

Proteolytic ^{18}O Labeling for Comparative Proteomics: Evaluation of Endoprotease Glu-C as the Catalytic Agent[†]

Kristy J. Reynolds, Xudong Yao, and Catherine Fenselau*

University of Maryland, Department of Chemistry and Biochemistry, College Park, Maryland 20742

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Recently, proteolytic ^{18}O labeling has been demonstrated as a promising strategy for comparative proteomic studies (Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. *Anal. Chem.* **2001**, *73*, 2836–42). In this approach, protein mixtures are digested in parallel in H_2^{16}O and H_2^{18}O and the ratios of isotopically distinct peptide products are measured by mass spectrometry. In the initial report from this laboratory, trypsin was shown to catalyze incorporation of two ^{18}O atoms into the carboxyl terminus of each new peptide formed by cleavage of the adenovirus proteome. In the present study, a second enzyme, endoprotease Glu-C, is evaluated as an agent for cleavage and labeling. Proteolytic ^{18}O labeling by Glu-C is shown to occur readily with phosphorylated and glycosylated proteins and with cysteine-alkylated and disulfide-linked proteins. A sequential double-labeling strategy is used to characterize N-linked glycopeptides. Labeled and unlabeled peptide pairs are found to coelute chromatographically, and measurements of isotope ratios by nanospray and capillary LC-MS are found to be accurate and precise.

Keywords: proteolytic labeling • comparative proteomics • relative quantitation • mass spectrometry • Glu-C

Introduction

A knowledge of the complexity and interaction of proteins, which will allow comprehension of dynamic cellular phenomena, is the central objective of proteomic strategies.^{2,3} Toward achieving this goal, fundamental methods are under development to identify changes in protein expression and post-translational modifications, in addition to obtaining protein identities. Such methods should be applicable to a full range of proteins with different physical properties, in a high-throughput manner.

To date, the most commonly used method for proteomic studies is two-dimensional gel electrophoresis (2DE).⁴ A 2DE experiment first separates proteins on the basis of charge and then separates them in a second dimension on the basis of molecular weight. In a comparative study, the gels are stained, and differentially displayed spots are excised, digested, and analyzed by mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Two-dimensional gel electrophoresis has a limited dynamic range, and hydrophobic proteins are often lost. In-gel digestion of the proteins is not efficient, and the whole procedure is time-consuming and tedious.^{4,5}

Recently, “shot-gun” strategies have been proposed for proteomics research.^{6–9} These strategies involve initial digestion of proteins in the mixture, followed by separation of peptides and detection by mass spectrometry. In such an experiment,

sequence information for peptides must be obtained by MS/MS analysis in order to search databases to obtain the identity of the precursor protein.^{10–12} Stable isotope labeling methods have provided to shot-gun strategies the capability for quantitation comparisons. Isotopes can be introduced by metabolic labeling,^{13–15} chemical labeling through derivatization,^{16–21} or enzymatic labeling.^{1,22–24} Isotope ratios are measured by mass spectrometry.

We have recently proposed proteolytic ^{18}O labeling as a quantitative method for comparative proteomics.¹ This strategy combines proteolysis and highly specific stable isotope labeling of proteins into one step. It involves digestion of one protein pool in H_2^{18}O to isotopically label each new C-terminus with two ^{18}O atoms and a second protein pool in H_2^{16}O . Since the ^{18}O label is stable except at pH extremes,²⁵ the peptide pools can be mixed and subjected to various separation procedures prior to mass spectrometric analysis. Sequence information from each peptide is used to identify the precursor protein, and the isotope ratio is measured to quantitate the relative amounts of precursor protein in the two pools. Since proteolytic labeling is comprehensive, the entire peptide mixture can potentially be examined. Among commonly used proteases, trypsin, endoprotease Lys-C, and endoprotease Glu-C have been reported to catalyze incorporation of two ^{18}O atoms into each new peptide C-terminus, resulting in a 4 Da mass increase.^{1,24,26–29} While attention has been paid to trypsin, Glu-C has not been well studied, particularly in the context of proteomic applications. In the present study, the use of endoprotease Glu-C is evaluated for proteolytic ^{18}O labeling, and figures of merit are determined for quantitative analysis

* To whom correspondence should be addressed. Phone: (301) 405-8614. Fax: (301) 405-8615. Email: fenselau@wam.umd.edu.

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of its products by capillary LC–MS. To establish viability for cellular applications, the effect of phosphorylation, glycosylation, and the reduction and alkylation of sulfhydryl groups on the proteolytic labeling is investigated. A sequential enzymatic ^{18}O labeling strategy is applied to study glycoproteins.

Experimental Section

Materials and Reagents. Chain B bovine insulin (oxidized), bovine serum albumin (BSA), chicken riboflavin binding protein (glycoprotein), chicken neurotensin, sequencing grade endoprotease Glu-C, and glycopeptidase F were purchased from Sigma (St. Louis, MO). The heat shock protein (HSP) fragment (CLNRQLPSSGVSEIR), which contains a phosphorylation site, was purchased from Bachem (Philadelphia, PA). Isotopically enriched H_2^{18}O , greater than 95%, was purchased from Isotech, Inc. (Miamisburg, OH). HPLC solvents and chemicals were purchased from Fisher Scientific (Fairlawn, NJ). Water (H_2^{16}O) used in all experiments was purified by a Millipore (Bedford, MA) water filtration system. All other reagents were acquired from commercial sources.

Sample Preparation. Protein reduction was carried out in 2 mM dithiothreitol (DTT) for 30–60 min in 6 M urea at room temperature. Reduced cysteine residues were alkylated by incubation with 20 mM iodoacetic acid or iodoacetamide for 30–60 min in darkness and at ambient temperature. After alkylation, samples were cleaned to remove excess reagents by 3-fold dilution with water and passage through a self-packed Poros R2 50 (Applied Biosystems, Framingham, MA) cartridge to capture the protein. A solution of 0.1% TFA was used to wash unbound components from the cartridge, and the protein was eluted with 50:50 methanol/water. Riboflavin binding protein samples were cleaned with a Bio-Rad Micro Bio-Spin 6 chromatography column (Hercules, CA), with the recovered protein in 10 mM Tris-HCl (pH 7.4).

Glu-C Catalyzed Labeling. Aliquots of proteins in solution were dried completely by SpeedVac (Thermo Savant, Holbrook, NY). Then the protein was digested by sequencing grade endoprotease Glu-C (1 μg per 10 μg of protein) in 100 mM Tris buffer (pH 8.0) and 2 M urea with a final volume of 100 μL at 37 °C. For larger proteins, additional Glu-C (0.5 $\mu\text{g}/10 \mu\text{g}$ of protein) was added after 1 day of digestion and incubated at 37 °C. Total digestion times varied from 18 to 48 h. One protein pool was digested in greater than 95% H_2^{18}O , and a second protein pool was digested in H_2^{16}O . The peptide pools were mixed and subjected to further separation and analysis.

Sequential Enzymatic Labeling of Glycoproteins. Following alkylation, 170 μg of chicken riboflavin binding protein was digested by Glu-C in H_2^{16}O or H_2^{18}O . Then the digest mixture was desalted with a self-packed Poros R2 50 cartridge. The peptides were eluted with 50:50 acetonitrile/water into 5 μL of 2 M Tris (pH 8) and then dried completely by SpeedVac. The peptides were resuspended in 35 μL of H_2^{16}O or H_2^{18}O and boiled for 3 min. Glycopeptidase F (9 units) and Triton-X 100 (1% v/v) were added, and the sample was allowed to digest overnight at 37 °C. The sample was desalted and analyzed by nanospray.

In another experiment, aliquots of chicken riboflavin binding protein were first deglycosylated in H_2^{16}O and H_2^{18}O , separately, by incubation with 9 units of glycopeptidase F in 100 mM Tris (pH 8.0) and 1% Triton-X 100 with a final volume of 35 μL for 24 h at 37 °C. Then urea and Glu-C were added to each sample. The volumes were increased to 100 μL with H_2^{16}O and H_2^{18}O , respectively, to match the normal parameters used for the

Glu-C proteolytic labeling method, and the solutions were incubated overnight at 37 °C. The H_2^{16}O and H_2^{18}O digests were mixed 1:1 and analyzed by nanospray and online LC–MS/MS.

QqTOF Mass Spectrometric Analysis. Mass spectra were obtained using an Applied Biosystems Q-Star (Foster City, CA). A Protana nanospray source (Odense, Denmark) was used for offline sample analyses with the following parameters: ion spray voltage (IS), 800 V; curtain gas (CUR), 25; declustering potential (DP), 50 V; focusing potential (FP), 220 V; declustering potential 2 (DP2), 10 V. Protana medium NanoES spray capillaries were loaded with approximately 3 μL of sample and placed in the source tip holder. Prior to analysis by nanospray, samples were desalted on Poros R2 50 packed in a gel-loader pipet tip and eluted with 50:50 methanol/water.

The electrospray source was used in conjunction with online capillary HPLC analyses with the following mass spectral parameters: IS, 5000 V; CUR, 35; GAS 1, 45; DP, 50 V; FP, 220 V; DP2, 10 V. Reversed-phase capillary HPLC was performed using Perkin-Elmer (Boston, MA) Series 200 micropumps with flow split to 3 $\mu\text{L}/\text{min}$ by an LC Packings (San Francisco, CA) Accurate splitter. Separation of peptides in 5 μL sample injections was performed on an LC Packings 3 μm , 0.3 \times 150 mm, C18 PepMap column coupled online to the mass spectrometer. Solvent A was 0.1% formic acid in 95/5 water/acetonitrile, and solvent B was 0.1% formic acid in 95/5 acetonitrile/water. Peptide elution was accomplished with a gradient of 0% B for 15 min, 0–50% B in 100 min, then B only for 15 min, and back to 0% B for 25 min. Data-dependent software (Analyst QS, Foster City, CA) was employed for the online analyses using a survey time-of-flight mass spectral (TOF-MS) scan (1 s) followed by a product ion (MS/MS) scan (3 s) of the most intense peak in the survey scan, with exclusion limits placed on previously selected m/z 's. The collision energy for MS/MS was dynamically selected by the software. The selection quadrupole window was set for low resolution. The Q-Star was externally calibrated using the doubly and triply charged peaks of chicken neurotensin.

Peptide Identification. Proteins were theoretically digested by PeptideMass,³⁰ and observed molecular mass data were used to identify the peptide by comparison with theoretical peptide masses. For isotopomers, identifications were made via interpretation of the MS/MS spectra by the manual tag function in the BioAnalyst (Applied Biosystems, Foster City, CA) software in addition to the molecular mass. Elution times of the peptides were acquired using the Extract Ions (Maximum) software function on the monoisotopic unlabeled (I_0) and labeled peaks (I_4).

Quantitation. Ratios of [$^{18}\text{O}/^{16}\text{O}$] peptides were calculated using the following equation:¹

$$\text{Ratio 1} = \frac{I_4 - \frac{M_4}{M_0}I_0 - \frac{M_2}{M_0}\left(I_2 - \frac{M_2}{M_0}I_0\right) + \frac{1}{2}\left(I_2 - \frac{M_2}{M_0}I_0\right)}{I_0} \quad (1)$$

where I_0 , I_2 , and I_4 are the observed peak areas for the monoisotopic peak for the peptides without ^{18}O label, the peak 2 Da higher, and the peak 4 Da higher (double ^{18}O labeled peak), respectively. M_0 , M_2 , and M_4 are the theoretical peak areas for the monoisotope peak of a peptide with known composition, the peak 2 Da higher and the peak 4 Da higher, respectively. Values for M_0 , M_2 , and M_4 were calculated for each peptide using IsoPro 3.0.³¹ The theoretical distribution of the

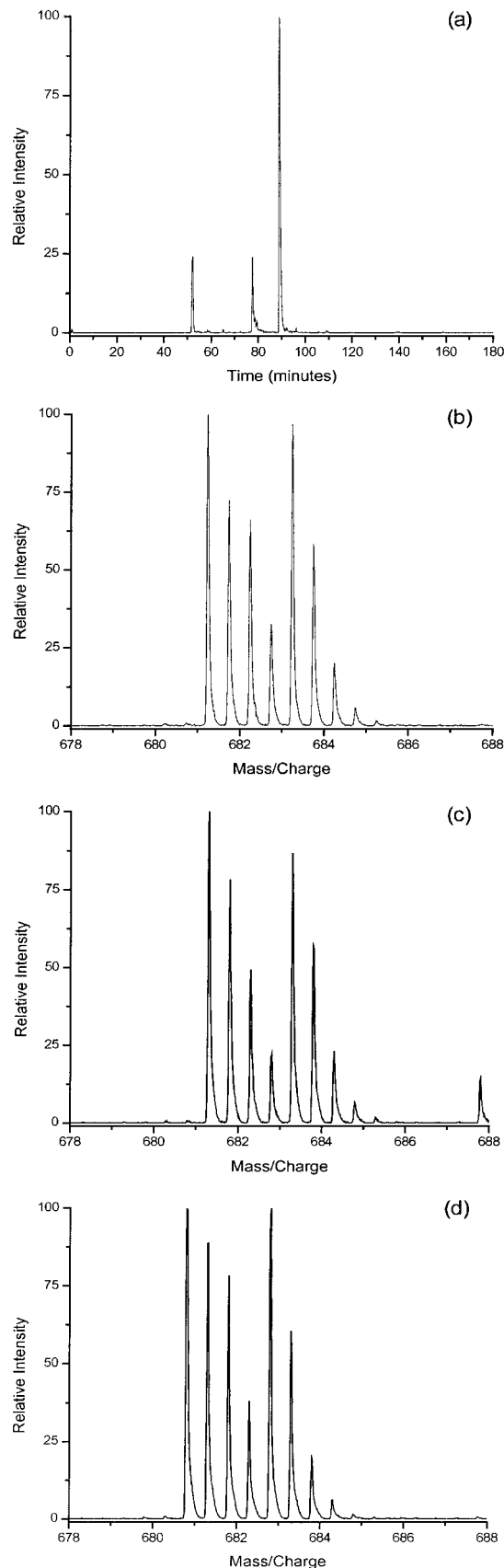


Figure 1. Analysis of digests of albumin prepared in H_2^{16}O and H_2^{18}O and combined: (a) chromatogram reconstructed by summing ions in the range m/z 680–682; (b) partial mass spectrum of the peak at 77.6 min; (c) partial mass spectrum of the peak at 52.1 min; (d) partial mass spectrum of the peak at 88.8 min.

Table 1. Ratio of [$^{18}\text{O}/^{16}\text{O}$] Consistency at Various Elution Times

BSA peptide sequence	sequence position	unlabeled (I_0) elution time (min)	labeled (I_4) elution time (min)	isotope ratio ^a	I_4/I_0 ^b
ACFAVE	589–594	46.48	46.48	0.95	0.87
KKFWGKYLVE	155–164	52.17	52.09	0.84	0.85
TYVPKAFDE	519–527	52.93	52.93	0.99	0.96
DKDVCKNYQE	335–344	58.43	58.43	0.99	0.97
DKGACLLPKIE	196–206	57.36	57.36	0.88	0.87
KQIKKQTALVE	544–554	67.70	67.70	0.90	0.88
LLYYANKYNGVFQE	177–190	75.32	75.32	0.99	1.07
YAVSVLLRLAKE	364–375	77.56	77.48	0.91	0.91
AKDAFLGSFLYE	345–356	88.78	88.78	0.86	0.81
DYLSLILNRLCVLHE	474–488	109.11	109.03	0.85	0.96
average				0.92	0.92
standard deviation				0.06	0.08

^a Calculated using eq 1. ^b Calculated using peak areas.

unlabeled and labeled peptides is assumed to be identical. Labeled to unlabeled peak area ratios (I_4/I_0) were also calculated.

Correlation Study. Digestions of 10, 50, and 100 μg of bovine insulin chain B (oxidized) were carried out in H_2^{16}O and H_2^{18}O , separately. Aliquots from a stock solution were transferred using a syringe and dried by SpeedVac prior to digestion. After overnight digestion, 10 μL of each ^{18}O labeled digest mixture was mixed with 10 μL of each unlabeled digest (9 mixtures total), desalted by Poros R2 50, and analyzed in duplicate by nanospray QqTOF.

Isotope ratios were calculated from the doubly and triply charged peaks of peptide FVNQHLCGSHLVE for all nine mixtures. UV absorbance was measured for the six digests at 216 nm. Three times, 5 μL of each sample was added to 400 μL of water, and the absorbance read at 216 nm. The readings were averaged to give an absorbance per microliter value for each sample. Ratios were calculated from the absorbance values associated with the same samples mixed for mass spectral analysis.

Results and Discussion

Glu-C Catalyzed Incorporation of Isotope Labels. Molar equivalents of BSA were digested separately in H_2^{16}O and H_2^{18}O . The peptide product mixtures were combined and analyzed by online capillary LC–MS/MS. Repetitive survey scans and a product ion scan of the most intense peak in each survey scan were recorded throughout the elution. Peptides YAVSVLLRLAKE, KKFWGKYLVE, and AKDAFLGSFLYE are predicted to have protonated monoisotopic masses of 1361.815, 1361.725, and 1360.678, respectively. An ion chromatogram of the range m/z 680–682, the mass range of the doubly charged ions, was reconstructed from the dataset to reveal three peaks (Figure 1a). Time-of-flight spectra (Figure 1b–d) confirm that about 50% of each peptide carries two atoms of ^{18}O . These and other spectra confirm that digestion by endoprotease Glu-C carried out in H_2^{18}O (>95% ^{18}O) reliably incorporates two ^{18}O atoms into each new peptide. Mechanistic considerations²⁸ and tandem mass spectrometry experiments^{1,27,32} confirm that these labels are introduced into the carboxyl terminus of each new peptide.

Chromatographic Coelution of Labeled and Unlabeled Peptides. The unlabeled and labeled variants of each peptide, differing in mass by 4 Da, were observed to coelute across the entire chromatographic separation. These observations are

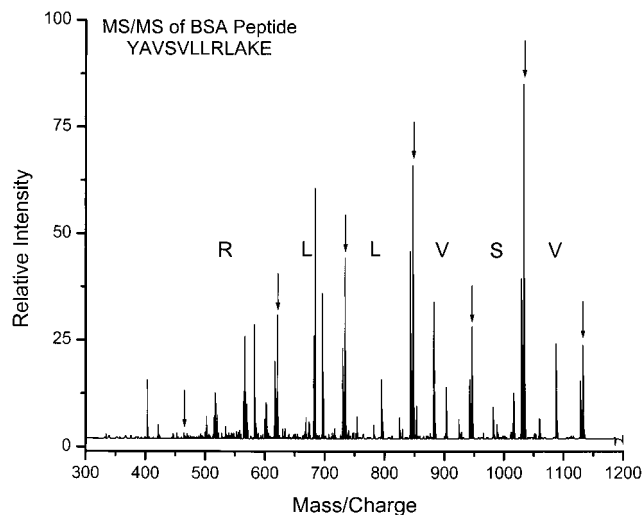


Figure 2. Tandem mass spectrum of precursor ions in the range m/z 680–682 obtained from the peak at 77.6 min of peptide YAVSVLLRLAKE in Figure 1a.

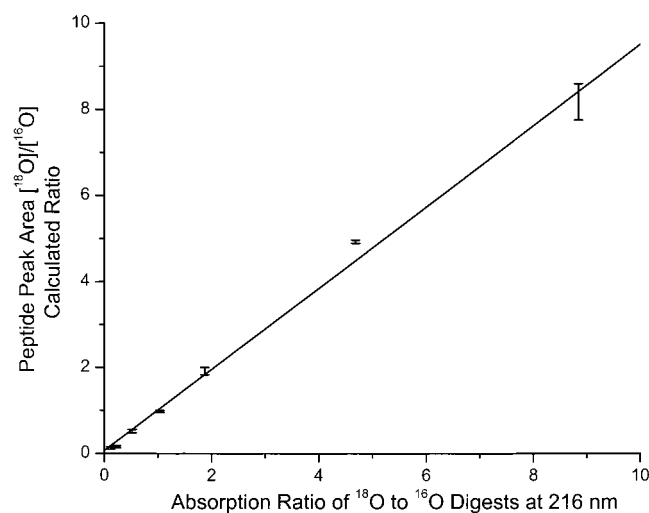


Figure 3. Comparison of the isotope ratios measured by mass spectrometry of insulin peptide FVNQHLCGSHLVE produced in $H_2^{16}O$ and $H_2^{18}O$, and mixed, with the ratios of spectroscopic adsorption determined separately on digestion mixtures. Slope: 0.94 ± 0.03 ($r^2 = 0.997 \pm 0.238$).

summarized in Table 1 for 10 peptides eluting between 46 and 110 min. Since there is no chromatographic separation of peptides labeled in this manner, relative peak intensities from a single MS scan can be easily used for quantitation of isotope ratios (see next section), without the need for the computer-aided data reconstruction reported for an alternate strategy.³³

The coelution of the peptide pairs also allowed concurrent selection of molecular ions for MS/MS analysis to generate sequence information. Fragmentation of each peptide pair produces y -ions that contain paired C-termini isotopes. This simplifies MS/MS interpretation.^{1,27,32} From the MS/MS spectra of the eluted peptides, partial sequences were obtained by recognizing isotopically paired y -ions. Figure 2 shows the MS/MS from the peak at 77.6 min, which yielded the sequence tag VSVLLR. This allowed the peptide to be identified as YAVSVLLRLAKE [364–375]. The isotope ratios were not quantitatively preserved in automated selection of precursor ions for tandem mass spectrometry.

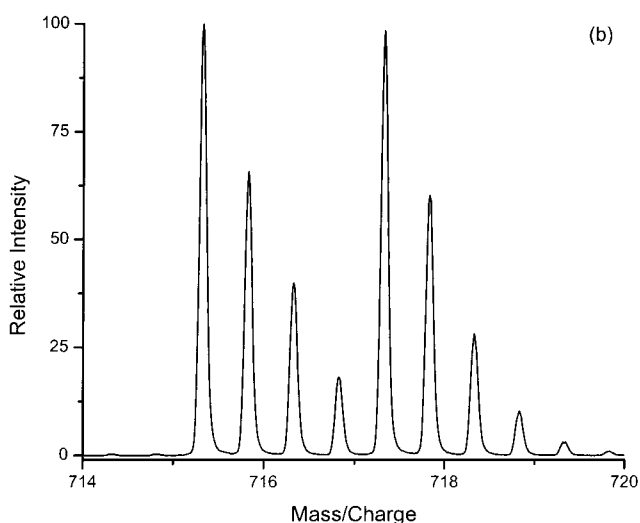
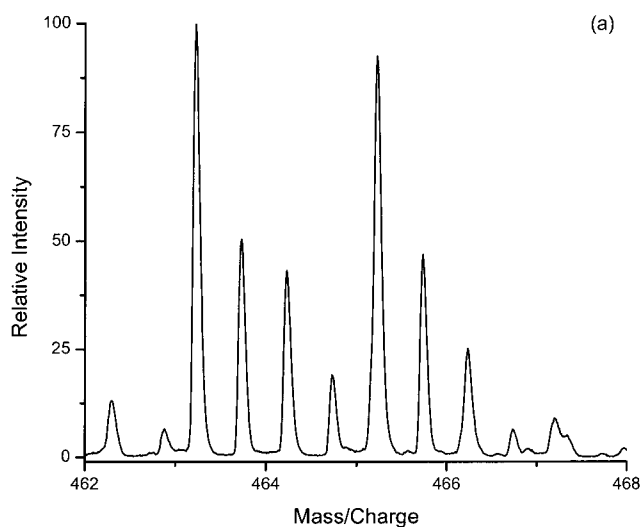


Figure 4. Partial mass spectra of labeled peptide pairs: (a) doubly charged insulin chain B peptide ALYLVCGE reduced and carboxymethylated; (b) doubly charged HSP peptide CLNRQLpSSGVSE, with the cysteine residue carboxyamidoethylated.

Online LC–MS Quantitation of Peptide Ratios. Isotope ratios for the 10 labeled and unlabeled BSA peptide pairs were calculated using eq 1 and also I_4/I_0 .¹ The average isotope ratio, calculated from eq 1, was 0.92 ± 0.06 , and the average I_4/I_0 ratio was 0.92 ± 0.08 (Table 1). It is proposed that if full sequence information is not available for an observed peptide, the I_4/I_0 ratio can be used in the relative concentration range used here. The analyzed peptides eluted from the capillary column at different times, over a 63-min interval. The small standard deviations in the ratios indicate both consistency of double ^{18}O incorporation for Glu-C peptides and reliable coelution of the isotope pairs throughout the separation.

In a comparative proteomic study, the ratios of labeled and unlabeled peptides in each pair are compared, to determine if a precursor protein has been up or down regulated. If the unlabeled peptide is present in great excess of the labeled peptide, its ^{13}C isotope pattern may interfere with the measurement of the labeled peptide peak. Wang et al. have proposed an inverse labeling strategy as a solution to this challenge.²⁴ They performed two converse labeling experiments in parallel.

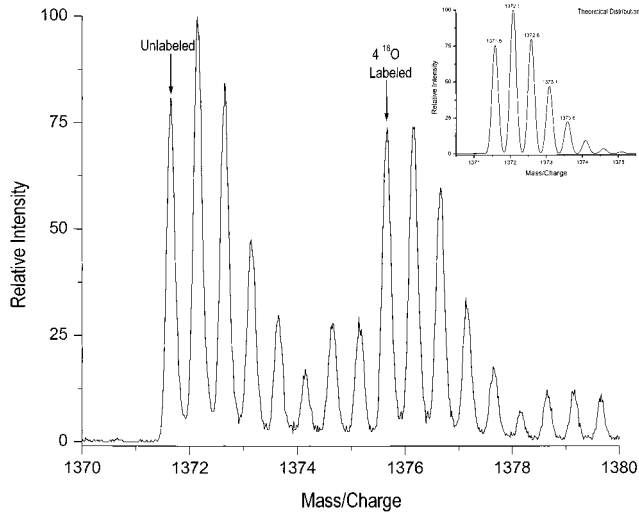


Figure 5. Partial spectrum of the labeled and unlabeled dimer of HSP peptide CLNRQLpSSGVSE. Inset shows theoretical distribution of molecular ion envelope for the unlabeled dimer.

In one experiment, the perturbed sample was proteolytically ^{18}O labeled, while the control was unlabeled; in a second

experiment, the perturbed sample was unlabeled and the control was labeled. After mixing, a characteristic inverse labeling pattern of mass shift was observed between the two parallel analyses for proteins that are differentially expressed.²⁴ An alternative solution is to analyze an aliquot of the ^{18}O labeled sample alone, for comparison to the spectrum of the mixture. In the absence of the ^{16}O peptide, interference is greatly reduced.

Correlation of MS and UV Quantitation. MS-based quantitation of the labeled and unlabeled peptides produced by incubation with endoprotease Glu-C was compared to the determination of relative amounts by UV absorbance, using oxidized insulin chain B as a standard. Using eq 1, the ratio of labeled to unlabeled sample was calculated for the doubly and triply charged isotope pairs of peptide FVNQHLCGSHLVE. Duplicate measurements for each charge state were averaged. The relative standard deviation was $\pm 7.6\%$. The UV ratios for pairs of peptide digests were plotted against the peak area ratios for FVNQHLCGSHLVE, shown in Figure 3, and a correlation of 0.94 ± 0.03 ($r^2 = 0.998$) was observed. The strong correlation between spectroscopic and mass spectral measurements and the small standard deviation supports the accuracy and precision of the mass spectrometric quantitation.

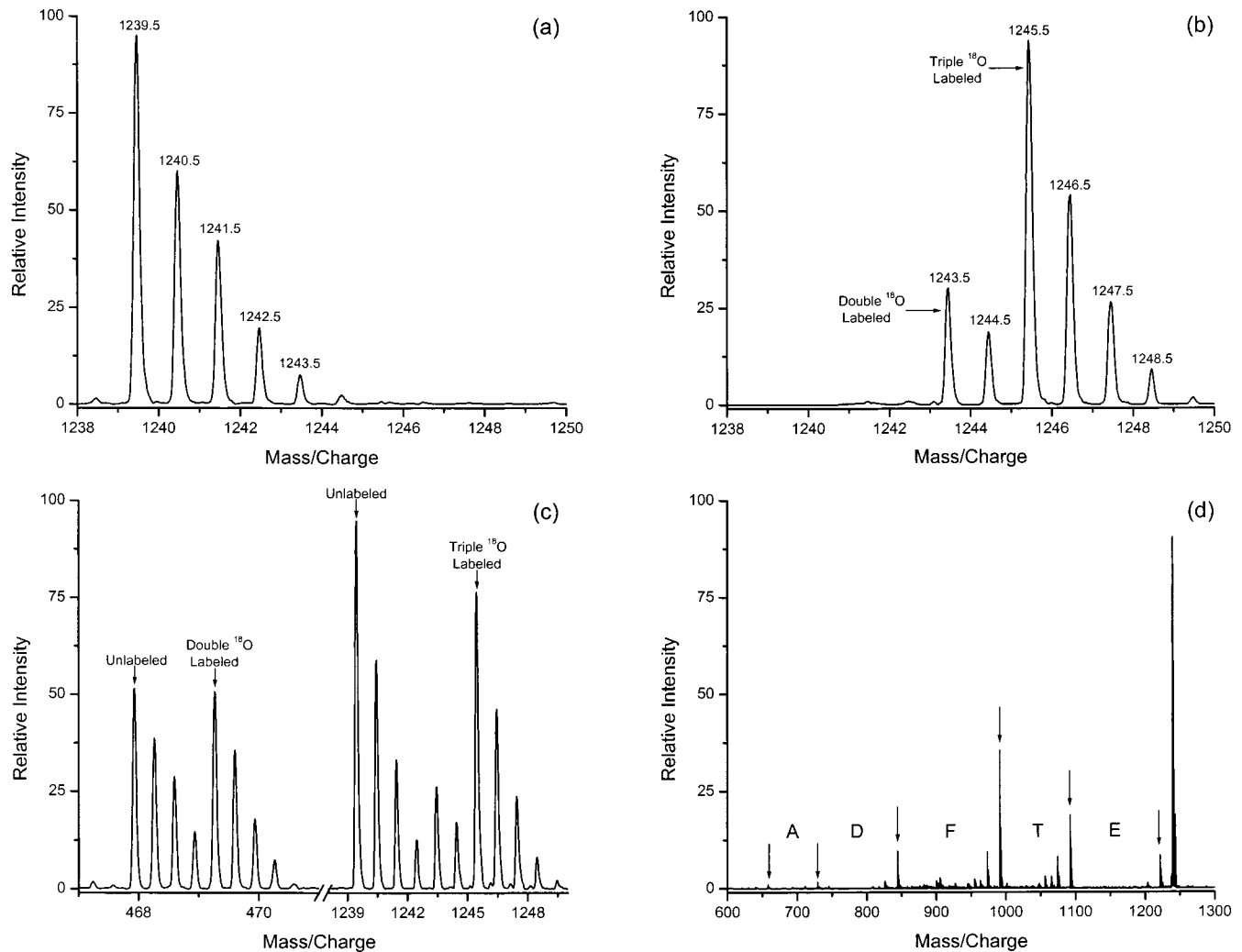


Figure 6. Partial spectra of the peptide SSCCYADLTE from riboflavin binding protein: (a) prepared by incubation with Glu-C and then glycopeptidase F in H_2^{16}O ; (b) prepared by incubation with Glu-C and then glycopeptidase F in H_2^{18}O ; (c) prepared by incubation with glycopeptidase F and then Glu-C in H_2^{16}O or H_2^{18}O and mixed; (d) activated for MS/MS analysis of the m/z 1239.5 precursor ion from the peptide in Figure 6c.

Effects on Proteolytic ^{18}O Labeling of Chemical and Post-Translational Modifications of Proteins. Proteins are often modified during biosynthesis, and they are often stabilized by chemical derivatization for analyses based on primary structures. As part of the foundation for application to more complex cellular proteomics, it is of interest to examine if such modifications of proteins affect the proteolytic labeling strategy. As a common practice, cysteine-containing proteins are reduced and alkylated before mass spectrometric (and other) analyses. Figure 4a shows a nanospray spectrum of an isotopic peptide mixture from bovine insulin chain B peptide that had been reduced, carboxymethylated and then proteolytically labeled. Figure 4b shows a spectrum of an isotopically labeled peptide pair from a heat shock protein (HSP) fragment (see below for sequence), whose cysteine residue had been alkylated with iodoacetamide. The observed isotope ratios, calculated using eq 1, are 0.99 and 0.98, respectively, both in good agreement with the mixing ratios. It appears that these routine chemical modifications do not interfere with subsequent proteolysis and double ^{18}O incorporation.

The carboxyamidomethylated HSP fragment, which contains a phosphorylated serine residue (CLNRQLpSSGVSEIR), was also examined as a model for phosphorylated proteins. Two ^{18}O labels were incorporated into the alkylated Glu-C product CLNRQLpSSGVSE, which was examined by nanospray MS following a standard 24 h incubation (Figure 4b). The observed monoisotopic molecular mass was 1429.63, measured with a resolution of 7000, which matched the theoretical mass of 1429.61 Da well. Tandem mass spectrometry experiments confirmed that these labels were located at the C-terminus. Thus, the phosphate group does not interfere with proteolytic double ^{18}O labeling of the carboxyl terminus.

It is also of interest to examine the effect of disulfide linkages between peptide products. Such disulfide-linked peptides should incorporate a total of four ^{18}O atoms, two at each C-terminus. The molecular mass of the disulfide-linked dimer of peptide CLNRQLpSSGVSE, recovered from digestion of the nonreduced HSP fragment in H_2^{18}O , is increased by 8 Da, compared to the disulfide product of the digestion carried out in H_2^{16}O (Figure 5). This mass increment can be used to recognize disulfide-linked peptides in proteolytically labeled mixtures.

Sequential Enzymatic ^{18}O Labeling of Glycoproteins. Chicken riboflavin binding protein was studied to evaluate the effect of glycosylation of proteins on the labeling method. This protein contains two N-linked sugar residues. It was digested first with endoprotease Glu-C, and excess Glu-C was denatured and then incubated with glycopeptidase F in H_2^{16}O and H_2^{18}O . Glu-C digestion produces a peptide SSCCYANFTE, which contains one of the glycosylation sites. Figure 6a shows the TOF-MS scan of the peptide obtained after sequential digestion in H_2^{16}O , and Figure 6b presents the H_2^{18}O analogue. It can be seen that the monoisotope peak is 6 Da higher, indicating that the presence of the sugar residues does not interfere with proteolytic labeling. The additional 2 Da increase is the result of incorporation of one atom of ^{18}O in the glycopeptidase reaction, which also converts asparagine (N) to aspartate (D).^{22,23} The same 6 Da increase in mass was observed when the order of enzymatic digestion was reversed (Figure 6c), which allowed for sequential addition of the enzymes in a single pot. Collisional activation yielded the sequence tag ADFTE (Figure 6d). This spectrum confirms that two ^{18}O atoms are localized at the carboxyl terminus and allows the site of the third label, i.e., the site of

N-glycosylation, to be identified. It is noteworthy that this peptide was easily recognized as having had a N-glycosylation site, because the isotope pair differ by 6 Da instead of 4 Da.

In summary, the present work supports the suitability of endoprotease Glu-C to provide globally labeled peptide products, the ability of capillary LC-MS to provide isotope ratios from such peptide mixtures, with good accuracy and precision, and the robustness of the approach in strategies that accommodate chemical and enzymatic (post-translational) modifications. Proteolytic labeling is being applied in this laboratory to comparative studies of protein levels in drug-resistant and drug-susceptible cancer cells.

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