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Acetone Precipitation of Proteins and the Modification of Peptides

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Acetone precipitation is a common method for precipitation and concentration of proteins. We show here that a trace amount of residual acetone in the precipitated protein, can, after proteolysis, lead to selective modification of peptides predominantly those in which a glycine residue is the second amino acid, probably generating a relatively stable derivative that, under gas phase conditions, generates a y_1 ion of the same mass as proline. This modification is detectable by either MALDI-ToF or ESI-ion trap mass spectrometry and under normal sample preparation conditions is incomplete. The derivatization occurs in the condensed phase and is sufficiently stable that the modified peptide can elute on reversed phase chromatography at a different time to the unmodified peptide. Acetone precipitation is such a commonly used procedure in protein sample preparation for proteomics that some caution may be warranted. A significant number of peptides (about 5% of a typical proteome) meet the requirements for this reaction and could, therefore, change the outcome of studies.

Keywords: Acetone modification • peptide modification • glycine enolates

Introduction

Quantitative mass spectrometry has become increasingly important in systems biology to deliver accurate quantification of sets of peptides/proteins. Accurate quantification of the components of a biological system is critical to analyses of mechanisms and rate control and for the generation of mathematical models that can then be used to predict how a system or pathway will behave following system perturbation. In mass spectrometry, proteins are often quantified using stable isotope labeled proteotypic peptides which act as surrogates for the proteins of interest. Selected reaction monitoring (SRM) methods are commonly used whereby precursor/fragment ion transitions are monitored in triple quadrupole instruments. This type of experiment provides increased selectivity and sensitivity, and if a stable isotope-labeled peptide present in the sample at a known concentration is also monitored, the absolute amounts of the unlabeled peptide and hence the target protein can be determined. Alternatively, label-free methods capitalize on ion statistics or protein coverage to generate estimates of protein abundance.[1−3]

Both approaches to quantification require that the peptides used for quantification are formally representative of the parent protein, being excised quantitatively and containing no variable or unexpected modifications. This is less of an issue in shotgun discovery proteomics experiments where the goal is the identification of proteins, a process that has a high degree of redundancy and which does not need complete coverage of the protein. Peptide modifications, while being undesirable, may not have a great impact on the success rate of such experiments, as the loss of a single peptide due to an unanticipated modification may not compromise the confidence score of the identification. At the most extreme, the increase in sample complexity due to modified peptides may increase false discovery rates or decrease the number of true positives.

However, for quantification proteomics, it is important to ensure that the targeted peptide is not partially converted into a modified form in an incomplete, irreproducible and unpredictable manner. Well-recognized peptide modifications occur at the side chains of methionine (+16 Da) and tryptophan (+16/+32 Da) which are prone to oxidation. Peptides containing glutamine or asparagine residues may deamidate spontaneously to glutamate (+1 Da) or aspartate (+1 Da) depending on the sequence context of these residues.[4,5] In addition, N-terminal glutamine residues are quickly transformed to pyroglutamate (−17 Da) under acidic conditions. Four novel types of *in vitro* peptide modifications introduced during sample preparation have recently been reported; these include ethylation of aspartate and glutamate (+28 Da), esterification of aspartate and glutamate by glycerol (+74 Da), loss of 19 Da from lysine, and addition of 108 Da to cysteine.[6]

In this report, we describe the modification of peptides by acetone, a modification dependent on sequence context whereby a glycine residue is the second amino acid in the peptide. Gas phase modification of glycine residues has been reported previously,[7] but here, we show this reaction occurs readily in the condensed phase and might, therefore, compromise proteomics studies in which the sample preparation includes acetone treatment.

Experimental Section

Sample Preparation. Carbamidomethylcysteine modification of a QconCAT protein (10 μg) was carried out as follows: samples were reduced by the addition of an equal volume of

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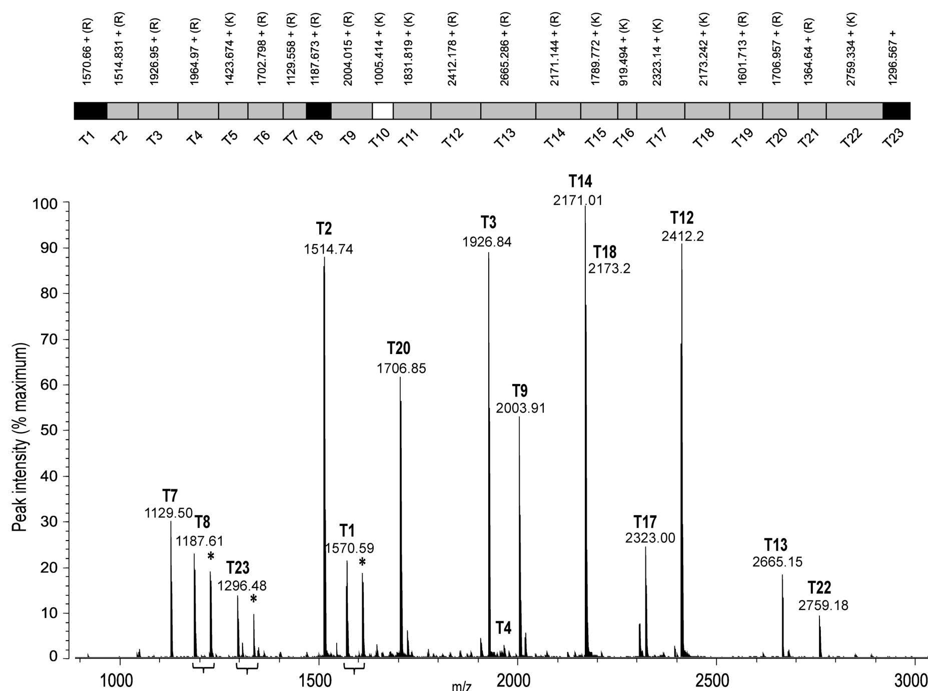

Figure 1. MALDI-ToF mass spectra of a QconCAT highlighting peptides susceptible to acetone modification. A recombinant QconCAT protein was reduced and alkylated and either precipitated by TCA or by acetone. TCA precipitated protein was washed with either acetone or ether. A tryptic peptide map of the protein illustrates peptide coverage of the protein in the spectrum (filled blocks) with modified peptides shaded in black. Each tryptic peptide (T1−23) is labeled with the monoisotopic $[M + H]^+$ mass. Satellite +40 Da peaks are denoted by an asterisk in the mass spectrum.

20 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 with incubation at 56 °C for 1 h. An equal volume of 110 mM iodoacetamide in 50 mM NH_4HCO_3 was added and samples were incubated at room temperature in the dark for 45 min. The protein was then precipitated by the addition of an equal volume of 35% (w/v) trichloroacetic acid (TCA) and the sample was held on ice for 2 h. Precipitated protein was isolated by centrifugation at 16 000*g* and the pellet was washed in either acetone or, alternatively, ether, to remove residual TCA. For comparison, reduced and alkylated protein was precipitated in 5 vol of acetone and the sample was held on ice for 2 h. Samples were digested to completion overnight at 37 °C with trypsin (0.2 μg) in 50 mM NH_4HCO_3.

MALDI-ToF MS Analysis. Samples were diluted in a 6 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile (ACN)/0.1% (v/v) trifluoroacetic acid (TFA). A total of 1 μL of sample was spotted onto the MALDI target and spectra were acquired over a mass range of 800−4000 m/z using an Axima ToF^2 in reflectron mode (Shimadzu Biotech, Manchester, U.K.).

ESI-MS Analysis. Samples (1 pmol) were desalted in-line using a (5 mm × 0.3 mm i.d.) C18 precolumn before chromatography on a reversed phase C18 Pepmap column (3 μm, 150 × 0.075 mm, 100 Å) using a U3000 chromatography system (LC Packings, Dionex, U.K.). The column was equilibrated in 0.1% (v/v) formic acid/2% (v/v) ACN (solvent A) and was developed with 90% ACN/0.1% formic acid (solvent B) 0−50% over 30 min at a flow rate of 300 nL/min into the source of an LTQ linear ion Trap (Thermo Electron, U.K.). The instrument was operated in a data-dependent mode whereby the three most abundant peptide ions were fragmented. For each full mass range scan, the zoom scan (mass window 10 Da) and subsequent MS/MS spectra were obtained sequentially for three peptide ions in a single cycle. Dynamic exclusion parameters were set to minimize repeated acquisition of intense ions (repeat count was 2, dynamic exclusion set at 30 s). The parent ion for each MS^2 scan was isolated with a mass window of 2.0 m/z units, fragmented using a collision energy of 35% with an activation time of 30 ms at $Q = 0.25$.

Direct infusion of a QconCAT digest, 1.25 pmol/μL in 50% (v/v) ACN/0.1% (v/v) formic acid was facilitated using a syringe driver (Harvard Apparatus Ltd., U.K.) at a flow rate of 0.5 μL/min. The metal-coated picotip (New Objective, Presearch, U.K.) was maintained at 1800 V relative to the source of a Q-ToF Micro (Waters, U.K.). Data was acquired over the mass range 400−1500 m/z and selective transmission of target ions to the collision cell was followed by manual optimization of collision energies for peptide fragmentation.

Results and Discussion

We have pioneered the use of artificial proteins (QconCAT) that are built by *de novo* gene synthesis to encode concatamers of tryptic peptides, such that after metabolic stable isotope labeling and codigestion with an unlabeled analyte, the released peptides act as a stoichiometrically equivalent set of quantification peptides.[8−10] In a study using a QconCAT to quantify proteins of the NF-κB signaling pathway, we noted inappropriate peptide masses in routine characterization of the QconCAT protein prior to deployment. The QconCAT which contains several cysteine residues was reduced, alkylated and precipitated with TCA (17% (v/v) final). After centrifugation, the residual TCA was removed by washing the pellet with acetone or ether prior to tryptic digestion.

Of the 23 peptides in the QconCAT, all but one were detected by MALDI-ToF (Figure 1). However, three additional ions, not apparently encoded in the QconCAT, were detected, each at a mass 40 Da heavier than one of the QconCAT peptides. These

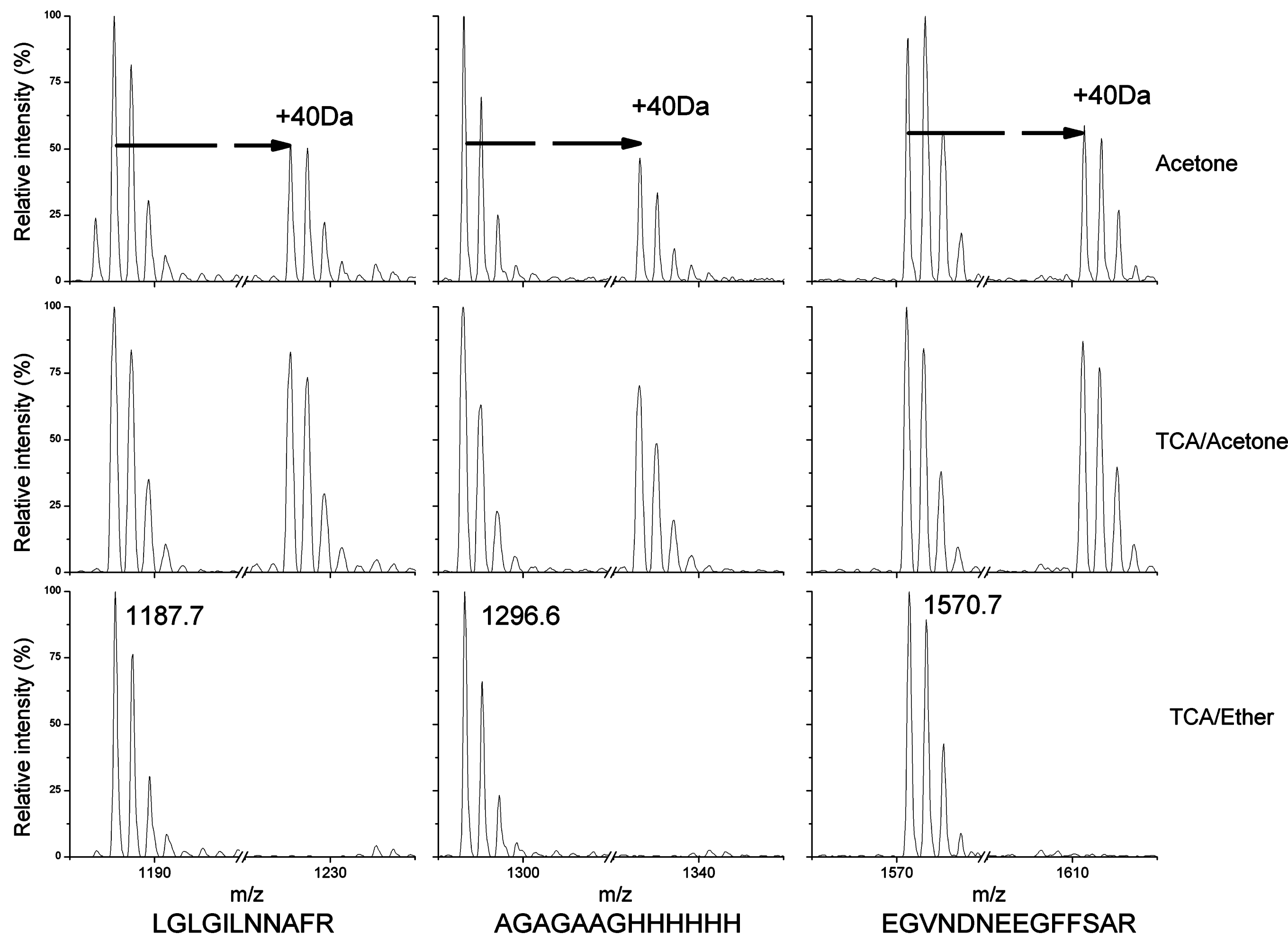

Figure 2. MALDI-ToF mass spectra of peptides susceptible to acetone modification. Zoom spectra of peptides observed to be modified by acetone: LGLGILNNAFR $[M + H]^+$ 1187.67, AGAGAAGHHHHHH $[M + H]^+$ 1296.57 and EGVNDNEEGFFSAR $[M + H]^+$ 1570.66. are aligned to compare the differences between acetone precipitation (top panel), TCA precipitation followed by acetone washes (middle panel), and TCA precipitation followed by ether washes (lower panel).

ions were clearly artifacts, and after systematic exploration of sample preparation and analysis conditions, the modification was attributed to the use of acetone in the sample preparation workflow. When acetone was used as a precipitant alone, or when TCA was used as a precipitant followed by acetone washes to remove TCA, the +40 Da adducts were highly evident. By contrast, when TCA precipitation was followed by diethyl ether washes, no adduction was observable, and the peptide mass was unchanged (Figure 2).

Closer examination of the MALDI-ToF mass spectrum of the three modified peptides from the QconCAT revealed that a common feature was the presence of glycine as the second amino acid: LGLGILNNAFR $[M + H]^+$ 1187.67, the C-terminal hexa-histidine peptide AGAGAAGHHHHHH $[M + H]^+$ 1296.57 and glu-fibrinopeptide EGVNDNEEGFFSAR $[M + H]^+$ 1570.66. Satellite peaks (+40 Da) are present in spectra acquired for samples where acetone was utilized to precipitate protein and when acetone was employed to remove residual TCA from the protein pellet (Figure 2). No satellite peaks were observed when acetone was substituted by ether. Additionally, two other peptides with an XGX_n sequence showed evidence of +40 Da satellite peaks (peptides 4 and 5, Table 1), although both peptides generated low intensity peaks by MALDI-ToF. No evidence of additional satellite peaks and therefore modifications to additional glycine residues were observed in the spectra of these, or of any other glycine containing peptides.

Acetone, to a final volume of 10% (v/v), was added to a tryptic digest of the NF-κB QconCAT in which precipitated protein had been washed with ether. Immediately after addition of the acetone, 2 μL of digest was removed and diluted 10-fold in matrix solution, and 1 μL was spotted onto the MALDI target. The acetone incubation with digest was sampled at 0.5 h and at hourly intervals up to 4 h. From the first time point of 0.5 h, satellite peaks (+40 Da) were present in the spectrum for all peptides of sequence context XGX_n where X represents any amino acid residue. The proportion of modified to unmodified peptide present for three peptides over the time course of the experiment is plotted in Figure 3. The proportion of modified peptide increased over time with a rate constant of 0.29 ± 0.01 h^{-1}. After overnight incubation, the proportion of modified to unmodified peptide was similar to that determined after 4 h (data not shown). The nonartifactual nature of this modification and its formation in the condensed as opposed to the gas phase was verified by LC-MS on an LTQ ion trap. The extracted ion chromatograms for both the unmodified and modified form of the peptide LGLGILNNAFR, $[M + 2H]^{2+}$ 594.34 and $[M + 2H]^{2+}$ 614.34, respectively, are illustrated in Figure 4A. The modified peptide has a longer retention time of 27.46 min

Table 1. Peptides Modified by Acetone[a]

	peptide	protein, accession	location
1	EGVNDNEEGFFSAR	[Glu]-fibrinopeptide B	
2	LGLGILNNAFR	NF-κB1, P19838	T79
3	HGTMDTESK	NF-κB1, P19838	T48
4	YGCEGPSHGGLPGASSEK	NF-κB2, Q00653	T7
5	HGDTALHVACQR	IκBϵ, O00221	T13
6	AGAGAAGHHHHHH	Purification tag	
7	AGIYYMNYDGFNTFSILK	MUP 3, P04939	L4
8	AGIYYLNYDGFNTFTILK	MUP 26, Q80YX8	L4
9	AGEYSVTYDGSNTFTILK	MUP 24, Q5FW60	L7
10	DGETFQLMELYGREPDLSSDIK	MUP 24, Q5FW60	L10
11	LGEYGFQNALIVR	BSA, P02769	T58

[a] Peptides 2−5 are unique tryptic peptides included in a QconCAT designed to quantify proteins in the NF-κB signalling pathway. Peptide 1 was included as a quantification peptide in QconCAT NF-κB. Peptides 7−9 are LysC peptides of mouse urinary protein (MUP) isoforms included in a QconCAT designed for polymorphism screening of MUPs. The parent protein, UniProtKB accession number and location of either the tryptic (T) or LysC (L) peptide within the protein are noted.

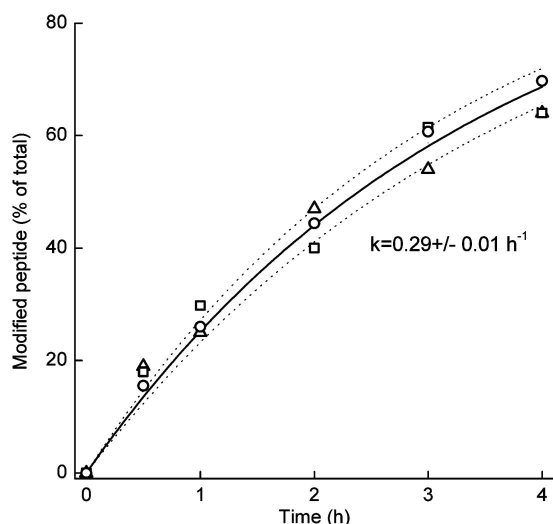

Figure 3. The proportion of modified peptide following the addition of acetone to peptide digests. Acetone was added to a final volume of 10% (v/v) to a digest of a recombinant protein in which precipitated protein had been washed with ether prior to trypsinization. Immediately after acetone addition, 2 μL of digest was removed and diluted 10-fold in matrix solution, and 1 μL was spotted onto the MALDI target. The digest was sampled at 0.5 h and at hourly intervals up to 4 h. Spectra were acquired over the mass range 800−4000 *m*/*z*. The intensities of each peak in the isotopomer profiles of both modified and unmodified peptides were summed and the proportion of modified to total peptide for three peptides was plotted over the time course of the experiment.

compared with 24.22 min for the unmodified peptide. The corresponding fragment ion spectra for these peptides are illustrated in Figure 4B, and while the spectra confirm the common identity of these peptides, the sequence cannot be fully interpreted. Accordingly, peptides were infused directly into the source of a Q-ToF Micro and spectra were acquired in the full-scan mode over the range 400−1500 *m*/*z*. Unmodified peptides and their corresponding +40 Da modified peptides were identified and the first quadrupole was set to transmit a single precursor for which the collision energy was manually optimized for fragmentation. A collision energy of 28 V was used to fragment both peptides. *De novo* sequencing of the *y*-ion series using the PepSeq utility of the MassLynx software interpreted "proline" (we use the term as a placeholder because the structure of the acetone adduct is not proven, but the mass is the same as a proline residue, and fragment ions maintain that behavior) as the second amino acid residue in the peptide pinpointing the site of modification to the second residue with a mass difference of +40 (G → "P") (Figure 5). The modification was also confirmed by the b-ion series, with an intense *b2* ion at 211.15 *m*/*z* consistent with the L"P" dipeptide (data not shown). High resolution (>30 000) spectra were obtained by infusion of a tryptic digest into the source of a high resolution mass spectrometer (MaXis Q-ToF, Bruker, U.K.). The mass difference between the modified and unmodified peptide was determined for two peptides and was consistent with a glycine to "proline" modification with a mass difference of 40.0304 and 40.0316 Da for LGLGILNNAR and HGTMDESK. respectively.

Modified peptides have been observed in other QconCAT/analyte mixes: polymorphism screening of mouse urinary proteins (MUPs) using QconCAT methodology also revealed peptides susceptible to acetone modification. Table 1 lists three peptides present in the QconCAT that were modified following TCA precipitation and acetone washes. The peptides exhibit poor ionization characteristics in MALDI-ToF MS so the +40 Da satellite peaks are not readily observed. ESI-LTQ trap analysis resolved the unmodified and modified peptides with identities confirmed by fragment ion spectra (Supporting Information). The corresponding analyte peptide for two of the quantification peptides were not detected in MUPs as the MUP isoforms, if expressed, were expected to be low-abundance proteins (Table 1, 7 and 8). The Q-peptide representing a male-specific MUP (Table 1, no. 9), and the corresponding analyte peptide were both modified by acetone (Supporting Information). From a total of 16 Q-peptides in the QconCAT, the three peptides with the XGX_n sequence exhibited the +40 Da modification. Following the same sample workup using BSA, the only peptide which contained glycine as the second residue was modified from a total of 54 BSA peptides observed in the spectrum (Table 1 and Supporting Information). This modification is, thus, highly favored in XGX_n peptides.

At present, we do not know the nature of the condensed phase glycine modification that gives rise to this adduct. Several observations are pertinent. The resolution of the unmodified and modified peptides on reversed phase chromatography suggest that the adduct is in place in the condensed phase and stable to the acid conditions of the buffers. The unaltered mobility of all other peptides, which would have the potential to form aldimine products, suggests that these are unstable

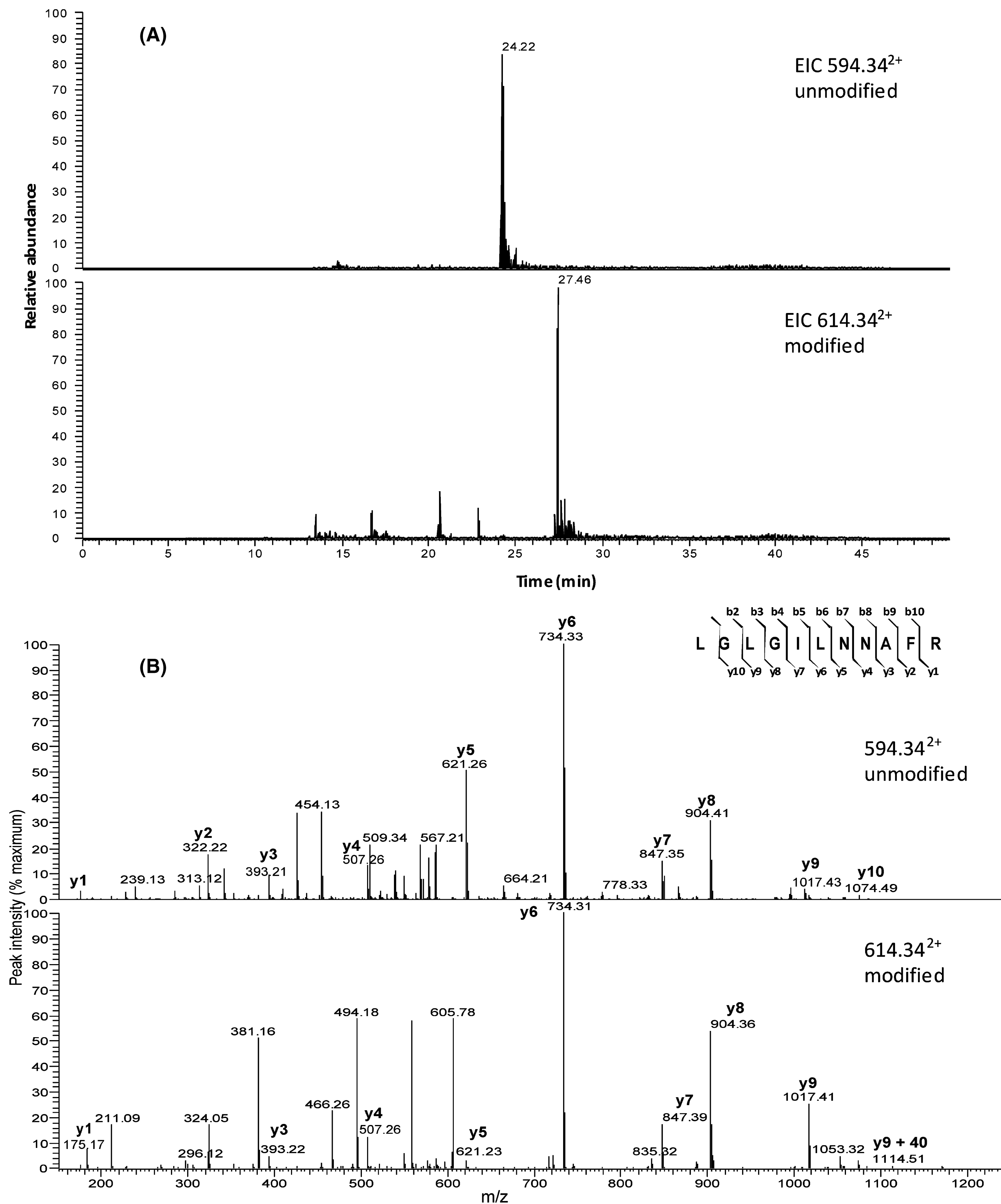

Figure 4. ESI-MS of unmodified and modified peptides. Peptides were chromatographed on a C18 capillary column directly into the source of an LTQ ion trap and fragmented in a data-dependent mode. (A) Extracted ion chromatograms of the unmodified peptide LGLGILNNAFR, $[M + 2H]^{2+}$ 594.34 and the corresponding modified peptide $[M + 2H]^{2+}$ 614.34 and (B) the corresponding fragment ion spectra for both peptides.

under the acidic conditions of chromatography. If the XGX_n peptides are acetylated first by reaction with NHS-acetate, no acetone adduct formation is observed, demonstrating a requirement for a free N-terminal amino group, and implying the role of aldimine formation ($(CH_3)_2C{=}NH\text{-}CHR\text{-}CO\text{-}NH\text{-}CH_2\text{-}CO\text{-}$) as the first step. The formation of the aldimine would

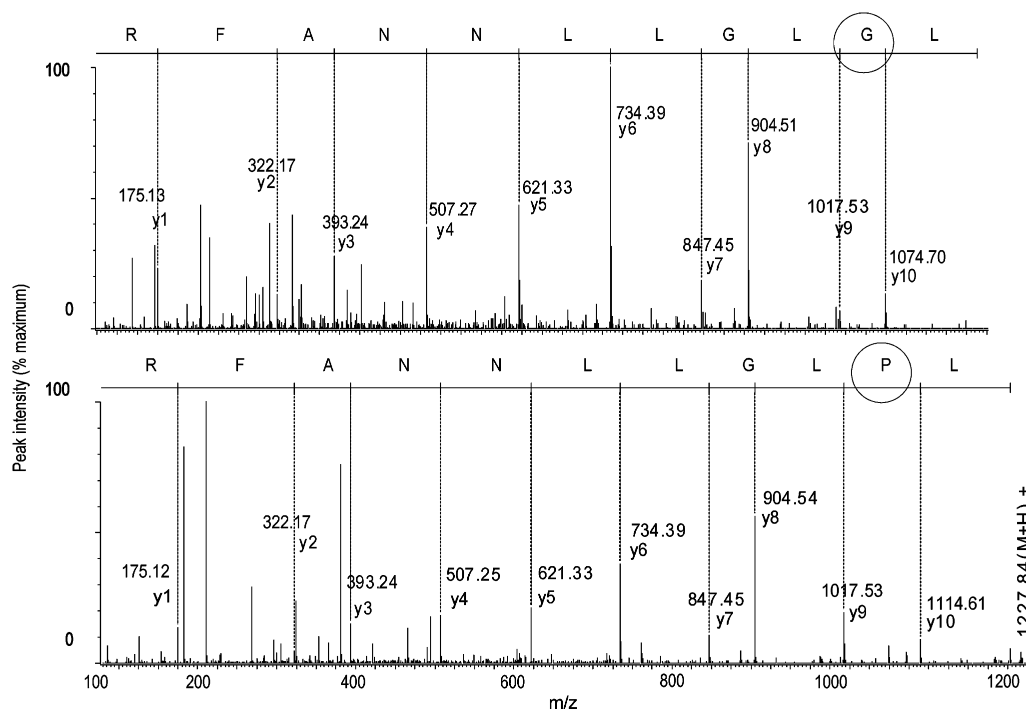

Figure 5. Fragmentation spectrum of a modified and unmodified peptide. A tryptic digest of a recombinant protein diluted into 50% (v/v) ACN/1% (v/v) formic acid was directly infused into the instrument at a flow rate of 0.5 μL/min. Q1 was set to transmit either the unmodified precursor $[M + 2H]^{2+}$ 594.3 or the modified precursor $[M + 2H]^{2+}$ 614.3 and a collision energy of 28 V was set for peptide fragmentation. One hundred spectra were acquired, combined and processed using the MaxEnt 3 utility of Masslynx 4.0. (Top panel) unmodified and (bottom panel) modified fragmentation spectrum.

Table 2. Proteome Abundance of XGX_n Peptides[a]

species	proteins	total peptides	XGX_n peptides	proteins with $\geq$1 XGX_n peptide
Homosapiens	25269	1493340	75218 (5.0%)	20449 (80.9%)
Gallus gallus	10792	425225	23355 (5.5%)	8129 (75.2%)
Saccharomyces cerevisiae	5883	335365	13972 (4.2%)	4678 (79.5%)
Escherichia coli	4341	133689	8605 (6.4%)	3235 (74.5%)

[a] The proteomes were processed using Transcript scripts (www.runrev.com) written by R.J.B., and available upon request. These scripts simply screened all tryptic peptides for the presence of glycine in the second position and generated the statistics above.

increase mass by 41 Da, not 40 Da, and such a structure would be acid-labile and, thus, not persistent under chromatographic conditions. It is likely, therefore, that a subsequent rearrangement must stabilize the adduct and yield a mass increase of 40 Da. The most likely candidate is the imidazolidinone (intermediate F, scheme 7, ref 7- in this paper, the reaction is given for a triglycine peptide, but the reaction would remain the same). Further evidence in support of this structure is the observation that the acetone adduct can no longer be acetylated by NHS acetate, even under conditions when the unmodified peptides can be readily reacted (Supplementary Figure 4).

Acetone modification of peptides is well-known, but is conventionally considered to involve aldimine formation between the ketone and the α- or ε-amino groups of a peptide. Indeed, a method of *in vitro* stable isotope mediated quantification has been proposed, based on this chemistry.[11] However, because of the acid lability of these adducts, the aldimine has to be stabilized by reduction, for example, with sodium cyanoborohydride. By contrast, the adduct we observe, specific to peptides of the XGX_n structure, does not require reduction for stability. As such, these may persist through any proteome analysis workflow that uses acetone as a treatment step. It is worth recalling that these adducts were observed after the use of acetone to remove residual trichloroacetic acid in a protein precipitation step. Acetone is frequently used to remove residual TCA following protein precipitation, and although the protein pellet is subsequently dried at 37 °C to drive off residual acetone, significant conversion of unmodified to modified peptide occurs. Acetone added to digests at a final concentration of 10% (v/v) mediated a conversion rate of 0.29 h^{-1}; therefore, with a trace amount of acetone (≤1%) in the sample, a rate constant of 0.03 h^{-1} would be expected resulting in a significant conversion to the modified peptide.

The impact of this modification is first as an artifact that can impede proteomics experiments, by creating adducts that split an analyte signal (typically by 50% in our experience), generating additional chemical noise that elutes differently to the parent peptide. The modification would have to be included in any search terms which might weaken the search. Lastly, there is the possibility of the aberrant amino acid being called by sequencing software as a proline rather than a glycine residue. All of these outcomes would only be of major significance if the frequency of XGX_n peptides was high enough to create a significant problem. We have analyzed several proteomes for the presence of such peptides (Table 2), although this analysis included no restriction based on, for example, size of the peptide. The frequency of tryptic peptides with a glycine

residue as the second amino acid position is between 4 and 6%. This accords well with the frequency of glycine in the proteome of the different organisms (between 6 and 7%) and there is little evidence for bias in favor or against peptides with a glycine as the second residue.

Although this observation is most readily seen as an undesirable outcome of acetone treatment of protein samples that should and can be avoided, it might also have some utility for selective tagging of a subset of a proteome. If all tryptic fragments are considered, between 75 and 80% of a proteome has at least one XGX_n peptide. However, this is an overestimate, and if an additional criterion is applied, of mass greater than or equal 800 Da, the representation drops from 79% to 63%, still a substantial population of peptides. While modification by acetone can at best provide *in vitro* isotope tagging for quantification, more complex ketones, provided they were able to undergo the same reaction, might also allow for selective isolation of a similar subset of peptides, creating an enriched data set that would be analogous to the enrichment achieved by ICAT, for example. Orthogonal juxtaposition of multiple overlapping selective steps could provide a route to an efficient quantitative proteome profiling.

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Supporting Information Available: LC-MS and LC-MSMS analysis of MUP peptides modified by acetone. MALDI-ToF MS analysis of BSA and of partially acetylated glufibrinopeptide following acetone treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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