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Proteolytic ^{18}O Labeling by Peptidyl-Lys Metalloendopeptidase for Comparative Proteomics

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The potential capabilities of a new proteolytic ^{18}O labeling method employing peptidyl-Lys metalloendopeptidase (Lys-N) have been demonstrated for use in comparative proteomics. Conditions (pH ≥ 9.5) have been found such that Lys-N incorporates only a single ^{18}O atom into the carboxyl terminus of each proteolytically generated peptide. This ^{18}O labeling method has a major advantage over current protelytic ^{18}O labeling methods that generate a mixture of isotopic isoforms resulting from the incorporation of one or two ^{18}O atoms into each peptide species by the proteases (trypsin, Lys-C, or Glu-C) used. We demonstrate that the single ^{18}O atom incorporation property of Lys-N overcomes the major problem of the current proteolytic ^{18}O labeling methods and provides accurate quantification results for isotopically labeled peptides.

Keywords: comparative proteomics • ^{18}O • Lys-N • isotope labeling • mass spectrometry • protein expression

Introduction

The completion of the genome sequencing of humans and other species and the emergence of new technologies in mass spectrometry have together fostered unprecedented opportunities for studying proteins on a large scale. It is expected that large scale quantitative measurements of protein expressions in different sets of samples (comparative proteomics) will advance our understanding of physiological processes and disease mechanisms. Comparative proteomic approaches have been applied to various biological samples to attempt to quantify proteins that are either up- or down-regulated in response to environmental changes. There are two primary strategies used in current comparative proteomics: two-dimensional gel electrophoresis (2D-PAGE) based methods and mass spectrometry methods based on in vitro stable isotope labeling strategies.[1,2]

In mass spectrometry methods based on in vitro stable isotope labeling methods, peptides from the control sample are labeled with naturally abundant (light) isotope(s), while peptides from the experimental sample are labeled with its heavier isotope(s) or vice versa. The samples are then mixed together in equal proportion and analyzed by mass spectrometry. The light- and heavy-peptide can be readily distinguished by mass spectrometry, since a peptide labeled with the light isotope and the same peptide labeled with the heavy isotope has a constant difference in molecular weight. The relative abundance of the two peptides can be determined by comparing the peak areas or intensities of the light- and heavy-peptide, which also equals the relative abundance of the parent protein in the original samples.

There are two ways to incorporate stable isotopes into peptides: (i) derivatization of peptides by a light- or heavy-isotope coded reagent (Isotope Coded Affinity Tag)[3−5] and (ii) incorporation of ^{16}O and ^{18}O atom(s) into the carboxyl termini of peptides from the proteolytic reaction involving $H_2{}^{16}O$ or $H_2{}^{18}O$ solvent molecules, respectively.[6−9] This second method is referred to as the proteolytic ^{18}O labeling method.

Although 2D-PAGE based methods have been the primary choice in comparative proteomics, 2D-gels are cumbersome to run, have a poor dynamic range, and are biased toward abundant and soluble proteins.[10] The mass spectrometry based stable isotope labeling strategy has the potential of overcoming most of the weaknesses of the 2D-PAGE based methods, as long as stable isotope labeling can be achieved that occurs with high and equal efficiency for both samples. Proteolytic ^{18}O labeling is the simplest of all of the stable isotope labeling methods, and therefore is expected to have the least methodological error (technical variations). Thus, the proteolytic ^{18}O labeling method has the potential of being a central method in comparative proteomics.

A major drawback of the protelytic ^{18}O labeling method, however, has been the generation of a mixture of isotopic isoforms resulting from the incorporation of either one or two ^{18}O atoms ($^{18}O_1/^{18}O_2$) into each peptide species by proteases (trypsin [EC 3.4.21.4], Lys-C [EC 3.4.21.50] or Glu-C [EC 3.4.21.19]).[11] Unfortunately, the ratios of the first and the second ^{18}O atom incorporation vary for a particular peptide sequence; hence the ratios of $^{18}O_1$- and $^{18}O_2$-peptides cannot be predicted. This makes quantification of the peptides complicated[12] and is likely to increase the error in the calculations of ^{16}O- and ^{18}O-labeled peptides ratios. The problem can be solved if only a single ^{18}O atom is incorporated into each peptide upon the

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proteolytic reaction. Nearly complete single ^{18}O atom incorporation has been achieved by trypsin in 50% ^{18}O water,[13] however this has not yet been achieved in highly enriched ^{18}O water (>95%), which is the preferred solvent in comparative proteomics.

Peptidyl-Lys metalloendopeptidase from *Grifola frondosa* (Lys-N, EC 3.4.24.20) cleaves peptidyl-lysine bonds (−X-Lys−) in proteins and peptides.[14] It is referred to as Lys-N in this article, indicating the substrate specificity of the protease. This protease contains one atom of zinc per molecule. It exhibits more than 50% of maximal activity within the pH range of 6−10.5, with maximum activity at pH 9.5.[15] The crystal structure of this metalloendopeptidase has been reported.[16]

Optimized conditions for Lys-N are reported in the present paper for controlling the reaction such that only a single ^{18}O atom is incorporated into each peptide. This technique does not generate the mixture of isotopic isoforms of ^{18}O-labeled peptides which plague other ^{18}O labeling methods, allowing for accurate quantification of isotopically labeled peptides.

Materials and Methods

Materials. Peptidyl-Lys metalloendopeptidase from *Grifola frondosa* (Lys-N) was obtained from Seikagaku Corp. (Tokyo, Japan). Oxygen-18 enriched water (>95%) was obtained from Spectra Stabe Isotopes (Columbia, MD). Bovine serum albumin (BSA), glutamate dehydrogenase (GDH) from bovine liver, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle, apomyoglobin from horse, aminoacylase-I (ACY-1) from porcine kidney, and creatine phosphokinase (CPK) from rabbit muscle were from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade or the highest quality that was commercially available.

Reduction and *S*-Carbamidomethylation. A protein mixture of BSA, GDH, GAPDH, ACY-I, CPK, and apomyoglobin (2 nmoles of each of the protein) in 200 μL of 2 M pH 8.0 buffer consisting of Tris-HCl, 5 M guanidine-HCl, and 2 mM ethylenediaminetetraacetic acid (EDTA) was reduced by adjusting the solution to 1mM dithiothreitol (DTT) and reacting for 60 min at 50 °C. An *S*-alkylation treatment was then done by adjusting the solution to 2.5 mM iodoacetamide and reacting for 30 min at 25 °C. The reaction reagents were then removed from the reduced/*S*-alkylated protein solution using a PD-10 gel filtration column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated with 0.1% formic acid. The protein fractions from the PD-10 column were combined and dried in a speed-vac and redissolved in 100 mM pH 10.0 buffer consisting of glycine-NaOH and 1 M urea. The protein concentration was determined by the modified Bradford method.[17]

Protein Digestion by Lys-N. Experiments studying the effect of pH on the digestion of proteins by Lys-N were carried out in the following buffer systems: 100 mM sodium phosphate at pH 6.0 and 8.0 (the pHs were adjusted with HCl and NaOH, respectively), and 100 mM glycine-NaOH at pH 9.0, 9.5, and 10.0. The digestion buffers were prepared measuring into eppendorf tubes the required aliquot of a $H_2{}^{16}O$ buffer/protein stock solution, drying them on a speed-vac concentrator and then reconstituting with either $H_2{}^{16}O$ or $H_2{}^{18}O$. The digestion of the treated protein mixture was carried out at 25 °C for 18 h at a Lys-N to protein ratio of 1:85 (w/w), unless otherwise stated. After the incubation, the various digests were diluted with 0.1% formic acid in $H_2{}^{16}O$ to the appropriate concentration dictated by the particular mass spectrometry experiment.

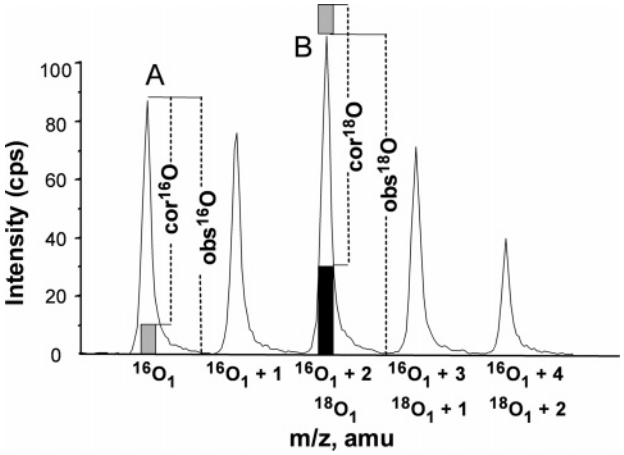

Figure 1. Calculation of cor^{16}O/cor^{18}O peptide ratio from a mass spectrum of a mixture of ^{16}O- and ^{18}O-labeled peptides. Peak A and B represent the monoisotopic peak of ^{16}O and ^{18}O labeled peptide, respectively. Gray bars represent ^{16}O-labeled peptide intensity arising from the 5% $H_2{}^{16}O$ present in the $H_2{}^{18}O$ water (0.05 · cor^{18}O). Black bar represents the M+2 isotopic peak intensity of the^{16}O-labeled peptide (obs^{16}O·X).

Liquid Chromatography−Mass Spectrometry (LC/MS) and Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) Analysis. All LC/MS and LC/MS/MS analyses were done using an UltiMate nano HPLC system (Dionex, San Francisco, CA) consisting of an isocratic pump, an autosampler, a gradient pump module and a column switching module, interfaced to a QStar quadrupole/time-of-flight mass spectrometer (Applied Biosystem-MDS Sciex, Foster City, CA) via a nano-electrospray ion source (Applied Biosystem-MDS Sciex, Foster City, CA) and a metal sprayer (GL Science, Tokyo, Japan). Protein digests (5 μL, ~1 pmol of total protein) were injected into a reverse-phase C18 trapping column (300 μm i.d. × 1 mm, Dionex, Sunnyvale, CA) equilibrated with 0.1% formic acid/2% acetonitrile (v/v) and washed for 5 min with the equilibration solvent at a flow rate of 10 μL/min, using the isocratic pump and autosampler. After the washing, the trapping column was switched in-line with the reverse-phase analytical column and the gradient pump module employed. The trapped peptides were chromatographed on a column (0.075 × 50 mm, New Objective Inc., Woburn, MA) packed with Jupiter C18 media (10 μm, 300 Å, Phenomenex, Torrance, CA) using a linear gradient of acetonitrile from 2% to 82% in water in the presence of 0.1% formic acid over a period of 80 min, at a flow rate of 200 nL/min.

The column effluent was passed directly into the nano-electrospray ion source. The total ion current was obtained in the mass range of m/z 300−2000 at 2100 and 65 V of electrospray voltage and orifice voltage, respectively, in the positive ion mode. AnalystQS software (version 1.1.0.6410, Applied Biosystem-MDS Sciex, CA) was used for instrument control, data acquisition, and data processing. In the LC/MS/MS analyses, the mass spectrometer was operated in the data-dependent MS to MS/MS switching mode, with the three most intense ions in each MS scan subjected to MS/MS analysis. The identities of the peptides were determined by submitting product ion spectra of the peptides to the Swiss Protein database using the Mascot database search software (Matrix Science, London, UK).

Calculation of $^{16}O/^{18}O$ Peptide Ratios. The isotope labeling method differentiates the sample from which the peptide originated. Theoretically, peptides with the ^{16}O label in the

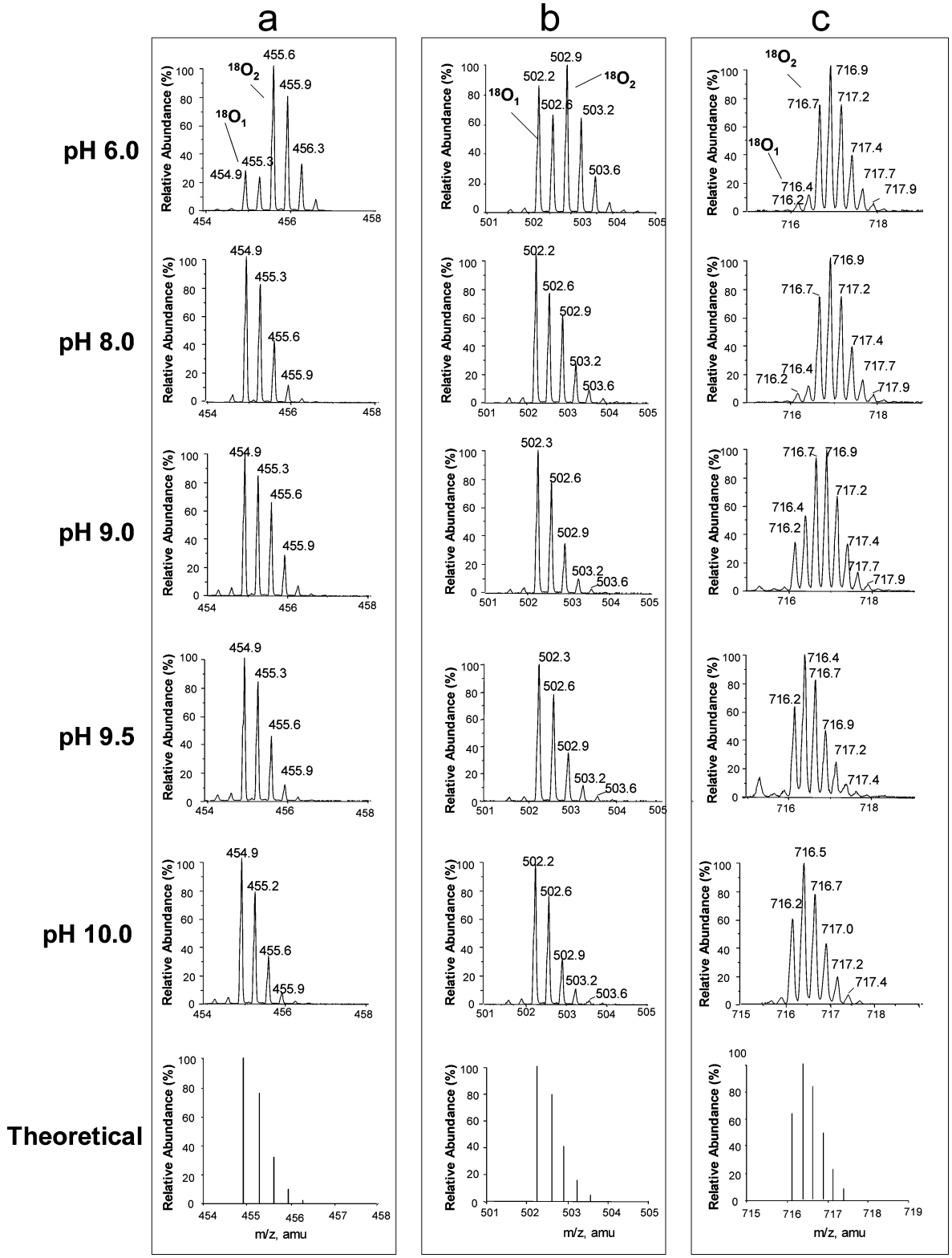

Figure 2. Mass spectra of apomyoglobin Lys-N peptides digested in $H_2{}^{18}O$ at various pHs. Apomyoglobin was digested by Lys-N in $H_2{}^{18}O$ at different pHs (6.0, 8.0, 9.0, 9.5, and 10.0) at 25 °C for 18 h and the resulting digests were analyzed by LC/MS. (a) $(M + 3H)^{3+}$ ions of peptide KALELFRNDIAA, (b) $(M + 3H)^{3+}$ ions of peptide KHPGDFGADAQGAMT, and (c) $(M + 4H)^{4+}$ ions of peptide KVEADIAGHGQEVLIRLFTGHPETLE, at different pHs. The bottom spectra are the theoretical abundances of the isotopes for each corresponding peptide containing one ^{18}O atom.

carboxyl termini are identified as coming from one sample (for example, the control) and peptides with the ^{18}O label in the carboxyl termini are identified as coming from the other sample (the experimental sample). In practice: (1) the MS peak corresponding to the ^{16}O labeled peptide must be corrected downward because 5% of the $H_2{}^{18}O$ digest incorporates ^{16}O (since the $H_2{}^{18}O$ solvent is 95% $H_2{}^{18}O$ and 5% $H_2{}^{16}O$); and (2) the MS peak corresponding to the ^{18}O labeled peptide must

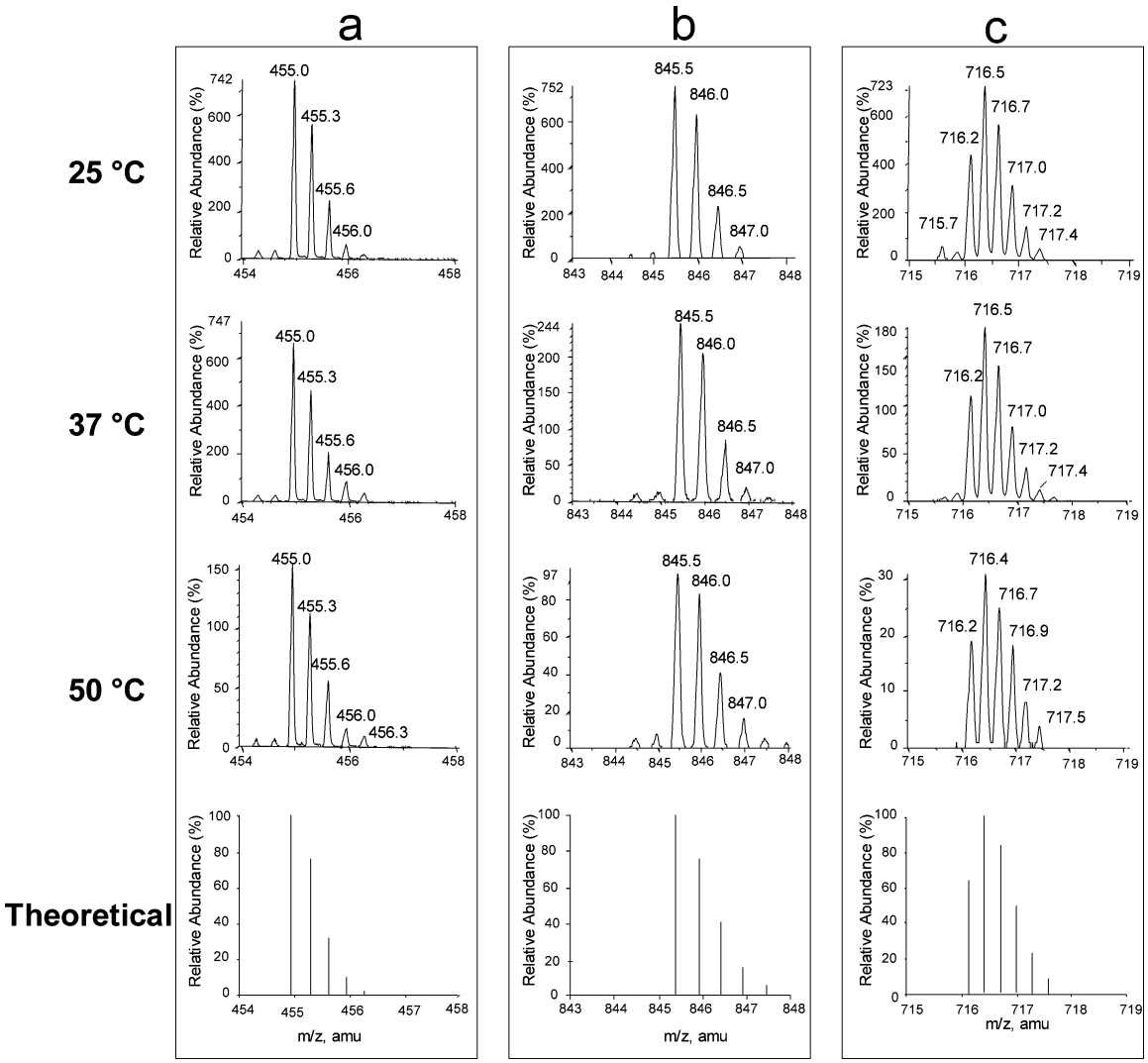

Figure 3. Mass spectra of apomyoglobin Lys-N peptides digested in $H_2{}^{18}O$ at various temperatures. Apomyoglobin was digested by Lys-N in $H_2{}^{18}O$ at different temperatures (25, 37 and 50 °C) for 18 h at pH 10.0 and the resulting digests were analyzed by LC/MS. (a) $(M + 3H)^{3+}$ ions of peptide KALELFRNDIAA, (b) $(M + 2H)^{2+}$ ions of peptide GLSDGEWQQVLNVWG, and (c) $(M + 4H)^{4+}$ ions of peptide KVEADIAGHGQEVLIRLFTGHPETLE, at different temperatures. The bottom spectra are the theoretical abundances of the isotopes for each corresponding peptide containing one ^{18}O atom.

be corrected upward 5% for the ^{16}O incorporation in the $H_2{}^{18}O$ digestion (again, the $H_2{}^{18}O$ solvent is 95% $H_2{}^{18}O$ and 5% $H_2{}^{16}O$) and corrected downward for the naturally occurring M+2 isotope of the peptide originating from the control sample. See Figure 1. The corrected (cor) ^{16}O and ^{18}O monoisotopic peak intensities for each peptide is calculated from the observed (obs) ^{16}O and ^{18}O monoisotopic peak intensities as given by eqs 1 and 2, where *X* is the theoretical fractional intensity of the M+2 isotopic peak of the ^{16}O-labeled peptide compared to the monoisotopic peak of the ^{16}O-labeled peptide. Note that $H_2{}^{16}O$ solvent was assumed to be 100% $H_2{}^{16}O$, because the contents of $H_2{}^{17}O$ and $H_2{}^{18}O$ in $H_2{}^{16}O$ solvent are insignificant.

$$\mathrm{cor}^{16}\mathrm{O} = \mathrm{obs}^{16}\mathrm{O} - 0.05 \bullet \mathrm{cor}^{18}\mathrm{O} \quad (1)$$

$$\mathrm{cor}^{18}\mathrm{O} = \mathrm{obs}^{18}\mathrm{O} - \mathrm{obs}^{16}\mathrm{O} \bullet \mathrm{X} + 0.05 \bullet \mathrm{cor}^{18}\mathrm{O} \quad (2)$$

Equations 1 and 2 can be rearranged, with a substitution of eq 2 into eq 1, to yield eqs 3 and 4

$$\mathrm{cor}^{16}\mathrm{O} = \mathrm{obs}^{16}\mathrm{O} - 0.05 \bullet \frac{\mathrm{obs}^{18}\mathrm{O} - \mathrm{obs}^{16}\mathrm{O} \bullet \mathrm{X}}{0.95} \quad (3)$$

$$\mathrm{cor}^{18}\mathrm{O} = \frac{\mathrm{obs}^{18}\mathrm{O} - \mathrm{obs}^{16}\mathrm{O} \bullet \mathrm{X}}{0.95} \quad (4)$$

Finally, the ratio of a particular protein (Y) concentrations of the two samples is equal to the ratio of the corrected monoisotopic peak intensities for a particular peptide in protein Y, as given in eq 5.

$$\text{protein Y in control/protein Y in sample} = \mathrm{cor}^{16}\mathrm{O} \,/\, \mathrm{cor}^{18}\mathrm{O} \quad (5)$$

Results and Discussion

Second ^{18}O Atom Incorporation by Lys-N is pH Dependent. Our preliminary experiments on the number of ^{18}O atom incorporated by Lys-N in 1% *N*-ethylmorpholine prepared in $H_2{}^{18}O$ resulted in a mixture of one and two ^{18}O atoms ($^{18}O_1$ and $^{18}O_2$) incorporated into the carboxyl terminus of each peptide, suggesting that there are two distinct ^{18}O incorporation mechanisms. This prompted us to carry out the digestion at different pHs. Apomyoglobin was digested in various pHs (6.0, 8.0, 9.0, 9.5, and 10.0) in $H_2{}^{18}O$ and the resulting digests were

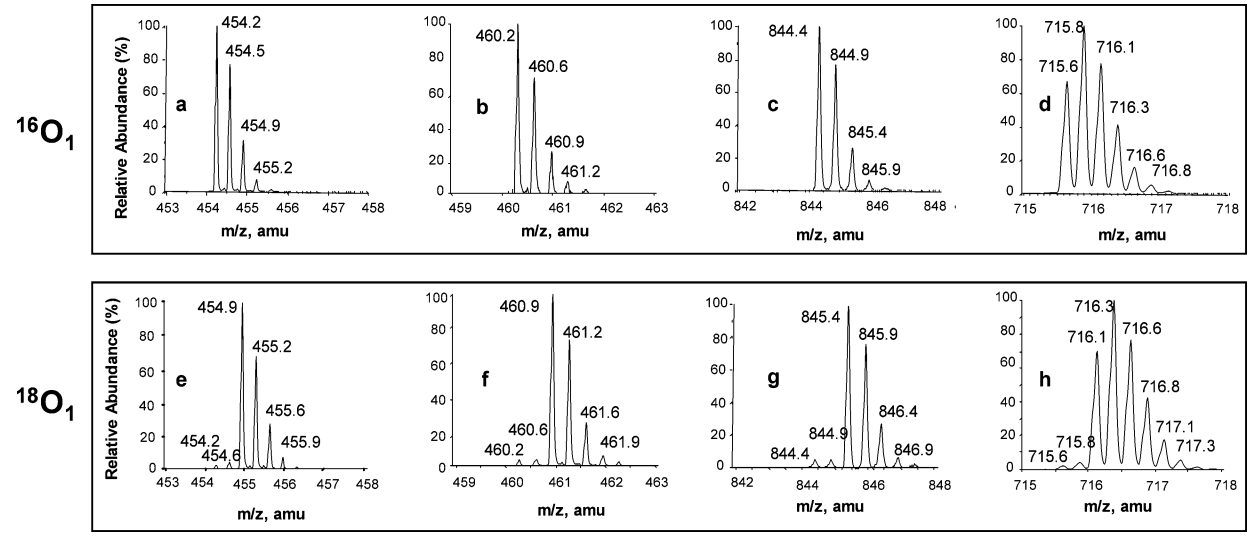

Figure 4. Comparison of mass spectra of apomyoglobin Lys-N peptides digested in $H_2{}^{16}O$ and $H_2{}^{18}O$. Apomyoglobin was digested by Lys-N in $H_2{}^{16}O$ or $H_2{}^{18}O$ at pH 10 in the presence of 1 M urea, and the resulting digests were analyzed by LC/MS. (a and e) $(M + 3H)^{3+}$ ions of peptide KALELFRNDIAA, (b and f) $(M + 3H)^{3+}$ ions of peptide KHGTVVLTALGGIL, (c and g) $(M + 2H)^{2+}$ ions of peptide GLSDGEWQQVLNVWG, and (d and h) $(M + 4H)^{4+}$ ions of peptide KVEADIAGHGQEVLIRLFTGHPETLE obtained by hydrolysis in $H_2{}^{16}O$ (a to d) or in $H_2{}^{18}O$ (e to h).

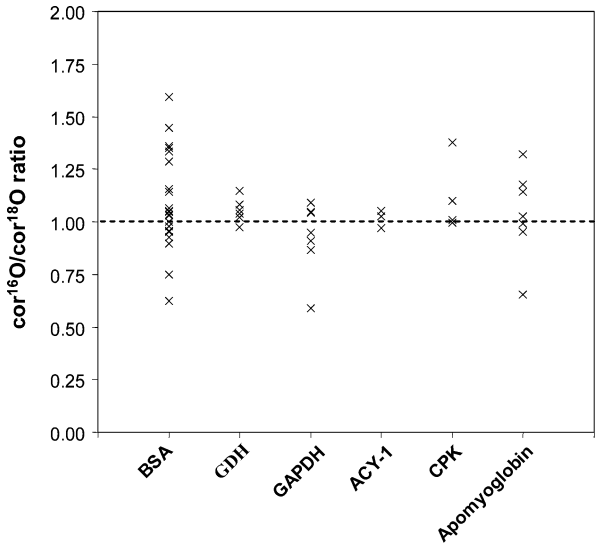

Figure 5. Distribution of the cor^{16}O/ cor^{18}O ratios of 50 Lys-N peptides. A protein mixture containing reduced and *S*-carbamidomethylated BSA, GDH, GAPDH, ACY-1, CPK, and apomyoglobin at equal molar concentration was digested in $H_2{}^{16}O$ or $H_2{}^{18}O$ and mixed in 1:1 ratio, and then 50 ng analyzed by LC/MS. The plot gives the cor^{16}O/cor^{18}O calculated from the observed peak intensities of the 50 different peptides using eq 5. The straight dashed line represents the theoretical $^{16}O/^{18}O$ ratio.

analyzed by LC/MS. Figure 2 shows the mass spectra of three representative apomyoglobin peptides hydrolyzed at different pHs. Interestingly, the results clearly showed that the $^{18}O_1$- and $^{18}O_2$-peptide ratios changed depending upon the pH. At pH 6.0, peptide peaks with two ^{18}O atoms ($^{18}O_2$) were abundant in all the three peptides. Increasing the pH led to a steady decrease in the incorporation of the second ^{18}O atom. Incorporation of the second ^{18}O atom was not observed at pH 9.5 and 10.0, as evidenced by the exact match of the relative intensities of the isotopes of the observed peptide mass spectrum compared to their theoretical abundances for one ^{18}O atom incorporated into the peptide structures (Figure 2, three bottom panels). This result suggests that the second ^{18}O atom incorporation is pH dependent and only one ^{18}O atom is incorporated at pH $\geq$ 9.5. The exact match of the relative intensities of the isotopes of the experimentally measured peptides to their theoretical plots also indicates that the nonenzymatic incorporation of ^{18}O atom during the digestion is negligible.

In a separate experiment, we also confirmed that there is no detectable nonenzymatic incorporation of ^{18}O atom into angiotensin II (DRVYIHPF) incubated in 100 mM glycine-NaOH buffer (pH 10.0) or 0.1% formic acid at 25 °C for 24 h (data not shown), confirming that significant oxygen back-exchange reaction does not take place during the incubation period and the LC/MS analysis.

Single ^{18}O Atom Incorporation Property of Lys-N at pH 10.0 is Not Affected by Temperature and Urea. It is important to know whether the single ^{18}O atom incorporation by Lys-N is affected by temperature and urea, as these considerations are important in the practical use of the protease. To examine the effect of temperature on ^{18}O atom incorporation, apomyoglobin was digested by Lys-N in 100 mM glycine-NaOH buffer (pH 10.0) prepared in $H_2{}^{18}O$ at different temperatures (25, 37, and 50 °C) and the resulting digests were analyzed by LC/MS. Figure 3 shows mass spectra of three representative apomyoglobin peptides hydrolyzed at different temperatures. The results showed that only one ^{18}O atom was incorporated into each peptide at all temperatures tested as evidenced by the exact match of the relative intensities of the isotopes of the observed peptide to their theoretical plots (Figure 3, bottom three panels). The activity of the enzyme was highest at 25 °C under the conditions employed, as judged by the observed ion intensities seen in Figure 3.

The effect of urea on ^{18}O atom incorporation by Lys-N was also examined. Apomyoglobin was digested in 100 mM pH 10.0 buffer prepared in $H_2{}^{18}O$ consisting of glycine-NaOH and various concentrations of urea (0.5, 2, 3, and 4 M) and the resulting digests were analyzed by LC/MS. Lys-N incorporated only one ^{18}O atom into the apomyoglobin peptides at all the urea concentrations tested (data not shown). The optimum enzymatic activity was observed at 1 M urea concentration,

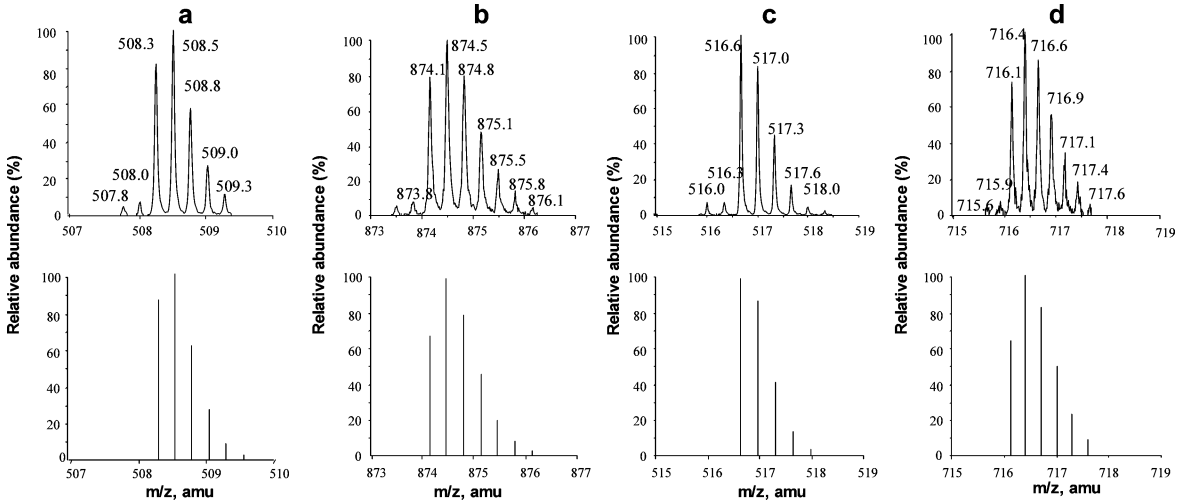

Figure 6. Mass spectra of four representative ^{18}O-labeled peptides whose cor^{16}O/ cor^{18}O ratios are more than ± 0.25 in Figure 5. A protein mixture containing reduced and *S*-carbamidomethylated BSA, GDH, GAPDH, ACY-1, CPK, and apomyoglobin at equal molar concentration was digested in $H_2{}^{18}O$ and 50 ng analyzed by LC/MS. (a) $(M + 4H)^{4+}$ ions of peptide KYLYEIARRHPYFYAPELLYYAN from BSA, (b) $(M + 3H)^{3+}$ ions of peptide KVIHDHFGIVEGLMTTVHAITATQ from GAPDH, (c) $(M + 3H)^{3+}$ ions of peptide KFEE ILTRLRLQ from CPK, and (d) $(M + 4H)^{4+}$ ions of peptide KVEADIAGHGQEVLIRLFTGHPETLE from apomyoglobin. The bottom spectra are the theoretical abundances of the isotopes for each corresponding peptide containing one ^{18}O atom.

determined by which urea concentration gave the maximum signal intensities for the peptides.

Figure 4 shows mass spectra of four representative apomyoglobin peptides hydrolyzed in $H_2{}^{16}O$ (Figure 4a−d) and in $H_2{}^{18}O$ (Figure 4e−h) in 100 mM pH 10.0 buffer consisting of glycine-NaOH and 1 M urea at 25 °C. The proportional abundances of the isotopes of the ^{16}O- and ^{18}O-labeled peptides (Figure 4a,e; Figure 4b,f, Figure 4c,g and Figure 4d,h) were identical, indicating that only one ^{18}O atom was incorporated into each peptide in the presence of urea. Taken together these results clearly show that the single ^{18}O atom incorporation property of Lys-N at pH 10.0 is not affected by either temperature or urea. This is significant because urea is frequently used to dissolve and denature proteins in proteomic applications.

The optimized digestion conditions that limit Lys-N to incorporating only one ^{18}O atom into peptides are 100 mM pH 10.0 buffer consisting of glycine-NaOH and 1 M urea at 25 °C for 18 h. These conditions were employed in all further experiments described in this article.

Single ^{18}O Atom Incorporation Property of Lys-N is General for Any Peptide Sequence. To confirm that Lys-N incorporates only one ^{18}O atom into any peptide generated, a protein mixture was made containing six reduced and *S*-carbamidomethylated proteins, at equal molar concentrations. Reduced/*S*-alkylated BSA, GDH, GAPDH, ACY-1, CPK, and apomyoglobin, were digested in $H_2{}^{16}O$ and $H_2{}^{18}O$. Both digests were then mixed in a 1:1 ratio. Fifty nanograms of this mixture was analyzed by LC/MS. A total of 50 Lys-N peptides from the six proteins were used to calculate the ratios of ^{16}O- and ^{18}O-labeled peptides (^{16}O/^{18}O). The identities (amino acid sequences) of the peptides were determined by submitting product ion spectra of the peptides to the Swiss Protein database using the Mascot database search software in a separate LC/MS/MS experiment.

Figure 5 shows the distribution of the cor^{16}O/cor^{18}O ratios (equation 5) of the 50 peptides. The cor ^{16}O/cor ^{18}O ratio agreed closely with the theoretical 1:1 ratio for most of the peptides. However, 12 peptides had cor ^{16}O/cor^{18}O ratios more than ±0.25 from the theoretical ratio. These peptides were from BSA (8 peptides), GAPDH (1 peptide), CPK (1 peptide) and apomyoglobin (2 peptide).

The protein mixture digested in $H_2{}^{18}O$ was further analyzed by LC/MS, to assess whether the discrepant results for the 12 peptides were due to an incorporation of a second ^{18}O atom. Figure 6 shows mass spectra of four of the discrepant peptides, each from a different protein. As can be seen in Figure 6, the relative intensities of the isotopes of the observed peptide peaks matched their theoretical plots (bottom row). The same results were obtained for the other 8 discrepant peptides (data not shown), supporting the conclusion that there was no incorporation of a second ^{18}O atom into these discrepant peptides. Thus, it is reasonable to assume that the single ^{18}O atom incorporation property of Lys-N under the conditions identified in this paper is general for any peptides. The discrepant results for the 12 peptides could arise from incomplete digestions at the sites of hydrolysis in either or both of the $H_2{}^{16}O$ and $H_2{}^{18}O$ solvent. Interestingly, we found that 9 peptides out of the 12 peptides were products of either a Glu-Lys or Pro-Lys bond cleavage, suggesting that the reaction rate of Lys-N in Glu-Lys and Pro-Lys bond cleavage is slower than other X-Lys bonds. Further work will need to be done to definitely identify the cause of these discrepancies.

The average cor^{16}O/cor^{18}O ratios for BSA, GDH, GAPDH, ACY-1, CPK, and apomyoglobin peptides were 1.08 ± 0.22 (n = 23), 1.05 ± 0.06 (n = 6), 0.92 ± 0.17 (n = 7), 1.01 ± 0.04 (n = 3), 1.12 ± 0.18 (n = 4) and 1.04 ± 0.21 (n = 7), respectively. The average and the standard deviation values were calculated using different peptides within a same protein.

Dynamic Range of Lys-N Based Proteolytic ^{18}O Labeling Method. To evaluate the feasibility of the Lys-N based proteolytic ^{18}O labeling method for comparative proteomics, apomyoglobin (3.4 μg) was digested separately in $H_2{}^{16}O$ and $H_2{}^{18}O$ under the optimized conditions, mixed in different ratios and analyzed by LC/MS. Figure 7 shows mass spectra of three representative apomyoglobin peptides (KALELFRNDIAA: Figure 7a, KHPGDFGADAQGAMT: Figure 7b and KHGTVVLTALGGILK: Figure 7c) mixed in different ^{16}O/^{18}O peptide ratios (0.11, 0.33, 1, 3, and 9). As can be seen in Figure 7, intensities

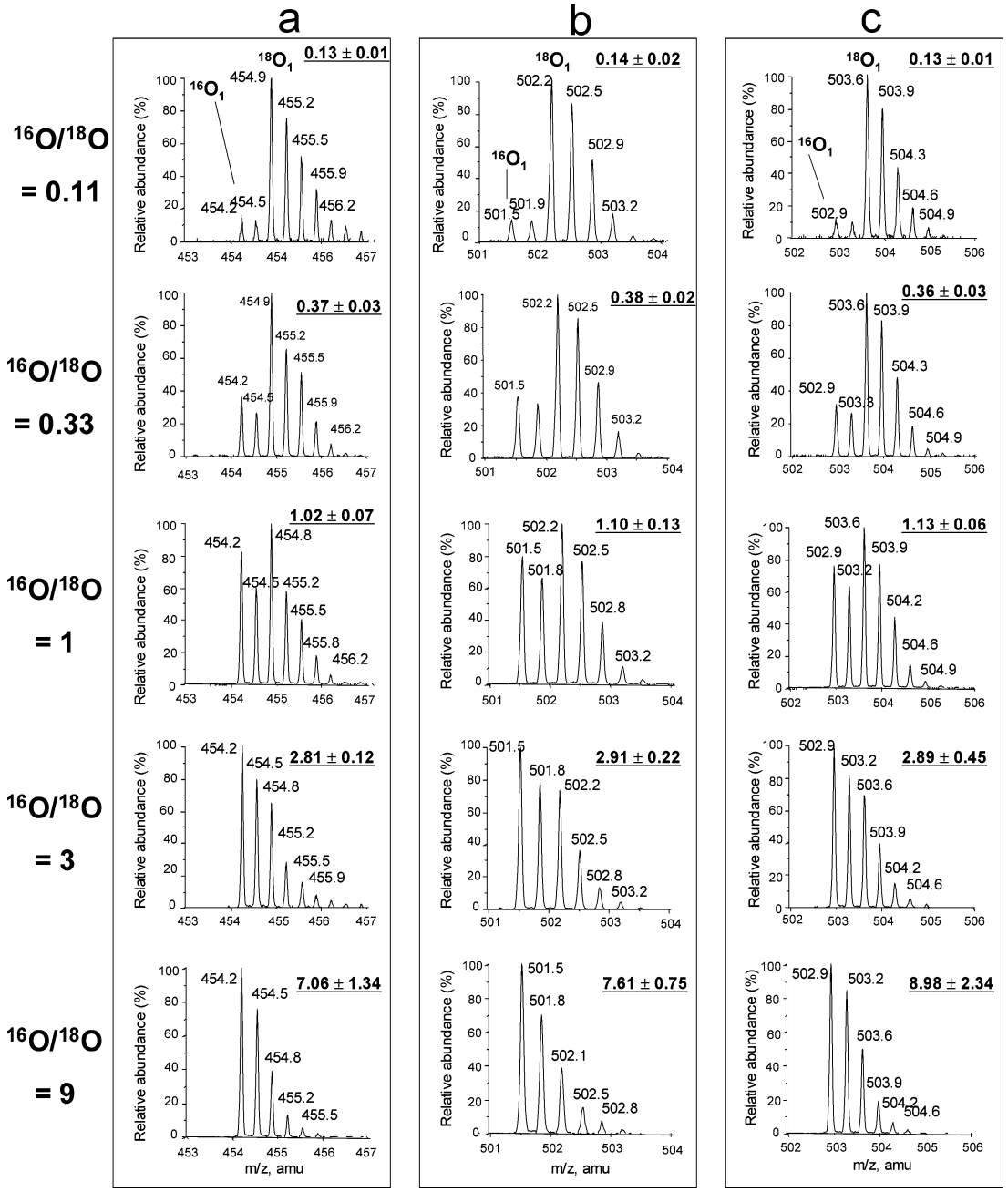

Figure 7. Mass spectra of ^{16}O- and ^{18}O-peptides mixed in different ratios. Apomyoglobin was digested by Lys-N in $H_2{}^{16}O$ or $H_2{}^{18}O$ at 25 °C for 18 h at pH 10.0 in the presence of 1M urea, mixed in ratios 0.11, 0.33, 1, 3 and 9, and then analyzed by LC-MS. (a) $(M + 3H)^{3+}$ ions of peptide KALELFRNDIAA, (b) $(M + 3H)^{3+}$ ions of peptide KHPGDFGADAQGAMT, and (c) $(M + 3H)^{3+}$ ions of peptide KHGTVVLTALGGILK, at different ^{16}O- and ^{18}O-peptide ratios. The average cor^{16}O/cor^{18}O ratios were calculated using the results from five independent LC/MS analyses and presented at the top right on each spectrum.

of the ^{16}O- and ^{18}O-peptide peaks changed depending upon the mixed ratio. The LC/MS analysis was repeated 5-times and the average cor^{16}O/cor^{18}O peptide ratios are presented at the top right on each spectrum, along with their SD values. The average cor^{16}O/cor^{18}O peptide ratios are also plotted against their theoretical ratios in Figure 8 to demonstrate the linearity of the quantification of $^{16}O/^{18}O$ peptide ratios. The correlation coefficients (r^2) of the linear regression lines for the three peptides were 0.9960 for KALELFRNDIAA, 0.9977 for KHPGDFGADAQGAMT, 0.9995 for KHGTVVLTALGGILK, respectively, indicating good linearity with respect to the $^{16}O/^{18}O$ peptide ratios over the range of 0.11 to 9. As can be seen in the Figure 8, the RSD values increased significantly as the $^{16}O/^{18}O$ peptide ratios increased. The RSD values at the $^{16}O/^{18}O$ peptide ratios of 1 or less were 5−12%, however the RSD values at the $^{16}O/^{18}O$ peptide ratio of 3 and 9 were 4−16% and 10−27%, respectively, suggesting that the accuracy of the method decreases as M+2 isotopic peak of ^{16}O-labeled peptide increases. This error at high ^{16}O values is also reflected in the slope being more than 10% away from the theoretical value of 1 for two of the plots in Figure 8 (0.7736 for KALELFRNDIAA and 0.8331 for KHPGDFGADAQGAMT, respectively). The slope values improve to be 0.9466 for KALELFRNDIAA and 0.9523 for KHPGDFGADAQGAMT when the data set at $^{16}O/^{18}O$ peptide

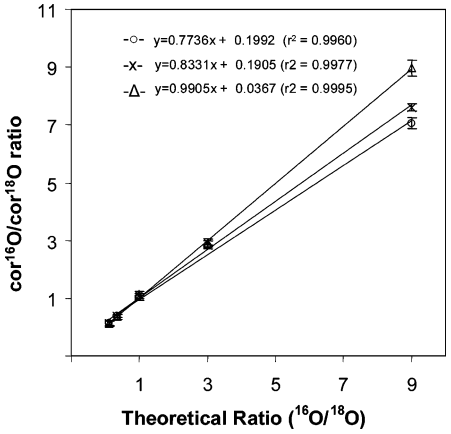

Figure 8. Plots of the cor^{16}O/cor^{18}O ratios of the three peptides in Figure 7 versus their theoretical ratios. Linear regression lines for KALELFRNDIAA (−○−), KHPGDFGADAQGAM (−×−), and KHGTVVLTALGGILK (−Δ−) were obtained from five different ratios ranging from 0.11 to 9 for each peptide. The correlation coefficients (r^2) of the linear regression lines for the three peptides are presented with the corresponding peptide lines. The vertical bar at each point indicates the RSD of the five repeated LC/MS analyses.

ratio of 9 are dropped. The phenomenon appears to be inherent in this method, because the obs^{16}O • X term is always larger than 0.05 • cor^{18}O term in eq 2, since a typical value of X for a 1500 Da peptide is about 0.4. Hence, the ^{16}O-labeled peptide has a significant impact on the calculation of ^{16}O/^{18}O peptide ratio through its M + 2 isotopic peak, therefore experimental error in ^{16}O/^{18}O peptide ratio becomes larger when ^{16}O/^{18}O peptide ratio is high, as was seen in our experimental results.

Concluding Remarks

In summary, we found that Lys-N incorporates only one ^{18}O atom into peptides at pH higher than 9.5. The single ^{18}O atom incorporation property of Lys-N overcomes the major problem of the current proteolytic ^{18}O labeling method: the generation of a mixture of isotopic isoforms resulting from the incorporation of either one or two ^{18}O atoms into each peptide species. We demonstrated that this Lys-N based comparative quantification method has linearity with good accuracy over a range of 0.11 to 9 for ^{16}O /^{18}O peptide ratios, and therefore can be used in comparative proteomic studies. The recommended protein digestion conditions for ^{18}O labeling using Lys-N is 100 mM pH 10.0 buffer consisting of glycine-NaOH and 1 M urea reacted at 25 °C for 18 h on proteins that have been reduced and *S*-alkylated.

We have shown that an enzyme catalyzed oxygen back-exchange reaction (^{18}O to ^{16}O or *vice versa*) does not occur with Lys-N. This is significant because any residual catalytic activity of Lys-N that may be present after the digestion does not change ^{16}O /^{18}O peptide ratios when the two digests in $H_2{}^{16}O$ and $H_2{}^{18}O$ are mixed together and analyzed in a $H_2{}^{16}O$ based solvent. On the other hand, trypsin, Glu-C, and Lys-C continually recognize the carboxyl termini of peptides produced by itself, further promoting the hydrolysis reaction of the generated peptides.[18] The enzymatic property of these serine proteases can lead to the enzyme catalyzed oxygen back-exchange upon the mixing of the $H_2{}^{16}O$ and $H_2{}^{18}O$ digests. A recent report demonstrated that trypsin catalyzes an oxygen back-exchange reaction leading to inaccurate results.[19] Thus, the absence of enzyme catalyzed oxygen back-exchange reaction is one advantage of using Lys-N in comparative proteomics.

Lys-N produces peptides that are larger as compared with trypsin, commonly used protease in proteomics, therefore the charge states of the peptide ions tend to be higher when electrospray is used for the ionization. Our experiments were performed on the quadrupole/time-of-flight type mass spectrometer, which provides high resolution of the measurement. Therefore, it was possible to analyze triply- and quadruply charged ions. Such experiments may not be reproduced using a quadrupole-based mass spectrometer.

Finally, we observed some peptides whose cor^{16}O/cor^{18}O ratios are more than ± 0.25 from the theoretical ratio (Figure 5). Most of these peptides appeared to be products of unfavorable peptide bond cleavage by Lys-N. This result points to the importance of achieving complete hydrolysis of all of the possible cleavage sites by proteases used in in vitro stable isotope labeling strategies.

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References

(1) Righetti, P. G.; Campostrini, N.; Pascali, J.; Hamdan, M.; Astner, H. Eur. *J. Mass Spectrom.* **2004**, *10*, 335−348.
(2) Conrads, T. P.; Issaq, H. J.; Hoang, V. M. *Adv. Protein Chem.* **2003**, *65*, 133−159.
(3) Gygi, S. P.; Rist, B., Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat Biotechnol.* **1999**, *17*, 994−999.
(4) Regnier, F. E.; Riggs, L.; Zhang, R.; Xiong, L.; Liu, P.; Chakraborty, A.; Seeley, E.; Sioma, C.; Thompson, R. A. *J. Mass Spectrom.* **2002**, *37*, 133−145.
(5) Julka, S.; Regnier, F. *J. Proteome Res.* **2004**, *3*, 350−363.
(6) Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. *Anal. Chem.* **2001**, *73*, 2836−2842.
(7) Stewart, I. I.; Thomson, T.; Figeys, D. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2456−2465.
(8) Reynolds, K. J.; Yao, X.; Fenselau, C. *J. Proteome Res.* **2002**, *1*, 27−33.
(9) Brown, K. J.; Fenselau, C. *J. Proteome Res.* **2004**, *3*, 455−462.
(10) Zhu, H.; Bilgin, M.; Snyder, M. *Proteomics. Annu. Rev. Biochem.* **2003**, *72*, 783−812.
(11) Schnolzer, M.; Jedrzejewski, P.; Lehmann, W. D.*Electrophoresis* **1996**, *17*, 945−953.
(12) Johnson, K. L.; Muddiman, D. C. *J. Am. Soc. Mass Specrom.* **2004**, *15*, 437−445.
(13) Shevchenko, A.; Chernushevich, I.; Ens, W.; Standing, K. G.; Thomson, B.; Wilm, M.; Mann, M. *Rapid Commun Mass Spectrom.* **1997**, *11*, 1015−1024.
(14) Nonaka, T.; Dohmae, N.; Hashimoto, Y.; Takio, K. *J. Biol. Chem.* **1997**, *272*, 30032−30039.
(15) Nonaka, T.; Hashimoto, Y.; Takio, K. *J. Biochem.* **1998**, *124*, 157−162.
(16) Hori, T.; Kumasaka, T.; Yamamoto, M.; Nonaka, T.; Tanaka, N.; Hashimoto, Y.; Ueki, T.; Takio, K. *Acta Crystallogr.* **2001**, *D57*, 361−368
(17) Ramagli, L. S. *Methods Mol. Biol.* **1999**, *112*, 99−103.
(18) Yao, X.; Afonso, C.; Fenselau, C. *J. Proteome Res.* **2003**, *2*, 147−152.
(19) Staes, A.; Demol, H.; Van Damme, J.; Martens, L.; Vandekerckhove, J.; Gevaert, K. *J. Proteome Res.* **2004**, *3*, 786−791.

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