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The pH Dependency of the Carboxyl Oxygen Exchange Reaction Catalyzed by Lysyl Endopeptidase and Trypsin

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Abstract

The pH dependency of the carboxyl oxygen exchange reaction catalyzed by lysyl endopeptidase (Lys-C) and trypsin has been studied. The reaction was quantitatively monitored by measuring the incorporation of ¹⁸O atom into the α-carboxyl group of *N*^α-acetyl-L-lysine from H₂ ¹⁸O solvent. The optimum pHs of the carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin were found to be pH 5.0 and 6.0, respectively, which were significantly shifted toward acidic pHs compared to the most favorable pHs of their amidase activities for *N*^α-acetyl-L-lysine amide in the pHs examined. Steady state kinetics parameters were also determined for both enzymes at two different pHs, one at the pH optimum for their carboxyl oxygen exchange activity (pH 5 - 6) and the other at the favorable pH for their amidase activity (pH 8 - 9). Significantly lower *K*_m (2-fold lower for Lys-C, 3-fold lower for trypsin), and higher *k*_{cat} values (1.5-fold higher for Lys-C, 5-fold higher for trypsin) were obtained at the acidic pHs compared to the alkaline pHs, suggesting that Lys-C and trypsin have higher substrate binding affinities and higher catalytic rates at the acidic pHs than at the alkaline pHs. The higher carboxyl oxygen exchange activities at the acidic pHs were also confirmed with peptide substrates derived from apomyoglobin. These findings are significant towards the goal of improving the efficiency of the Lys-C and trypsin catalyzed ¹⁸O labeling reactions and are thus pertinent to improving the accuracy and reliability of quantitative proteomic experiments utilizing ¹⁸O labeling.

Keywords

carboxyl oxygen exchange; ¹⁸O incorporation; mass spectrometry; lysyl endopeptidase; trypsin; quantitative proteomics; comparative proteomics

Introduction

Hydrolysis of a protein in H₂ ¹⁸O solvent by serine proteases results in incorporation of two ¹⁸O atoms into the carboxyl terminus of each proteolytically generated peptide, if sufficient time was given for the reaction to reach equilibrium.¹⁻⁴ Studies done by Yao and coworkers clearly demonstrated that the two ¹⁸O atoms are incorporated by trypsin at equilibrium via the following two step mechanism:⁴

1. $RC^{16}ONHR' + H_2^{18}O \rightarrow RC^{16}O^{18}O^- + ^+H_3NR'$
2. $RC^{16}O^{18}O^- + H_2^{18}O \rightarrow RC^{18}O^{18}O^- + H_2^{16}O$

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The first ^{18}O atom is incorporated from the $\text{H}_2\ ^{18}\text{O}$ solvent upon proteolytic cleavage of a peptide bond, as shown in reaction 1. The second ^{18}O atom incorporation is essentially a carboxyl oxygen exchange reaction as shown in the reaction 2, which occurs following the proteolytic cleavage. The protease continues to interact with the peptide product and exchange the carboxyl oxygen, which results in two ^{18}O atoms incorporation, if the first ^{18}O atom retains (if not, which incorporates another ^{18}O atom, but concomitantly loose the first ^{18}O atom as a leaving $^{18}\text{OH}^-$, results in a net single ^{18}O atom incorporation). Thus, the reaction 2 is required to occur multiple times on the peptide to achieve complete incorporation of two ^{18}O atoms. If the reaction continues, both the oxygens in the C-terminal carboxyl group of the peptide should eventually come to equilibrium with oxygen from the $\text{H}_2\ ^{18}\text{O}$ solvent.

There has been an increasing interest in the use of protease catalyzed ^{18}O labeling for quantitative proteomics.^{5,6} However, this method often suffers because of the generation of a mixture of isotopic isoforms resulting from the variable incorporation of either one or two ^{18}O atoms ($^{18}\text{O}_1/^{18}\text{O}_2$) into each peptide species,⁷ presumably because the second ^{18}O atom incorporation reaction (carboxyl oxygen exchange reaction) is extremely slow under the conditions commonly used for protein digestion, leading to incomplete and variable exchange in the time frame of the proteolytic reaction. This complicates the quantification of the peptides and increases the error in the calculations of ^{16}O - and ^{18}O -labeled peptide ratios.⁸ Complete incorporation of two ^{18}O atoms would obviate this difficulty. Finding reaction conditions to achieve this would be a significant step towards improving quantitative proteomics methods utilizing Lys-C and trypsin catalyzed ^{18}O labeling.

The carboxyl oxygen exchange reaction was found by Rittenberg and Sprinson for chymotrypsin fifty years ago.⁹ Shortly after the finding, Doherty and Vaslow demonstrated that the binding of acetyl-3,5-dibromo-L-tyrosine to chymotrypsin is tighter at acidic pH than alkaline pH by an enzyme-substrate equilibrium experiment.¹⁰ The kinetic parameters of chymotrypsin-catalyzed carboxyl oxygen exchange reaction have also been reported by Vaslow¹¹ for acetyl-3,5-dibromo-L-tyrosine at pH 7.2 and by Bender and Kemp¹² for benzoyl-L-phenylalanine and acetyl-L-tryptophane at pH 7.8. Recently, Yao and coworkers have reported kinetic parameters of trypsin-catalyzed carboxyl oxygen exchange reaction at pH 8.0 for short peptide substrates.⁴ However, pH studies were not performed in these kinetic studies. A recent study done by Zang and coworkers showed that trypsin-catalyzed carboxyl oxygen exchange reaction at pH 6.75 is more efficient than at pH 8.50 based on an experiment that measured the changes of isotopic peaks of the labeled peptides after 20 h of labeling reactions.⁵ Also, a study performed by Staes and coworkers showed that complete two ^{18}O atoms incorporation can be achieved after overnight reaction at pH 4.5.¹³ These two earlier works suggested that the optimum pH of trypsin catalyzed carboxyl oxygen exchange reaction is shifted toward acidic pH compared to the favorable pH for its amidase activity. However, these earlier works did not measure the initial rate of the reaction, therefore no quantitative information on the rate of the reaction were obtained. As far as we know the effect of pH on the kinetic parameters has not been studied before.

The purpose of this study is to better understand the catalytic mechanism of carboxyl oxygen exchange reaction promoted by serine proteases. Lys-C and trypsin were chosen, because they have been the most utilized proteases in ^{18}O labeling experiment. A better understanding of the mechanism will lead to the development of a more reliable quantitative proteomic method. Since the substrate for carboxyl oxygen exchange reaction (RCOO^-) has a negative charge at the C-terminus at physiological pH, while the corresponding substrate for amide hydrolysis reaction (RCONH_2) does not, it seems reasonable to assume that the mechanism of enzyme-substrate complex formation in the carboxyl oxygen exchange reaction is somewhat different from the mechanism in the amide bond hydrolysis reaction. For this reason, we examined the pH dependency of the carboxyl oxygen exchange reaction for N^{α} -acetyl-L-lysine (Ac-Lys-OH)

catalyzed by Lys-C and trypsin, utilizing H_2^{18}O as a carboxyl oxygen labeling agent. The single ^{18}O atom incorporated reaction product, Ac-Lys- ^{18}OH , was quantified by stable isotope dilution tandem mass spectrometry. Steady state kinetics parameters for the carboxyl oxygen exchange reaction were also obtained. Based on these results conditions for use of Lys-C and trypsin to efficiently incorporate two ^{18}O atoms into the carboxyl termini of peptides are suggested.

Materials and Methods

Materials

N^α -acetyl-L-lysine (Ac-Lys-OH), N^α -acetyl-L-lysine amide (Ac-Lys-NH₂) and N^ϵ -tert-butyloxycarbonyl-L-lysine (H-Lys(Boc)-OH) were purchased from Bachem AG (Torrance, CA). Horse apomyoglobin and [$^{13}\text{C}_4$]acetic anhydride were obtained from Sigma-Aldrich (St. Louis, MO). Oxygen-18 enriched water (>95%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Lys-C from *Achromobacter lyticus* was purchased from Wako Chemicals USA (Richmond, VA). Sequencing grade modified porcine trypsin was obtained from Promega (Madison, WI). All other chemicals and materials were either reagent grade or were of the highest quality that was commercially available.

N^α -[$^{13}\text{C}_2$]acetyl-lysine ([$^{13}\text{C}_2$]Ac-Lys-OH) was synthesized by acetylating H-Lys(Boc)-OH (50mM) by [$^{13}\text{C}_4$]acetic anhydride (2mM) for 16 h at 25 °C in 0.8 mM N-ethylmorpholine, 50% acetonitrile in water. After the reaction, the reaction mixture was dried in a speed-vac, and then the Boc group was cleaved by 3 M HCl in ethyl acetate for 30 min at 25°C. The product, [$^{13}\text{C}_2$]Ac-Lys-OH, was extracted in water and purified by reverse phase HPLC. The molecular weight of the purified [$^{13}\text{C}_2$]Ac-Lys-OH was determined by high resolution mass spectrometry [$^{13}\text{C}_2\text{C}_6\text{H}_{17}\text{N}_2\text{O}_3$ (M + H⁺): calculated, 191.1306; found, 191.1261]. [$^{13}\text{C}_2$]Ac-Lys-OH in water was quantified by spectrophotometry at 215 nm using a molar absorption coefficient for Ac-Lys-OH of 877 M⁻¹•cm⁻¹ and used for the following experiments.

Measurement of the pH dependency of the carboxyl oxygen exchange reaction for Ac-Lys-OH and the amidase activity for Ac-Lys-NH₂

The initial rate of carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin was determined by monitoring the rate of ^{18}O atom incorporation into the carboxyl terminus of Ac-Lys-OH. For the pH studies, a 10 mM solution of Ac-Lys-OH in aqueous buffers at different pHs (specified below) was incubated with 1 μM Lys-C or trypsin at 25 °C in 95% H_2^{18}O buffered at various pHs. The buffer solutions used were 50 mM citrate at pH 4.0, 5.0 and 6.0 (pH adjusted by addition of NaOH), 50 mM phosphate at pH 7.0 and 8.0 (pH adjusted by addition of NaOH) and 50 mM Tris at pH 9.0 (pH adjusted by addition of HCl). The duration of the reaction was 5 min for Lys-C and 20 min for trypsin. All the reactions were confirmed to be linear within the time range. The total volume of the reaction mixture was 50 μL . The reaction was stopped by adding 4-times the volume of 80% acetonitrile/1% formic acid (v/v) in water containing a constant amount of internal standard, [$^{13}\text{C}_2$]Ac-Lys-OH (0.75 μg), dried in speed-vac concentrator, and redissolved in 50 μL of 5% acetonitrile/0.1% heptafluorobutyric acid (HFBA) (v/v) in water. The single ^{18}O atom incorporated reaction product, Ac-Lys- ^{18}OH , in the reconstituted reaction solution was then quantified by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate.

The amidase activity of Lys-C and trypsin was measured by monitoring the initial rate of hydrolysis of Ac-Lys-NH₂ in H_2^{18}O . Ac-Lys-NH₂ (10 mM) was incubated with 0.2 μM Lys-C or 1 μM trypsin at 25 °C in 95% H_2^{18}O at various pHs. The buffer solutions used were the same as described above for the carboxyl oxygen exchange reactions. The duration of the reaction was 5 min for Lys-C and 20 min for trypsin. The single ^{18}O atom incorporated reaction

product, Ac-Lys-¹⁸OH, was quantified after addition of a [¹³C₂]Ac-Lys-OH internal standard (0.75 µg) by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate.

Measurement of the steady-state kinetic parameters for the carboxyl oxygen exchange reaction

The steady-state kinetic parameters for the carboxyl oxygen exchange reaction by Lys-C and trypsin were obtained at two different pHs (pH 9.0 and 5.0 for Lys-C and pH 8.0 and 6.0 for trypsin). Ac-Lys-OH (0.0625 – 10 mM) was incubated with 1 µM Lys-C or trypsin at 30 °C in 95% H₂ ¹⁸O buffered at various pHs (described above). The total volume of the reaction was 20 µL. The duration of the reaction was 10 min for Lys-C and 20 min for trypsin. The single ¹⁸O atom incorporated reaction product, Ac-Lys-¹⁸OH, was quantified after addition of a [¹³C₂]Ac-Lys-OH internal standard (0.75 µg) by stable isotope dilution tandem mass spectrometry, as described below. All reactions were carried out in triplicate.

K_m and V_{max} values were calculated by fitting the Michaelis-Menten equation to the experimental data using SigmaPlot 9.01 (Systat Software, Richmond, California). k_{cat} values were calculated by dividing the obtained V_{max} values by the total enzyme amount added to the system.

Quantification of Ac-Lys-¹⁸OH by stable isotope dilution tandem mass spectrometry

An Agilent 1100 HPLC system (Palo Alto, California) coupled to an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ ion source (Applied Biosystems-MDS-Sciex, Foster City, CA) was used to quantify Ac-Lys-¹⁸OH. The reconstituted reaction mixtures were injected (typically 5 µL) onto a reversed-phase C18 column (2.1 × 150 mm, 5 µm, 300 Å, Vydac) equilibrated with 5% acetonitrile/0.1% HFBA (v/v) in water and then eluted with the same solvent at a flow rate of 200 µL/min. A switching valve (Valco Instrument, Houston, TX) was placed between the HPLC system and the mass spectrometer to divert non-volatile salt fractions from the mass spectrometer at the beginning of the run. The switching valve was switched in-line with the mass spectrometer at 3 min after the injection. The elution time of Ac-Lys-¹⁸OH was 3.8 min.

The ion spray voltage and the ion source probe temperature were set at 5 kV and 400 °C, respectively. Nitrogen was used both as a nebulizer and as an auxiliary gas, at flow rates of 3 and 8 L/min, respectively. A multiple reaction monitoring mode was used for monitoring Ac-Lys-¹⁸OH and [¹³C₂]Ac-Lys-OH. The precursor and fragment ions monitored were m/z 191 (Ac-Lys-¹⁸OH + H) and 149 (H-Lys-¹⁸OH + H) for Ac-Lys-¹⁸OH and m/z 191 ([¹³C₂]Ac-Lys-OH + H) and 147 (H-Lys-¹⁶OH + H) for [¹³C₂]Ac-Lys-OH, respectively. The collision energy was 20 eV for both the analyte and internal standard. Nitrogen was used as the collision gas.

Amounts of Ac-Lys-¹⁸OH in the reaction mixture were determined from the chromatographic peak area ratios of the analyte over the internal standard, assuming identical ionization efficiencies and identical yields of fragment ions for Ac-Lys-¹⁸OH and [¹³C₂]Ac-Lys-OH. The experimentally determined amounts of Ac-Lys-¹⁸OH were corrected upward 5 %, accounting for ¹⁶O incorporation resulting from the H₂ ¹⁸O solvent having a 5% H₂ ¹⁶O content.⁸ The assay method showed good linearity over the concentration range of 1.95 – 1,000 µM ($r^2 > 0.999$). A S/N=20 was obtained for 1.95 µM Ac-Lys-OH.

Measurement of the carboxyl oxygen exchange reaction for Lys-C and tryptic peptides of apomyoglobin

Apomyoglobin (34 μg) was digested for 16 h at 25 $^{\circ}\text{C}$ with either 1 μg of Lys-C or trypsin in 100 mM ammonium bicarbonate in H_2^{16}O solvent. The digestion was stopped by adding formic acid to a final concentration of 1% and by boiling the reaction mixture for 2 min. The resulting Lys-C and tryptic peptides were lyophilized and used in the following experiments.

The rate of the carboxyl oxygen exchange reaction into the Lys-C and tryptic peptides by Lys-C and trypsin, respectively, were semi-quantitatively measured by looking at the rate of ^{18}O atom incorporation into the carboxyl termini of the peptides by LC/MS (described below). The Lys-C and tryptic digests (both 1.5 μg) were incubated with Lys-C and trypsin (both 0.03 μg), respectively, in H_2^{18}O at 30 $^{\circ}\text{C}$ buffered (see above) at pH 5.0 or 9.0 for Lys-C and pH 6.0 or 8.0 for trypsin for various reaction times (1 min–60 min). The total reaction volume was 50 μL . Aliquots (5 μL) of the reaction mixtures were taken after 1, 5, 10, and 60 min. The reactions were stopped by mixing with 40 μL of 10% formic acid and by heating at 95 $^{\circ}\text{C}$ for 2 min. After the heat treatment, the solvents were evaporated in the speed-vac concentrator and the samples were stored at -20 $^{\circ}\text{C}$ until use.

The stored ^{18}O labeled peptide mixtures were reconstituted in 20 μL of aqueous 0.1% trifluoroacetic acid (TFA), desalted by a C18 ZipTip as per the manufacturer's instructions (Millipore, Bedford, MA), and subjected to LC/MS analysis using an UltiMate nano HPLC system (Dionex, San Francisco, CA) interfaced to a QStar quadrupole/time of flight mass spectrometer (Applied Biosystems-MDS Sciex, Foster City, CA), as described previously.⁷ Briefly, the peptide mixture (5 μL) was injected directly into a reverse-phase analytical column (0.075 $\times$ 60 mm, New Objective Inc., Woburn, MA) packed with Jupiter C18 media (Phenomenex, Torrance, CA), washed with 2% acetonitrile/0.1% formic acid (v/v) in water for 15 min at flow rate of 2 $\mu\text{L}/\text{min}$. The peptides were then eluted with a 40 min linear gradient of 2% acetonitrile/0.1% formic acid (v/v) in water to 40% acetonitrile/0.1% formic acid (v/v) in water at a flow rate of 200 nL/min. The column effluent was directed on-line to a nano-electrospray ion source. The total ion current was obtained in the mass range of m/z 400–1600 in the positive ion mode. The electrospray and orifice voltages were 2050V and 65V, respectively.

Results and Discussion

pH dependency of the carboxyl oxygen exchange reaction for Ac-Lys-OH

The pH dependency of the carboxyl oxygen exchange reaction measured by the incorporation of an ^{18}O atom into Ac-Lys-OH catalyzed by Lys-C and trypsin is shown in Figure 1a and b, respectively. The pH dependency of their amidase activities obtained for the hydrolysis of Ac-Lys-NH₂ are also shown in the figure. The optimum pHs of the carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin were found to be pH 5.0 and 6.0, respectively, which were 3–4 pH units lower than the most favorable pHs of their amidase activities (pH 9) within the pH range examined.

Steady-state kinetic parameters for the carboxyl oxygen exchange reaction

To better understand the catalytic mechanism of carboxyl oxygen exchange reaction catalyzed by Lys-C and trypsin, we obtained steady-state kinetic parameters for the carboxyl oxygen exchange reaction of Ac-Lys-OH in H_2^{18}O solvent, assuming negligible kinetic isotope effect of H_2^{18}O solvent. The reactions were performed at the acidic and alkaline pH optimums of the carboxyl oxygen exchange and amidase reactions, respectively. Figure 2a shows the effects of substrate concentration on the initial velocity of the carboxyl oxygen exchange reaction catalyzed by Lys-C at pH 5.0 and 9.0. Similarly, Figure 2b shows the effects of substrate

concentration on the initial velocity of the carboxyl oxygen exchange reaction catalyzed by trypsin at pH 6.0 and 8.0. From the plots in Figure 2a and b, it is noticeable that the K_m values decrease and the V_{max} values increase with a decrease in pH. Note that the high pH trypsin catalyzed reaction was done at pH 8.0, because the rate of the carboxyl oxygen exchange was too low at pH 9.0 (most favorable pH for the amidase activity in the pHs examined) to accurately measure the kinetic parameters.

Table I summarizes the K_m and k_{cat} values calculated by fitting the Michaelis-Menten equation to the experimental data for the two enzymes at acidic and alkaline pHs. For Lys-C at pH 5.0, the K_m was about 2-fold smaller, the k_{cat} was about 1.5-fold higher, and the k_{cat}/K_m was about 2.5-fold higher than the K_m , k_{cat} , and k_{cat}/K_m , respectively, at pH 9.0. For trypsin at pH 6.0, the K_m was about 3-fold smaller, the k_{cat} was about 5-fold higher, and the k_{cat}/K_m was about 16-fold higher than the K_m , k_{cat} , and k_{cat}/K_m , respectively, at pH 8.0. These results show that Lys-C and trypsin have higher substrate binding affinities and higher catalytic rates at the acidic pHs. The higher substrate binding affinities of Lys-C and trypsin to the substrate for carboxyl oxygen exchange reaction at the acidic pHs are consistent with the result obtained for chymotrypsin by Doherty and Vaslow,¹⁰ therefore, this enzymatic property may be a common feature of serine proteases.

The experimentally determined k_{cat}/K_m value for carboxyl oxygen exchange reaction by Lys-C at pH 5.0 was $1.42 \text{ mM}^{-1}\cdot\text{s}^{-1}$ (Table 1), which is only 3.4-fold lower than the reported k_{cat}/K_m value for amide hydrolysis of N^α -benzoyl-L-lysine amide at pH 8.5 ($4.81 \text{ mM}^{-1}\cdot\text{s}^{-1}$),¹⁴ suggesting that the carboxyl oxygen exchange catalytic activity of Lys-C at the acidic pH is almost equivalent with its amidase activity at alkaline pH. Similarly, for trypsin at pH 6.0, the experimentally determined k_{cat}/K_m value for carboxyl oxygen exchange reaction was $0.083 \text{ mM}^{-1}\cdot\text{s}^{-1}$ (Table 1), which is 5.1-fold lower than the reported k_{cat}/K_m value for amide hydrolysis of N^α -benzoyl-L-lysine amide at pH 8.0 ($0.41 \text{ mM}^{-1}\cdot\text{s}^{-1}$),¹⁵ also the difference of both the values are less than an order of magnitude.

The higher value of k_{cat}/K_m for Lys-C at pH 5.0 compared to the value at pH 9.0 is due mainly to the effect of the decreased value of the K_m component on the ratio, while both the K_m and k_{cat} significantly contribute to the higher k_{cat}/K_m of trypsin at the acidic pH. The results suggest that Lys-C catalyzes the reaction more efficiently than trypsin at alkaline pH by as yet unknown mechanism.

It was also found that k_{cat}/K_m of Lys-C at pH 5.0 was about 17-fold higher than that of trypsin at pH 6.0. This is attributed to the approximate 2-fold lower K_m and 10-fold higher k_{cat} of Lys-C for Ac-Lys-OH. This indicates that Lys-C has higher carboxyl oxygen exchange activity than trypsin.

Rate of the carboxyl oxygen exchange reaction for peptide substrates

We semi-quantitatively measured the rate of ^{18}O atom incorporation into Lys-C and tryptic peptides derived from apomyoglobin. This was done to test whether the increased rate of carboxyl oxygen exchange noted for the reaction of Ac-Lys-OH at acidic pH could be generalized to peptide substrates. Lys-C and tryptic digest of apomyoglobin prepared in H_2^{16}O solvent were incubated with Lys-C and trypsin in H_2^{18}O solvent at the favorable pHs of the carboxyl oxygen exchange and amidase reactions for various reaction times. The resulting ^{18}O labeled peptides were analyzed by LC/MS. Figure 3 shows the mass spectra of a representative ^{18}O -labelled apomyoglobin peptide that has a lysine residue at the C-terminus (Lys-peptide). The Lys-C results clearly show that there was complete incorporation of two ^{18}O atoms (m/z 502.9) within 1 min of incubation at pH 5.0 (Fig. 3a), while the dominant result at pH 9.0 was the incorporation of only one ^{18}O atom (m/z 502.3) (Fig. 3b). In the case of trypsin, complete incorporation of two ^{18}O atoms was achieved within 10 min of incubation

at pH 6.0 (Fig. 3c), while the dominant result at pH 8.0 was the incorporation of only one ^{18}O atom, and complete incorporation of two ^{18}O atoms was not achieved even after 60 min of incubation (Fig. 3d). All the other Lys-peptides showed similar results (data not shown). Note that a small fraction of one ^{18}O atom incorporated peak (m/z 502.3) exists even after achieving complete incorporation of two ^{18}O atoms because of the purity of H_2 ^{18}O solvent (95% H_2 ^{18}O and 5% H_2 ^{16}O) used in the experiments.

These results demonstrate that the enhanced rate of carboxyl oxygen exchange reaction by Lys-C and trypsin at acidic pH for Ac-Lys-OH can be generalized to peptide substrates. They also show that the carboxyl oxygen exchange activity of Lys-C for peptide substrates is significantly higher than that of trypsin, consistent with the results obtained in the Ac-Lys-OH experiments.

Figure 4 shows the mass spectra of a representative ^{18}O -labelled peptide that has an arginine residue at the C-terminus (Arg-peptide), which was also labeled by trypsin as described above. Complete incorporation of two ^{18}O atoms (m/z 537.6) was achieved within 10 min of incubation at pH 6.0 (Fig. 4a), while it took 60 min at pH 8.0 (Fig. 4b), showing that the higher carboxyl oxygen exchange rate at the acidic pH found for Lys-peptides can be expanded to Arg-peptides.

It is noticeable that the rate of ^{18}O atoms incorporation into the Arg-peptide by trypsin is obviously faster than the rate into the Lys-peptide (Fig. 4a and b for Arg-peptide and Fig. 3c and d for Lys-peptide). After 1 min of incubation, dominant peak is two ^{18}O atoms (m/z 537.6) incorporated peak at both pH 6.0 and 8.0 for the Arg-peptide (Fig. 4a and b), while the dominant result for Lys-peptide was the incorporation of only one ^{18}O atom (m/z 502.3) (Fig. 3c and d). All the other Arg-peptides derived from apomyoglobin showed faster ^{18}O atom incorporation rates than Lys-peptides (data not shown). The faster oxygen exchange reaction by trypsin for Arg-peptide has also been demonstrated by Yao and coworkers for peptide substrates, YGGFMK and YGGFMR, and for tryptic peptides derived from apomyoglobin.⁴

Concluding Remarks

Our study demonstrated that the rate of carboxyl oxygen exchange reaction by Lys-C and trypsin can be accelerated significantly by carrying out the labeling reaction at the acidic pH conditions used in the present work. The catalytic activities ($k_{\text{cat}}/K_{\text{m}}$) of Lys-C and trypsin at the acidic pHs were 2.5-fold and 17-fold higher than those at the alkaline pHs, respectively. This finding is significant towards improving the accuracy of ^{18}O labeling in quantitative proteomic experiments; by identifying the conditions that lead to the complete incorporation of two ^{18}O atoms into peptides. We recommend carrying out ^{18}O labeling by Lys-C and trypsin under the acidic pH conditions specified in the present work. This study strongly supports the use of the separate experiments for digestion of proteins and ^{18}O labeling of the generated peptides,⁴ each can be optimized separately (the digestion at pH 8, and the labeling at pH 5-6). Endoproteinase Glu-C and chymotrypsin have also been shown to be effective catalysts to incorporate two ^{18}O atoms into peptides.¹⁶ Based on the studies obtained with Lys-C and trypsin, the optimum pHs of the carboxyl oxygen exchange reaction catalyzed by these two proteases is assumed to be acidic. However, the optimum pHs need to be confirmed.

It was found that the activity of carboxyl oxygen exchange reaction of Lys-C ($k_{\text{cat}}/K_{\text{m}}$) at pH 5.0 was 17-fold higher than that of trypsin at pH 6.0 when Ac-Lys-OH was used as a substrate. The amidase activities of Lys-C for N^{α} -benzoyl-L-lysine amide and N^{α} -benzoyl-lysine-*p*-nitroanilide have been reported to be an order of magnitude higher than that of trypsin.^{14,17} Thus, it is apparent that the use of Lys-C as a catalyst in ^{18}O labeling experiment has an advantage over trypsin in terms of efficiency of ^{18}O labeling in both the first and the second ^{18}O atom incorporation (the reaction 1 and 2, respectively, in Introduction). However, Lys-C produces peptides that are larger as compared with peptides produced by trypsin,

therefore a high resolution mass spectrometer may be required for the analysis of the peptides because of the tendency of larger peptides to produce higher charge states than smaller peptides when electrospray is used.

A number of kinetic studies of serine proteases have shown that the unprotonated state of the active site histidine residue is required to express the amide hydrolysis catalytic activity.^{18, 19} This unprotonated histidine state promotes the nucleophilic attack by an active site serine hydroxyl group on the carbonyl carbon of the substrate, leading to amide bond hydrolysis. The pK_a of the active site histidine residues of Lys-C and trypsin have been reported to be 6.0 and 6.6,^{20,21} respectively. Therefore, a significant portion of the active site histidine residues of both proteases are expected to be in the protonated state at the optimum pHs of the carboxyl oxygen exchange reaction (pH 5.0 for Lys-C and pH 6.0 for trypsin). Thus, a different mechanism is implied for the carboxyl oxygen exchange reaction at acidic pH compared to the amide bond hydrolysis reaction. Further work is necessary to elucidate the detailed catalytic mechanism of carboxyl oxygen exchange reaction.

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Figure 1.

Effects of pH on the initial velocities of the carboxyl oxygen exchange and amidase activities of Lys-C and trypsin. For carboxyl oxygen exchange activity, 10 mM solution of Ac-Lys-OH in aqueous buffers at different pHs was incubated with 1 μM Lys-C or trypsin at 25 °C in 95% H₂ ¹⁸O buffered at various pHs. For amidase activity, Ac-Lys-NH₂ (10 mM) was incubated with 0.2 μM Lys-C or 1 μM trypsin at 25 °C in 95% H₂ ¹⁸O at various pHs. The single ¹⁸O atom incorporated reaction product, Ac-Lys-¹⁸OH, was quantified by stable isotope dilution tandem mass spectrometry as described in Materials and Methods. (a) pH dependency of the carboxyl oxygen exchange and amidase activities of Lys-C. (b) pH dependency of the carboxyl oxygen exchange and amidase activities of trypsin. -○-: carboxyl oxygen exchange activity, -●-: amidase activity. All reactions were carried out in triplicate.

Figure 2.

Effects of substrate concentration on the initial velocity of the carboxyl oxygen exchange reaction. Various concentrations of Ac-Lys-OH were incubated with Lys-C or trypsin, both 1 μ M, in 95% H_2^{18}O solvent at 30 $^\circ\text{C}$ at pH 5.0 and 9.0 for Lys-C reaction and at pH 6.0 and 8.0 for trypsin reaction. The single ^{18}O atom incorporated reaction product, Ac-Lys- ^{18}OH , was quantified by stable isotope dilution tandem mass spectrometry as described in Materials and Methods. (a) Effect of the substrate concentration of the carboxyl oxygen exchange reaction catalyzed by Lys-C. (b) Effect of the substrate concentration on the carboxyl oxygen exchange reaction catalyzed by trypsin. -○-: acidic pH, -●-: alkaline pH. All reactions were carried out in triplicate.

Figure 3.

Mass spectra of a representative ^{18}O labeled Lys-peptide catalyzed by Lys-C and trypsin. Apomyoglobin Lys-C and tryptic digest (both 1.5 μg) prepared in H_2^{16}O solvent were incubated with Lys-C and trypsin (both 0.03 μg), respectively, in 95% H_2^{18}O solvent at 30°C for various reaction times (1 min-60 min). After the reaction, the resulting ^{18}O labeled peptides were analyzed by LC/MS as described in Materials and Methods. (a) and (b): $(M + 3\text{H})^{3+}$ ions of peptide HPGDFGADAQGAMTK labeled with ^{18}O by Lys-C at pH 5.0 and 9.0, respectively, for different reaction times. (c) and (d): $(M + 3\text{H})^{3+}$ ions of the same peptide labeled with ^{18}O by trypsin at pH 6.0 and 8.0, respectively, for different reaction times. m/z 501.6 is a peptide peak incorporated no ^{18}O atom, m/z 502.3 is a peptide peak incorporated only one ^{18}O atom, and m/z 502.9 is a peptide peak incorporated two ^{18}O atoms.

Figure 4.

Mass spectra of a representative ^{18}O labeled Arg-peptide catalyzed by trypsin. Apomyoglobin tryptic digest (1.5 μg) prepared in H_2^{16}O solvent were incubated with trypsin (0.03 μg) in 95% H_2^{18}O solvent at 30°C for various reaction times (1 min-60 min). After the reaction, the resulting ^{18}O labeled peptides were analyzed by LC/MS as described in Materials and Methods. (a) and (b): $(M + 3\text{H})^{3+}$ ions of peptide VEADIAGHGQEVLR labeled with ^{18}O by trypsin at pH 6.0 and 8.0, respectively, for different reaction times. m/z 536.3 is a peptide peak incorporated no ^{18}O atom, m/z 536.9 is a peptide peak incorporated only one ^{18}O atom, and m/z 537.6 is a peptide peak incorporated two ^{18}O atoms.

Table 1

Kinetic parameters for carboxyl oxygen exchange reaction

	Lys C			trypsin		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
acidic pH	0.88	1.24	1.42	1.48	0.123	0.083
	(± 0.06) ^a	(± 0.05)	(± 0.07)	(± 0.21)	(± 0.007)	(± 0.007)
alkaline pH	1.59	0.90	0.57	4.89	0.025	0.005
	(± 0.12)	(± 0.05)	(± 0.02)	(± 0.91)	(± 0.003)	(± 0.001)

^aNumbers in the parentheses are standard deviations of the mean values.