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*Anal Chem*. Author manuscript; available in PMC 2010 June 28.

Published in final edited form as:
*Anal Chem*. 2009 April 1; 81(7): 2804–2809. doi:10.1021/ac802484d.

# Acid-Catalyzed Oxygen-18 Labeling of Peptides for Proteomics Applications

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## Abstract

In enzymatic $^{18}O$-labeling strategies for quantitative proteomics, the exchange of carboxyl oxygens at low pH is a common, undesired side reaction. We asked if acid-catalyzed back exchange could interfere with quantitation and whether the reaction itself could be used as an alternative method for introducing $^{18}O$ label into peptides. Several synthetic amino acid sequences were dissolved in dilute acid containing 50% (v/v) $H_2{}^{18}O$ and incubated at room temperature. Aliquots were removed over a period of 3 weeks and analyzed by tandem mass spectrometry (MS/MS). $^{18}O$-incorporation ratios were determined by linear regression analysis that allowed for multiple stable isotope incorporations. At low pH, peptides exchanged their carboxyl oxygen atoms with the aqueous solvent. The isotope patterns gradually shifted to higher masses until they reached the expected binomial distribution at equilibrium after ~11 days. Reaction rates were residue and sequence specific. Due to its slow nature, the acid-based back exchange is expected to minimally interfere with enzymatic $^{18}O$-labeling studies provided that storage and analysis conditions minimize low pH exposure times. On its own, acid-catalyzed $^{18}O$ labeling is a general tagging strategy that is an alternative to the chemical, metabolic and enzymatic isotope-labeling schemes currently used in quantitative proteomics.

Stable-isotope labeling strategies are commonly used in mass spectrometry-based differential proteomics approaches.[1–3] For example, adding known amounts of chemically identical, isotopically labeled internal standards enables accurate quantification.[4] Typically, isotopes are either incorporated metabolically (*e.g.*, SILAC5), chemically (*e.g.*, iTRAQ6, ICAT7, GIST8), synthetically (*e.g.*, AQUA9, 10) or enzymatically (*e.g.*, tryptic digestion in $^{18}O$-enriched water).[11–15] Recently, we used a modification of the enzymatic approach to identify endogenous salivary proteinases by virtue of their $^{18}O$-labeled peptide products.[16]

Each labeling technique has its limitations. For example, metabolic labeling requires culturing cells in isotopically labeled medium, an experimental system with limited biological applications.[5, 17] This approach has been extended to *in vivo* applications at the whole-animal

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level,[18] an expensive and time-consuming process. Chemical labeling is often limited to peptides that provide the requisite functional groups, *e.g.*, cysteine for ICAT-based labeling, a requirement that was recently eliminated by the introduction of iTRAQ-labeling strategies to tag primary amine groups.[5] Furthermore, side reactions may introduce additional labels at other reactive sites, or inefficient reaction kinetics may lead to underlabeling. With enzyme-catalyzed $^{18}$O-transfer, most peptides are labeled except those derived from the carboxyl (C) termini of proteins. However, the efficiency of the incorporation of a second $^{18}$O atom in the carboxyl exchange reaction, which depends on the enzyme-substrate interaction, can vary.[15, 19] Isotopically labeled synthetic peptides are expensive and require individual synthesis.

Acid-mediated oxygen exchange has been reported by many investigators to be an undesirable side reaction occurring when peptides are stored at low pH, *e.g.*, in 0.2% trifluoroacetic acid.[20–22] Here we report on the kinetics of the back exchange reaction and how it can be used as an alternative means to label peptides with $^{18}$O, reminiscent of a strategy originally devised by Murphy and Clay for the stable isotope labeling of amino acids and carboxylic acids.[23, 24] We have termed this methodology ALeO—acid-catalyzed labeling employing $^{18}$O. This approach has numerous applications in both quantitative and qualitative proteomics studies.

## MATERIALS AND METHODS

### Time-Course Experiment

Sequazyme peptide mass standards kit (Applied Biosystems) calibration mixture 1 (Cal Mix 1) is composed of des-Arg1-bradykinin (PPGFSPFR, *m/z* 904.5, 1 pmol/μL), angiotensin I (DRVYIHPFHL, *m/z* 1296.7, 1.3 pmol/μL), [Glu1]-fibrinopeptide B (EGVNDNEEGFFSAR, *m/z* 1570.7, 1.3 pmol/μL) and neurotensin (qLYENKPRRPYIL, *m/z* 1672.9, 50 fmol/μL). Peptide standards kit P7720S (New England Biolabs) is a set of five individual peptides: angiotensin I, neurotensin, adrenocorticotropic hormone (ACTH) [1–17] (SYSMEHFRWGKPVGKKR, *m/z* 2093.1), ACTH [18–39] (RPVKVYPNGAEDESAEAFPLEF, *m/z* 2465.2) and ACTH [7–38] (FRWGKPVGKKRRPVKVYPNGAEDESAEAFPLE, *m/z* 3657.9). Aliquots of the peptide mixture Cal Mix 1 (2 μL) and individual peptides (50 ng) were incubated 1:1 (v/v) with $^{18}$O-enriched water (95%, Sigma Isotec) in the presence or absence of 1% (v/v) final trifluoroacetic acid (total volume 30 μL). The reaction products were sampled over 18 days by spotting an aliquot of the mixture directly onto a stainless steel matrix-assisted laser desorption ionization (MALDI) target with α-cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid) at a 1:1 (v/v) ratio.

### MALDI–Time of Flight Tandem Mass Spectrometry (TOF/TOF MS)

Peptides were analyzed in positive-ion mode on a 4800 Proteomics Analyzer mass spectrometer (Applied Biosystems) equipped with TOF/TOF ion optics and a 200 Hz Nd:Yag laser. Prior to analysis, the instrument was externally calibrated with a mixture of peptide standards. For monitoring of the incorporation of $^{18}$O at individual carboxyl groups by tandem MS (MS/MS), the width and center of the precursor ion mass window were manually selected to permit the submission of peptide corresponding to the entire isotopic envelope to fragmentation. MS data were analyzed using a laboratory information system created in-house that utilizes Mascot Distiller software (Matrix Science) for spectral processing and peak detection.

### Calculation of Isotopic Incorporation Ratios

The relative quantities of unlabeled and peptide species carrying multiple $^{18}$O isotopes were calculated using an algorithm similar to that described by Eckel-Passow *et al.*[25] Specifically, the maximum number of $^{18}$O incorporations for a given peptide was determined by doubling

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the number of aspartic and glutamic acid residues within the peptide sequence and adding two for the C-terminal carboxyl group. Individual $^{18}O$ incorporations were independent events and their rates were peptide and site specific. Replacing an oxygen atom with $^{18}O$ resulted in a mass shift of +2 Da, which generated a mixed population of isotopomers for a given peptide with masses *m*, *m*+2,… *m*+2*N*, where *N* represents the total number of oxygen atoms in the peptide available for exchange with $^{18}O$ in the solvent. $A_0$, $A_1$, … $A_N$ corresponded to the fraction of the whole population represented by the subpopulation of molecules at mass *m*, *m* +2, … *m*+2*N*, respectively. With such a mixed population of molecules, one would expect the mass spectrum, then, to be a sum of spectra $A_0C_{+0} + A_{1-} C_{+2} \ldots + A_NC_{+2N}$, where $C_{+2N}$ represents the spectrum of the unlabeled peptide species shifted by 2*N* Da. We used a least-squares method to compute approximate values for the unknown parameters $A_N$ as follows. The profile data were extracted from the respective mass spectra in the proximity of the theoretical mass-to-charge ratio (*m*) of the targeted peptide, which corresponded to the mass ranging from (*m*-0.5) to (*m* + 2*N*+2) Da. The isotope cluster *C* for the unlabeled peptide was modeled using the method described by Kubinyi.[26] Then, an *N*+2-parameter model was fit to the measured contour data using least-squares regression. The model consisted of *N*+2 parameters $A_0$,… $A_N$, representing the inferred ion currents resulting from peptide subspecies that incorporate various numbers of $^{18}O$ atoms ranging from 0 to *N*, and *K*, a parameter that estimates the baseline of the spectrum. The model used was $y = A_0C + A_1 C_{+2} + \ldots + A_N C_{+2N} + K$. Thus, the modeled contour data consisted of a sum of copies of the theoretical cluster *C* duplicated at 0, +2, … and +2*N* Da, each with an unknown multiplier. The regression calculation yielded the values for $A_0$, … $A_N$, for which the model fit the measured contour data with least-square difference. The parameters *A* were then normalized by dividing by ($A_0$ + … + $A_N$). These normalized values $a_0$, … $a_N$ could be interpreted as representing the relative quantities of peptide molecules that incorporated the respective numbers of $^{18}O$ atoms.

In time-course experiments in which the samples were incubated with $^{18}O$ water for various times, the normalized $a_0$, … $a_N$ values were computed for each time point. Thus, the $a_0$ values measured at different time points represented a time series measuring the “decay” of the unlabeled peptide subspecies as more and more $^{18}O$ atoms were incorporated into the peptide molecules.

The procedure described above can also be used to analyze MS as well as MS/MS spectra. In the case of MS/MS data, it is feasible to calculate the relative $^{18}O$ incorporation for any of the observed fragment ions, allowing for the monitoring of individual $^{18}O$-incorporation reactions at a given acidic residue within the peptide or the peptide C terminus. The simplest example of such a measurement is the monitoring of a fragment ion containing a single labeling site, *e.g.*, *y*-series fragment ions containing the C-terminal peptide residue but no additional acidic residues, or *b*-series fragment ions containing a single acidic residue. To monitor $^{18}O$ incorporation into sites located between other labeling sites, it would be necessary to observe the incorporation into the two flanking fragment ions and then to solve for the incorporation into the site that was flanked.

### Absolute Quantification Using ALeO Strategy

Two versions (one labeled, one unlabeled) of the Cal Mix 1 peptide standard were mixed in ratios of 100:1, 50:1, 20:1, 10:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100 in $H_2O$ and analyzed by MALDI-TOF MS. The relative ratios (unlabeled/labeled) were calculated by determining the $^{16}O/^{18}O$ peak area ratios by linear regression as described above.

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### Calculation of the Overall Size and Acid Residue Content of Tryptic Peptides

All human protein entries in SwissProt were digested *in silico* by trypsin, allowing for no missed cleavage. The total number of peptides for a given length (6–30 amino acids) and the percentage that contained 0 to 4 glutamic or aspartic amino acid residues were calculated.

## RESULTS AND DISCUSSION

### Acid-Mediated $^{18}O$ Labeling of Peptides

At low pH, peptides exchange their carboxyl oxygen atoms with the aqueous solvent; this reaction can be used as a general method to introduce the stable isotope $^{18}O$ from $H_2{}^{18}O$. MALDI-TOF MS/MS is an ideal analytical platform for the acquisition of spectra generated by isotopically labeled peptides; after co-crystallization with matrix on the stainless steel plate, acid-catalyzed oxygen exchange stops owing to the lack of solvent, and the isotopic distribution state is frozen. Accordingly, we dissolved several model peptides in a solution containing 50% (v/v) $H_2{}^{18}O$ and used this MS approach to monitor their isotope incorporation over time (Figure 1). As expected, we observed transfer of up to two $^{18}O$ atoms for each carboxyl group, resulting in the incorporation of up to ten $^{18}O$ atoms in [Glu1]-fibrinopeptide B (3 glutamic acid residues, 1 aspartic acid residue and the C-terminal carboxyl group), two $^{18}O$ atoms in des-Arg1-bradykinin (C-terminal carboxyl group) and four $^{18}O$ atoms in neurotensin (1 glutamic acid residue and the C-terminal carboxyl group). The carboxyl exchange reaction under acidic conditions occurred more slowly than enzyme-catalyzed exchanges,[16] and the predicted binomial distribution at equilibrium was reached after ~11 days. MS/MS analyses revealed that the isotopic envelopes of the peptide products displayed the characteristic +2 Da shift, as expected. The width and shape of the envelope at equilibrium depended on the actual numbers of potential labeling sites (Figure 1D-I). In cases of peptides with multiple acidic residues (*e.g.*, [Glu1]-fibrinopeptide B, ACTH [18–39]), the magnitude of the mass shift was sufficient to prevent overlap with the natural isotopic envelope. Despite prolonged exposure to low pH, we did not observe significant degradation of peptides judging from the mass spectrometric data.

### MS/MS Analyses of $^{18}O$ Incorporation

When peptides with acidic side chains were selected for fragmentation by collision-induced dissociation, it was possible to monitor the sites of $^{18}O$ incorporation on the fragment ion level. Figure 2A shows the MALDI-TOF/TOF MS/MS spectrum of the $^{18}O$-labeled peptide [Glu1]-fibrinopeptide B and the time course of $^{18}O$ incorporation at individual sites. For example, the isotopic envelope of fragment ions *y*1 and *y*6 reflected the incorporation of $^{18}O$ atoms at the C terminus, whereas the *y*7 envelope revealed $^{18}O$ incorporation at the C terminus and a single glutamic acid residue (position 8; EGVNDNE**E**GFFSAR). For peptides of unknown sequence, $^{18}O$ incorporation as monitored by MS/MS can be used to determine the presence and position of acidic amino residues for *de novo* sequencing applications. By default, fragment ions belonging to the *y*-ion series can be identified by a characteristic 1:2:1 (unlabeled: singly labeled: doubly labeled) isotopic envelope. Within the *y*-ion series, sites of acidic residues are indicated by additional $^{18}O$ incorporations (*e.g.*, fragment ions *y*7, *y*8, *y*10). Furthermore, it is possible to measure $^{18}O$ incorporation rates at individual sites by monitoring individual fragment ions. Analysis of [Glu1]-fibrinopeptide B MS/MS data indicated that aspartic acid residues incorporated $^{18}O$ at a slower rate than the C-terminal or glutamic acid residues. To further investigate this phenomenon, we monitored acid-catalyzed $^{18}O$ incorporation into angiotensin 1 by MS/MS (Figure 2B). By tracing the isotopic envelope of the *b*6 and *y*7 ions of the sequence DRVYIHPFHL, we were able to simultaneously measure incorporation into the N-terminal aspartic acid residue and the C-terminal carboxyl group. Again, the incorporation at the aspartic acid was the slowest process and did not reach equilibrium during the 18 days of incubation.

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### The Rate of $^{18}O$ Incorporation Is Sequence and Residue Dependent

To further investigate the kinetics of acid-catalyzed $^{18}O$ incorporation into carboxyl groups, we used MS/MS to monitor individual sites across several synthetic peptides. Specifically, we measured the distribution of the isotopic envelope of the respective fragment ion species (Figure 3). Exchange rates, which were inferred by fitting exponential decay functions to the experimental data, are summarized in Table 1. The exchange reaction at the C terminus was faster for polar residues (*e.g.*, arginine of des-Arg1-bradykinin, [Glu1]-fibrinopeptide B) than for nonpolar residues (*e.g.*, leucine residues of angiotensin I, neurotensin). Interestingly, the side chain carboxyl groups of aspartic residues showed the slowest incorporation rate by far, as compared to the carboxyl groups of glutamic acid residues and the C termini.

### Applications of Acidic $^{18}O$ Labeling to Quantitative Proteomics

To demonstrate one possible application, we mixed $^{16}O$- and $^{18}O$-labeled peptide mixtures at various ratios covering 5 orders of magnitude. The titration curve (Figure 4) showed a high correlation of the theoretical and experimentally observed peptide ratios. In contrast to enzymatic reactions, which exclusively incorporate $^{18}O$ into C-terminal carboxyl groups, the number and positions of $^{18}O$ atoms in acid-catalyzed labeling were sequence dependent because additional exchange reactions occur in the side chains of glutamic and aspartic acid residues. To roughly estimate the occurrence of acidic side chains, we calculated the percentage of tryptic peptides of human proteins that contain 0 to 4 aspartic or glutamic acid residues as a function of peptide size (Figure 5). This analysis shows that about 50% of tryptic peptides have at least one acidic side chain, with glutamic acid residues being slightly more frequent. Accordingly, the isotopic envelopes of ALeO-labeled peptides, which have a greater span than the 2–4 Da typically observed in enzymatic $^{18}O$ labeling, do not have significant overlap with the isotopic envelopes that are due to natural abundance (e.g., Figure 1F,I).

## CONCLUSION

Acid-catalyzed incorporation of $^{18}O$ into peptides offers several advantages over enzymatic labeling. First, most peptides are labeled, including C-terminal protein fragments that are not recognized by trypsin or other proteinases. On average, peptides contain at least one additional acidic side chain residue (glutamic or aspartic acid) and can, therefore, incorporate multiple $^{18}O$ atoms with a resulting incremental increase of 4 Da per site. For some peptides, the number of acidic residues is large enough to fully separate the isotope envelope of unlabeled and labeled species. For peptides whose identity is unknown, the absence of information regarding sequence and potential incorporation sites may make quantitative comparisons of labeled and unlabeled peaks difficult and cause a significant increase in data complexity. However, incorporation of $^{18}O$ does not shift peptide elution time in a reversed-phase HPLC separation; thus, the separation step(s) can be exploited in the matching process.

Individual oxygen exchange rates are influenced by amino acid identity and environment: Exchange at aspartic acid residues is significantly slower than the one observed at glutamic acid residues or C-terminal carboxyl groups, which do not reach equilibrium even after prolonged incubation time. However, by capturing the labeling profile of individual peptides, it is possible to compensate computationally for underlabeling. Overall, acid-catalyzed back exchange of $^{18}O$ is a slow reaction and should minimally alter isotope ratios of enzymatically labeled peptides provided that samples are analyzed in a reasonable time frame or stored under properly controlled conditions.

In summary, we showed that acid-catalyzed $^{18}O$ labeling is an alternative to the chemical, enzymatic and synthetic isotopic labeling strategies that are commonly employed in quantitative proteomics-type approaches. Using a mixture of model peptides, we showed that

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linear quantitation is possible over 3 orders of magnitude. However, the absolute limit of detection will depend on the peptide and mass spectrometer used. This general labeling strategy has the added advantage of using an inexpensive reagent ($H_2{}^{18}O$). Furthermore, the fragment ion data of isotopically labeled side chains permit the determination of the number and location of acidic residues within a peptide. The reaction is simple, broadly applicable and quantitative, but owing to the long incubation time required, careful planning is necessary.

## Acknowledgments

This work was supported by U01 DE016274, U24 CA126477, the Sandler New Technology Fund and a UCSF CTSI-SOS Pilot Study Award.

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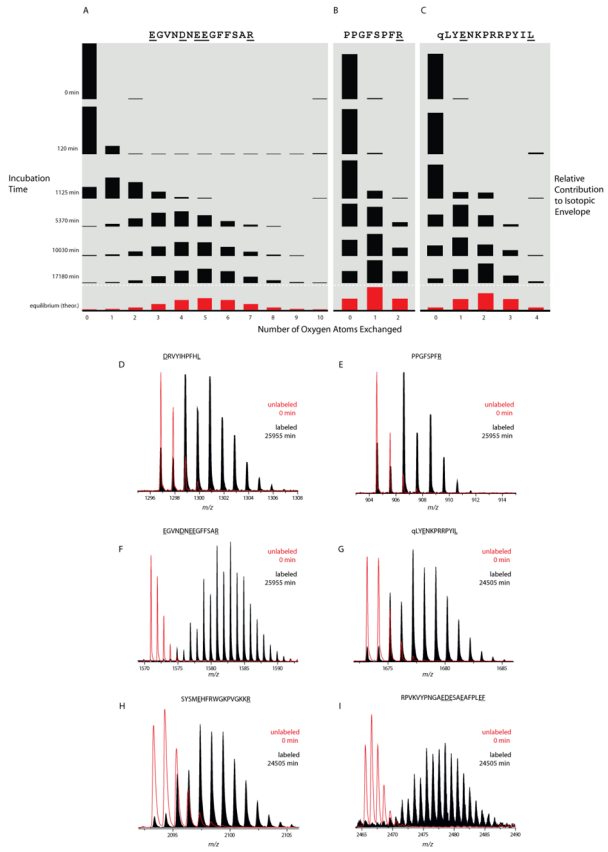


**Figure 1.**

At low pH, peptides exchange their carboxyl oxygen atoms with $H_2{}^{18}O$, resulting in the incorporation of two $^{18}O$ atoms into the terminal carboxyl group and two $^{18}O$ atoms into the side chain carboxyl groups of acidic amino acid residues (glutamic or aspartic acid). The progression of $^{18}O$ incorporation for three model peptides—(A) [Glu1]-fibrinopeptide B, (B) des-Arg1-bradykinin, (C) neurotensin—over 12 days is shown in the black bar graphs. Over time, $^{18}O$ incorporation reaches an equilibrium that is determined by the binomial distribution $B(n,p)$, where $n$ is the number of oxygens that can exchange and $p$ is the concentration of $H_2{}^{18}O$ in the solvent. $n$ is typically twice the number of acidic/C-terminal residues, as each such residue can exchange two oxygen atoms. Theoretical distributions given by $B(n,p)$ are shown in the red bar graphs. Isotopic envelopes of peptides are shown before (red trace) and after (black trace) incubation in dilute acid and an equal volume of 95% $H_2{}^{18}O$ for 18 days. (D) Angiotensin I, (E) des-Arg1-bradykinin, (F) [Glu1]-fibrinopeptide B, (G) neurotensin, (H) ACTH [1–17], (I) ACTH [18–39].

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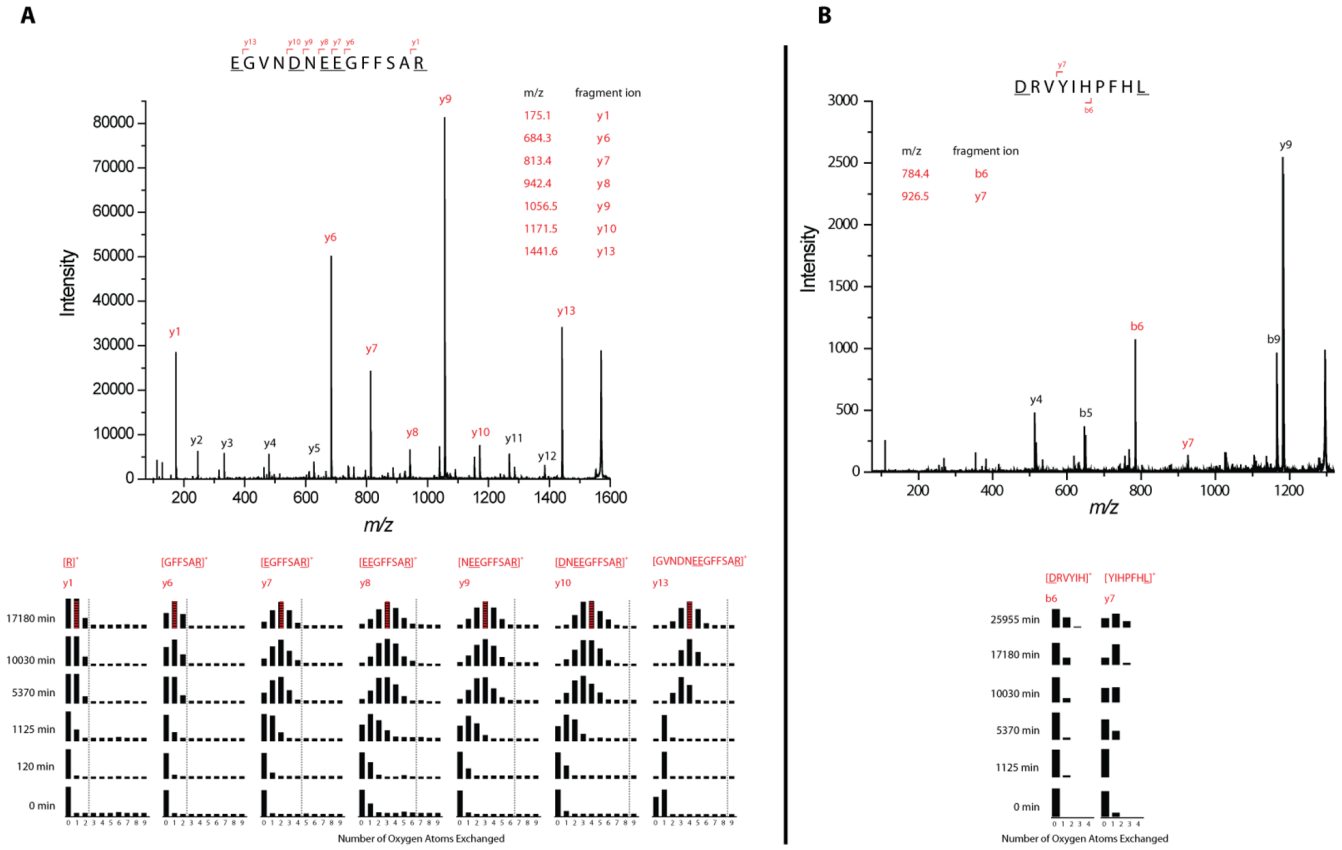


**Figure 2.**

(A) MALDI-TOF/TOF MS/MS spectrum of [Glu1]-fibrinopeptide B and isotopic envelopes of fragment ions (*y*1, *y*6, *y*7, *y*8, *y*9, *y*10 and *y*13) of MS/MS spectra of [Glu1]-fibrinopeptide B that were acquired at various times during the 18-day incubation. (B) MALDI-TOF MS/MS spectrum of angiotensin 1 and isotopic envelopes of fragment ions (*b*6, *y*7) of MS/MS spectra of angiotensin 1 that were acquired at various times during the 18-day incubation.

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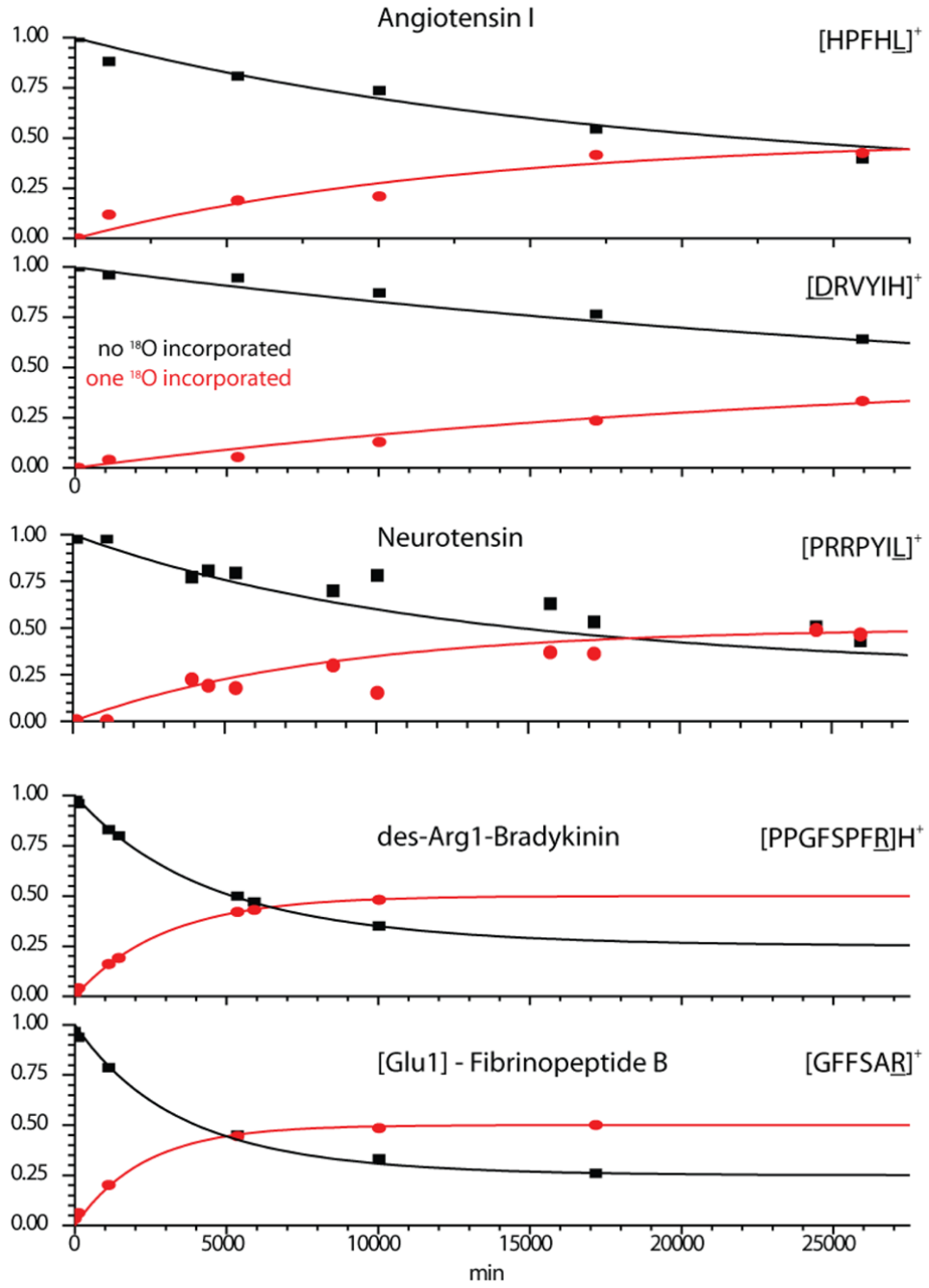


**Figure 3.**

MS/MS-based monitoring of $^{18}O$ incorporation at individual sites across several synthetic peptides. Black data points indicate the measured fraction of the respective fragment ions that had no $^{18}O$ incorporation into the selected residue; red data points indicate the measured fraction that had one $^{18}O$ incorporation (the fraction having two $^{18}O$ incorporations is not shown). At equilibrium, 25% of the molecules had no $^{18}O$ incorporations, 50% had one, and 25% had two. Curves follow the functional form given by exponential decay, which allows one to infer individual oxygen exchange rates. C-terminal $^{18}O$ incorporation was slower at leucine residues (angiotensin I, neurotensin) than at arginine residues (des-Arg1-bradykinin,

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[Glu1]-fibrinopeptide B). The slowest incorporation was typically observed at the side chain carboxyl groups of aspartic residues.

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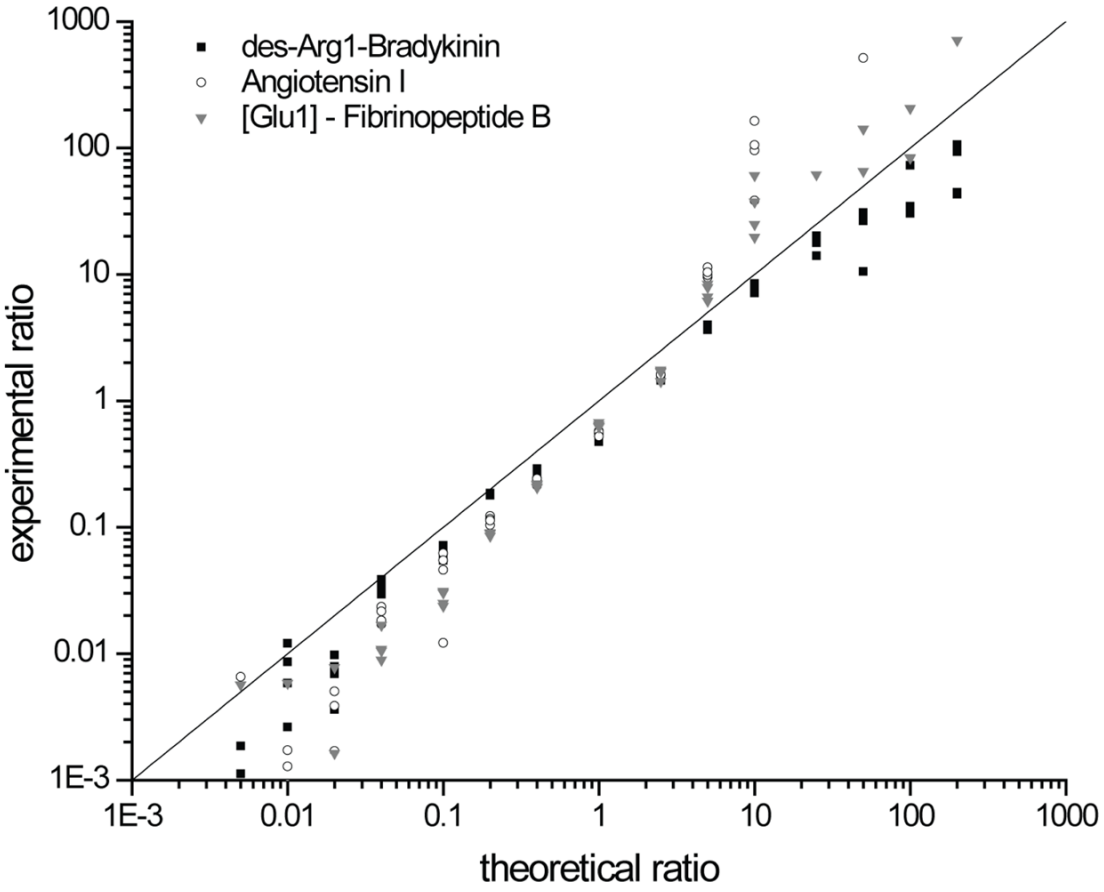


**Figure 4.**

Absolute quantification using ALeO peptides. Various amounts of peptides were diluted in $H_2O$, combined with known amounts of ALeO peptides labeled with $^{18}O$ under acidic conditions and analyzed by MALDI-TOF MS. The isotope envelope signals from the unlabeled/labeled peptides were integrated, and their ratio was used to estimate the amount of peptide added. Titration curves were obtained by plotting the theoretical ratio of unlabeled/labeled amounts of peptides against the experimentally observed ratios for peptides des-Arg1-bradykinin, angiotensin 1, and [Glu1]-fibrinopeptide B.

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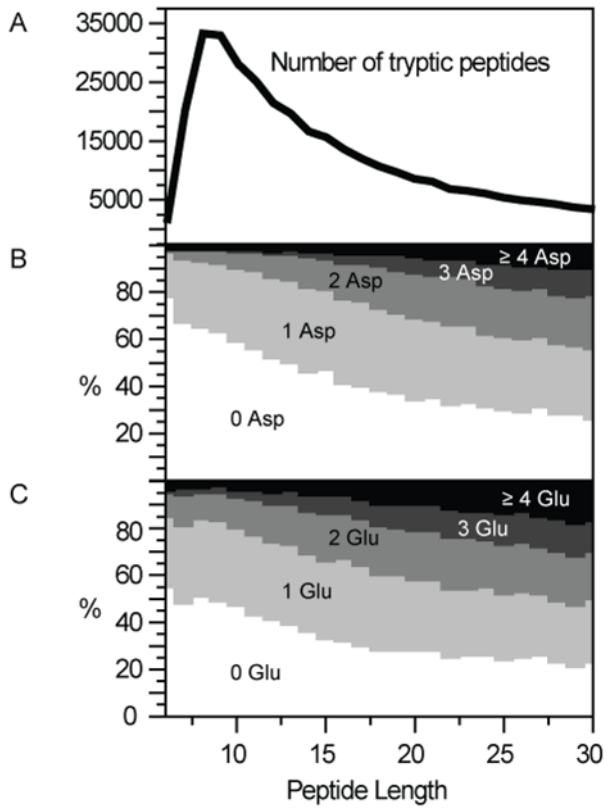


**Figure 5.**

(A) Size distribution of peptides generated from an *in silico* tryptic digest of all human proteins in SwissProt. The relative distribution of peptides that contain 0, 1, 2, 3, or more than 4 aspartic acid residues (B) or glutamic acid residues (C) is shown as a function of peptide length.

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**Table 1**

$^{18}O$ exchange rates (k) at individual sites across several synthetic peptides.

| *m/z* | sequence | peptide name | | site | k (× $10^{-3}$) |
|---|---|---|---|---|---|
| 904.5 | PPGFSPFR | des-Arg1-bradykinin | | C-terminal R | 0.17 |
| 1296.7 | DRVYIHPFHL | angiotensin I | *b*6 | N-terminal D | 0.02 |
| | | | *y*5 | C-terminal L | 0.04 |
| 1570.7 | EGVNDNEEGFFSAR | [Glu1]-fibrinopeptide B | *y*6 | C-terminal R | 0.22 |
| 1672.9 | qLYENKPRRPYIL | neurotensin | *y*7 | C-terminal L | 0.06 |

*Anal Chem*. Author manuscript; available in PMC 2010 June 28.