxidation method used in this study utilized iron, these observations are reasonable. The MS/MS spectrum for the peptide KCSTSSLLEAC**T**F is shown in Figure 7. As was expected, no fragmentation of the Girard P tag was observed. All the y and some b ions are present. The difference between y1 and y2 in the y ion series shows loss of the derivatized amino acid.

Identification of Oxidized Proteins from Yeast. It has been shown previously that under normal conditions the concentration of protein carbonyls in yeast is ~1.5 nmol/mg of protein. The concentration of protein carbonyls is only increased 1.7-fold after oxidization with hydrogen peroxide for 1 h.[24] To further evaluate the efficacy of this method, GRP was used to label naturally oxidized yeast proteins. Exponentially growing yeast cells in YPD liquid medium were harvested and lysed. Yeast lysate was labeled with GRP and reduced with sodium cyanoborohydride. Two milligrams of the protein pellet was redissolved in digestion buffer and tryptic digested. The tryptic digest pH was adjusted to 6.00, and SCX chromatography was performed using 200 μL of the digest to select labeled peptides. The selected peptides were further separated on a C_{18} reversed-phase column, and fractions were collected. The collected fractions were speed vacuum-dried and were analyzed using the 4700 MALDI mass spectrometer. The acquired MS/MS spectra were submitted for database searches using the Mascot search engine. The list of identified peptides and corresponding proteins is included in Table 2.

Additional Benefits of GRP Derivatization of Oxidized Protein. β-Casein was extensively oxidized according to Stadtman et al.[3] using a metal-catalyzed oxidation system consisting of iron(III) chloride and ascorbic acid. Measurements of β-casein concentration before and after oxidation using the Coomassie blue protein assay indicated a 2.8-fold decrease in protein concentration after oxidation. One explanation for this phenomenon is that oxidation increases hydrophobicity by altering the tertiary structure of proteins. Other possibilities are that proteins are fragmented and cross-link. Fragments produced as a result of oxidative damage would be lost during dialysis, and cross-linked proteins

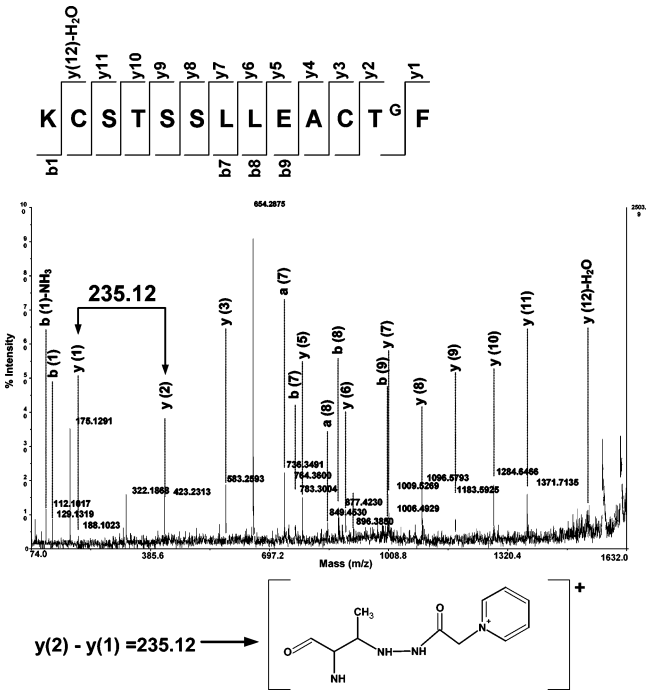

Figure 7. MS/MS spectrum of the peptide KCSTSSLLEAC**T**F acquired by MALDI-TOF-TOF instrument. All y ions and some b ions are assigned. The difference between y (2) and y (1) indicates the loss of GRP-tagged threonine.

Table 2. List of Girard P-Labeled Peptides Identified from Yeast Naturally Oxidized Proteins[a]

no.	protein	NCBI gi|	Girard P-labeled peptides
1	translational activator of COB mRNA	6320403	1653.87^{+} SY^{4}PSLNT^{1}NPNY^{4,5}
2	mitochondrial glutamyl-tRNA synthetase	625182	1529.81^{+} SKRK^{1}GDM^{3}SISD^{6}L
3	involved in telomere length regulation	6320287	1907.10^{+}D^{6}K^{1}YGH^{2}IAHQEGDVCY
4	permease	453646	1377.74^{+}EK^{1}FVY^{4}PFIR
5	human SAP homologue 49	6324895	1907.09^{+}VGNID^{6}P^{1}RIT^{1}K^{1}EQL
6	loss of rDNA silencing	6320647	1907.09^{+} NSKAAH^{2}TSKP^{1}TIHLL
7	ubiquitin fusion degradation protein	6321485	1377.73^{+} IRK^{1}D^{6}DANFGGK 1776.97^{+} APKAEPKQD^{6}IKDMK^{1,5}
8	Sir3p	6323475	1776.98^{+}KACSSSGRE^{6}T^{1}ILSNF^{5}
9	cytoplasmic alanyl-tRNA synthetase	609338	2088.16^{+} HDGT^{1}NFVDEITE^{6}P^{1}GK 2105.18^{+}HDGT^{1}NFVDEITE^{6}P^{1}GKK^{5} 2627.28^{+} AGNDP^{1}E^{6}GR1VAHGC7Y^{4}ISNAALAK^{1}
10	chain A, hat domain Of Gcn5	5822444	1907.09^{+} DYEGGT^{1}LMQC^{7}SM^{3}LPR
11	PHO85 requiring; Ylr114cp	6323143	1515.79^{+} GIC^{7}LVDFHHK^{1}R
12	peroxisomal NADP-dependent isocitrate dehydrogenase; Idp3p	6324319	1529.82^{+} CATITPD^{6}E^{6}AR^{1}M^{3}K
13	pyruvate decarboxylase	4118	3579.74^{+} HRMSANISET^{1}T^{1}SM^{3}ITDIATAP SE^{6}ID^{6} RLIR^{5} 2088.17^{+} H^{2}H^{2}TLGNGDFTVFHR 2105.18+ H^{2}H^{2}T^{1}LGNGD^{6}FTVFHR
14	Bin2p	493574	1907.14^{+} ACT^{1}IMLRGGSKD^{6}IL
15	CCR4 associated factor; Caf16p	14318491	3039.47^{+}SPLSMNQVD^{6}DDESVE^{6}D^{6}ST^{1} NYQTTTY
16	protein required for cell viability	6322364	1907.11^{+} IP^{1}GFPMLFLHM^{3}VAQR
17	MER2	758284	1444.88+ M^{3}M^{3}VTR^{1}SMKR
18	Cin8p	3542	2105.12+ LKSK^{1}E^{6}TIQSQNCQIESL
19	defective F1F0-ATPase α subunit precursor	2447013	1166.69+ AVD^{6}ALVP^{1}IGR
20	protein forms dimers in vivo and in vitro	6681846	2088.17^{+}KIIYD^{6}E^{6}DGKPC^{7}R^{1} 2105.18^{+} KIIYD^{6}E^{6}DGK^{1}PC^{7}R^{1,5}
21	transacetylase; Ayt1p	6322965	1485.47^{+} REDLSAP^{1}TM^{3}DSL
22	excises 7,8-dihydro-8-oxoguanine	6323580	1485.46^{+}YNALPISRKK^{1,5}
23	adheres VOraciously (to TOR2); Avo1p	6324494	1156.62^{+} SSTLHPP^{1}GAR
24	nonhomologous end-joining regulator 1	6323295	1114.68^{+} SD^{6}AE^{6}WCVK^{1}
25	deubiquinating enzyme	706835	1114.68^{+} AH^{2}IAP^{1}INTK
26	phosphatidylserine synthase	218415	1444.83^{+}ATFNVTVAQLPK^{1,5}

[a] This includes proteins that are oxidized as a result of naturally occurring exogenous and endogenous ROS. The modifications coded 1−6 are (1) GRP-labeled lysine, argentine, proline, or thereonine, (2) hydroxylated histidine or tryptophan, (3) oxidized methionine, (4) tyrosine oxidation to nitrotyrosine, (5) sodiation at the C terminus, and (6) sodiation of aspartic and glutamic acid.

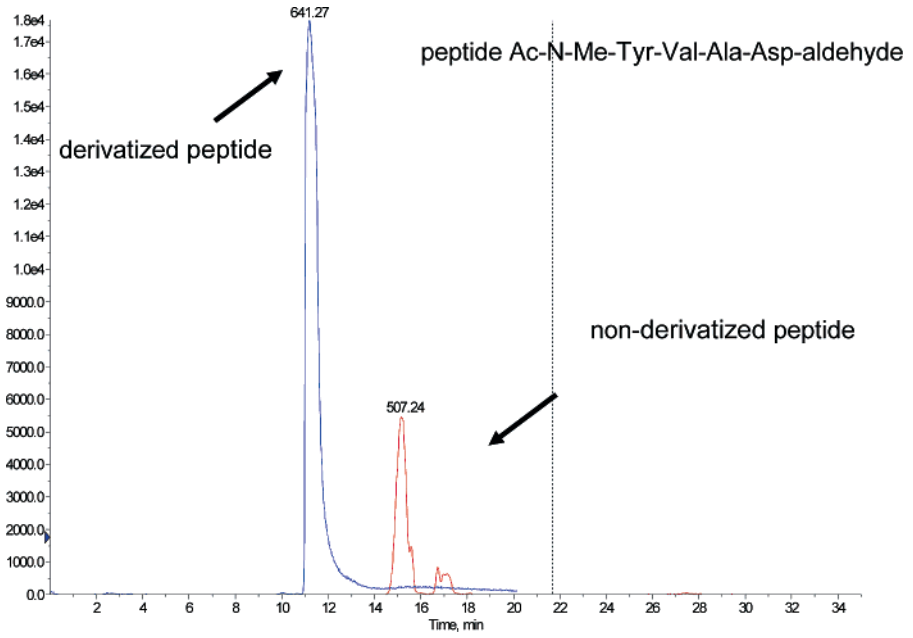

Figure 8. Increase in ionization efficiency of model peptide Ac-N-Me-Tyr-Val-Ala-Asp-aldehyde after derivatization. The reconstructed ion chromatogram of the derivatized and nonderivatized peptide mixed in 1:1 ratio indicates 3.7-fold increase in ionization efficiency. Derivatized peptide carries one permanent positive charge that enhances the ionization.

are less assessable to staining. During the study of GRP derivatization, oxidized β-casein was derivatized with GRP and the mixture filtered and dialyzed to eliminate salts that could interfere with protein concentration measurements. Again using the Coomassie blue assay to measure concentration, it was determined that there was a 1.8-fold decreases in protein concentration after oxidization and GRP derivatization. The reduction of protein in this second case is thought to be due to a solubility-enhancing effect of adding positive charge to cross-linked or denatured proteins. Increasing protein solubility would be a secondary benefit of GRP derivatization.

It has been noted in the literature and comments above that increasing the positive charge in peptides increases detection efficiency. Evidence of this with GRP is seen in the model peptide Ac-N-Me-Tyr-Val-Ala-Asp-aldehyde (Figure 8). The other model peptides used in these studies showed increased ionization efficiency as well after derivatization with GRP (data not shown). A general increase in detection sensitivity is still another advantage of using this reagent.

CONCLUSION

There is a need to identify posttranslational modifications. The unique feature of posttranslational modifications involving protein oxidation to the aldehydes and ketone level is that the process is nonenzymatic and not genetically coded. It is therefore difficult to find a binding protein that recognizes this posttranslational modification specifically.

It is concluded from the studies presented here that derivatization of aldehydes and ketones in proteins with GRP is an effective way to specifically tag oxidized proteins. Moreover, the derivatization process introduces additional positive charge at the site of oxidation. After trypsin digestion, these GRP-containing peptides can be enriched from proteolytic digests by cation exchange chromatography at near-neutral pH. This simplifies proteome digests and aids in the identification of protein oxidation sites. An additional advantage of GRP derivatization is that the ionization efficiency of derivatized peptides in increased. A disadvantage of the method is that selection is not absolute. Some nonderivatized peptides are selected as well, but they are easily recognized by search engines. The ease with which this method can be used with existing proteomics tools and instrumentation will be a valuable asset in the study of oxidative stress and oxidative stress diseases.

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