Use of ^{18}O Labels to Monitor Deamidation during Protein and Peptide Sample Processing

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Nonenzymatic deamidation of asparagine residues in proteins generates aspartyl (Asp) and isoaspartyl (isoAsp) residues via a succinimide intermediate in a neutral or basic environment. Electron capture dissociation (ECD) can differentiate and quantify the relative abundance of these isomeric products in the deamidated proteins. This method requires the proteins to be digested, usually by trypsin, into peptides that are amenable to ECD. ECD of these peptides can produce diagnostic ions for each isomer; the $c_{\cdot} + 58$ and $z - 57$ fragment ions for the isoAsp residue and the fragment ion $((M + nH)^{(n-1)+\cdot} - 60)$ corresponding to the side-chain loss from the Asp residue. However, deamidation can also occur as an artifact during sample preparation, particularly when using typical tryptic digestion protocols. With ^{18}O labeling, it is possible to differentiate deamidation occurring during trypsin digestion which causes a +3 Da ($^{18}O_1$ + 1D) mass shift from the pre-existing deamidation, which leads to a +1-Da mass shift. This paper demonstrates the use of ^{18}O labeling to monitor three rapidly deamidating peptides released from proteins (calmodulin, ribonuclease A, and lysozyme) during the time course of trypsin digestion processes, and shows that the fast (~4 h) trypsin digestion process generates no additional detectable peptide deamidations. (J Am Soc Mass Spectrom 2008, 19, 855–864) © 2008 American Society for Mass Spectrometry

Deamidation is a spontaneous nonenzymatic post-translational modification of proteins. It plays an important role in protein degradation and is postulated to function as a timer in aging [1–4]. Deamidation occurs on asparagine (Asn) and glutamine (Gln) residues and has been observed and characterized in a wide variety of proteins both in vivo and in vitro. The Asn deamidation takes place much more rapidly than that of Gln (up to ten times faster), because the formation of a six-membered cyclic imide is entropically less favorable [5, 6]. Upon deamidation, the asparaginyl residue is converted to a mixture of isoaspartyl (isoAsp) and aspartyl (Asp) residues.

Many factors can influence deamidation rates, such as protein sequence [5, 7, 8], secondary structure [9], local three-dimensional structure [10], pH, temperature, ionic strength, buffer ions, turnover of the protein, and other solution properties [11, 12]. In many cases, the deamidation rate is influenced by the primary structure. It is well known that Gly or Ser located on the C-terminal side of Asn greatly accelerates Asn deamidation. Ser and, to a lesser extent, Thr and Lys preceding Asn (at its N-terminal side) can also facilitate the Asn deamidation [11, 12]. The small side chains of Gly and Ser allow extensive conformational changes, whereas amino acids with branched, bulky, hydrophobic side chains or Asn/Gln located close to intramolecular disulfide bonds reduce the conformational flexibility necessary for the intermediate formation [5]. Furthermore, the secondary and tertiary structures usually determine whether deamidation actually occurs [6]. Stabilization of Asn residues by higher-order structures has been observed, which may result from conformational restrictions and the reduced nucleophilic reactivity of the backbone NH centers due to hydrogen bonding [9, 13]. Also, the structural change induced by one deamidation site may further influence the deamidation rates at other sites [5].

Deamidation may occur via two different pathways depending on the pH of the solution, which can affect the abundance of products. In an acidic solution (pH <5), deamidation proceeds via the acid-catalyzed pathway, where direct hydrolysis of the Asn residue side-chain amide group results in the formation of Asp as the only product. At pH >5, deamidation primarily occurs via a base-catalyzed pathway, in which the Asn residue is converted to a succinimide intermediate that can then hydrolyze rapidly to produce L-Asp and L-isoAsp, typically in a 1:3 ratio for random-coil peptides [8] (Scheme **1**). This reaction is reversible in aqueous solution. The deamidation rate reaches a minimum at approximately pH of 5. In basic conditions, the rate-limiting step is the intramolecular nucleophilic attack on the side-chain carbonyl by the nitrogen. In acidic conditions, the rate-limiting

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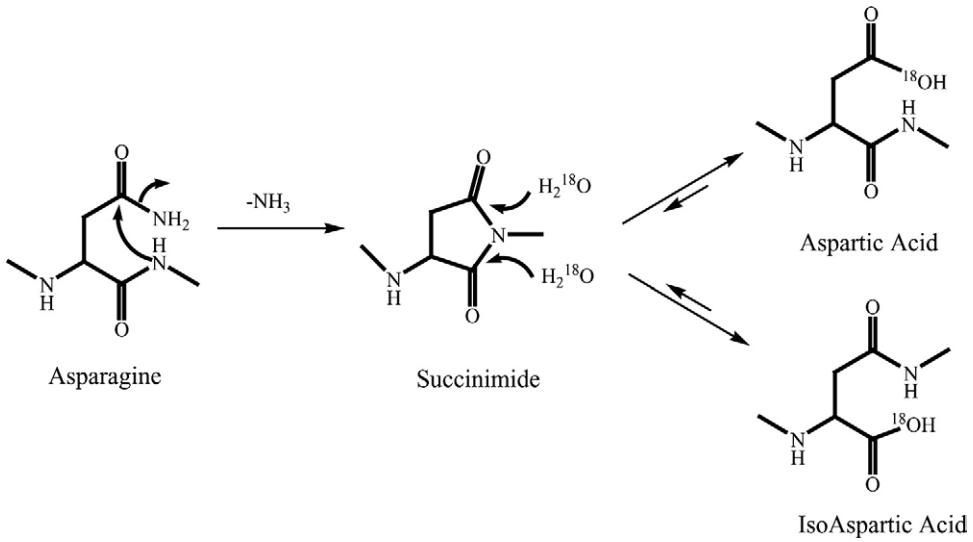

Scheme 1. The mechanism of the asparagine residue deamidation in $H_2{}^{18}O$.

step is the direct elimination of NH_2^- from the tetrahedral intermediate [1, 12, 14, 15].

Methods for detection of deamidation are usually based on the charge-sensitive techniques or mass spectrometry analysis. Deamidation introduces negative charges to a protein that shifts its isoelectric point (pI). It also results in a +0.984-Da mass shift from Asn to Asp/isoAsp, which can be detected and the extent measured using the mass defect and the envelope deconvolution method [16–19]. The advantages of these techniques are that protein samples can be introduced into a Fourier transform mass spectrometer without sample pretreatment (e.g., digestion, separation, absorption, and ionization) and that protein deamidation can be measured quantitatively without tandem mass spectrometry (MS/MS) analysis. Although determining the conversion of Asn to Asp/isoAsp is relatively straightforward, distinguishing the products Asp and isoAsp is more challenging. Several methods for the Asp/isoAsp differentiation exist, including NMR [20], HPLC [21], Edman-based sequencing [22], and antibody detection [23], although they all have certain limitations: the former three typically require relatively large quantity of proteins and the latter requires highly specific antibodies.

MS/MS methods can facilitate the detection and quantification of deamidation while only requiring femtomolar to picomolar amount of samples. In addition, they can also provide very specific information on the deamidation sites and help differentiate and quantify the ratio of the Asp and isoAsp products. In collisionally activated dissociation (CAD), the two isomers have different, identifiable side-chain fragmentation patterns for the N- and C-terminal ions [22, 24, 25]. In electron capture dissociation (ECD) [26–29] and electron-transfer dissociation (ETD) [30, 31], their fragmentations also produce different diagnostic ions: the $[(M + nH)^{(n-1)+\cdot} - 60]$ fragment ion for the detection of the Asp, and the $c^{\cdot} + 58$ and $z - 57$ ions for the detection and location of the isoAsp [32–35]. However, ECD of intact proteins is often inefficient because the number of available fragmentation channels is large and the resulting fragments frequently remain bound by noncovalent interactions and are thus undetectable. In most cases, the proteins need to be digested to form small peptides, most commonly by trypsin, before they are tested [36]. Trypsin digestion, which usually occurs at pH of about 8 and results in many small random coil peptides, is well known to accelerate base-mediated deamidation. Thus, it is important to distinguish the artifactual deamidations introduced during the sample processing steps from those that have occurred naturally to decrease the uncertainty about the biological relevance of any observed modifications.

One common method to monitor artificial, spontaneous reactions in mass spectrometry is to incorporate stable isotopic mass labels. For example, proteolytic ^{18}O labeling and hydrogen/deuterium exchange have been used extensively in studies of protein modification, such as comparative proteomics [37–40], quantitative proteomics [41], protein conformational studies [42], protein dynamics analysis [43], protein–ligand interactions [44], and protein aggregates research [45, 46]. Proteolytic ^{18}O labeling, in particular, has been used in the identification and quantification of succinimide [47] and citrullination [48] in proteins. The mechanism of protease catalyzed incorporation of ^{18}O into peptide fragments has been studied extensively [49, 50]. For trypsin, it usually results in up to two ^{18}O atoms incorporation into the peptide C-terminus, causing a mass shift of +2 Da per ^{18}O substitution. Since deamidation involves hydrolysis, sample preparation procedures performed in $H_2{}^{18}O$ will also offer the possibility of direct incorporation of a mass label during the reaction. If a peptide deamidates in $H_2{}^{18}O$, it not only gets the +0.984-Da mass shift from the deamidation reaction, but also incorporates an ^{18}O atom on the

newly formed Asp/isoAsp residue to get a total mass shift of +2.988 Da. This paper demonstrates the use of this simple mass labeling procedure for distinguishing the artificial deamidation that occurred during the tryptic digestion process, which leads to a +3 Da mass shift, from the preexisting deamidation in the sample that causes a +1-Da mass shift. Furthermore, fragment ions containing the artificial deamidation site will also be 2 Da heavier than those containing the natural deamidation site, providing additional insight into the origin and location of the deamidation. Time course studies monitoring the extent of deamidations in three rapidly deamidating peptides released from tryptic digestions of proteins were also performed, which showed no detectable artificial deamidation during fast (~4 h) tryptic digestions at 37 °C and pH of about 8.3.

Experimental

Materials

Sequencing grade trypsin was purchased from Roche Applied Science (Indianapolis, IN). HPLC grade $H_2{}^{16}O$ was purchased from Honeywell/Burdick & Jackson (Muskegon, MI). All other chemicals, proteins, and $H_2{}^{18}O$ (95% ^{18}O) were purchased from Sigma (St. Louis, MO).

Reduction and Alkylation

Ribonuclease A (RNase A) and lysozyme each have four disulfide bonds that were reduced and alkylated before analysis as described previously [33]. Briefly, proteins were reduced in 6 M urea/50 mM ammonium bicarbonate at pH 6, with tenfold molar excess of dithiothreitol over disulfide bonds and the resultant mixtures were incubated for 1 h at 37 °C. Iodoacetamide was then added in fivefold molar excess over cysteine residues and the resultant mixtures were incubated for 1 h in the dark at room temperature. The samples were dried and purified by home-made Poros 50 R1 packed solid-phase microextraction tip (Applied Biosystems, Foster City, CA). At each stage of sample processing, the sample was monitored by electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (ESI FTICR-MS). The reduced and alkylated RNAse A and lysozyme were then dried for use in the time course digestion.

^{18}O-Labeled Time Course Digestion

Calmodulin (20 μg), RNase A, and lysozyme (both 20 μg, denatured and purified) were each dissolved into 50 μL of 0.1 M ammonium bicarbonate buffer (pH 8.3) prepared using $H_2{}^{18}O$. Trypsin (1 μg) was added to each solution, yielding a wt/wt ratio of 1:20, purged with N_2 gas, sealed, and incubated at 37 °C. Aliquots were taken at 2, 4, 6, 8, and 24 h, with an additional aliquot taken at 48 h for RNAse A and lysozyme, due to their slower deamidation rates. Each aliquot was immediately frozen at −80 °C to stop the reaction and washed later by an equal volume of $H_2{}^{18}O$ twice to desalt before mass spectrometry analysis.

^{16}O-Labeled Controls

Control experiments were done by using the same proteins and methods described earlier in the ^{18}O-labeled time course digestion section, except that $H_2{}^{16}O$ was used in place of $H_2{}^{18}O$.

Calmodulin ^{18}O-Labeled Triplicate Experiments

The digestion of 30 μg of calmodulin powder with 1.5 μg dried trypsin in 75 μL of 0.1 M ammonium bicarbonate buffer (pH 8.3) was performed in triplicate and incubated at 37 °C. Sample aliquots were taken according to the time course, frozen, washed, and analyzed as described earlier.

Mass Spectrometry Analysis

ESI FTICR-MS was performed on a custom qQq-FT-MS instrument equipped with an external nanospray ion source [51, 52]. ECD experiments used an indirectly heated dispenser cathode placed about 3 cm from the cell [53–55]. The control samples were electrosprayed at approximately 10^{-5} M concentration in 49.5:49.5:1 of methanol:water:formic acid spray solution, whereas the ^{18}O-labeled samples were electrosprayed at the same concentration, but in 24.5:74.5:1 of methanol:water:formic acid spray solution. The different spray solution used was necessary to avoid ESI tip clogging, so that stable spray could be achieved for the ^{18}O-labeled samples. Multiply charged precursor ions were isolated using the front-end resolving quadrupole (Q1), followed by external accumulation in the CAD cell (Q2) before being transferred to the ICR cell and trapped by gated trapping. These ions were then irradiated with low-energy electrons (~0.2 eV) for time periods ranging from 50 to 120 ms to generate ECD fragments. A conventional FTMS excitation/detection sequence was used and the signal was averaged over 20 to 50 scans. All ECD spectra were internally calibrated and the peak lists are available in the supplementary data section.

Results and Discussion

Calmodulin Tryptic Peptide ESI FTMS Spectra (^{16}O versus ^{18}O)

Figure 1 shows the mass spectra of the triply charged calmodulin tryptic peptide (^{91}VFDKDG**NG**YISAAELR$_{106}$) ion in a tryptic digestion time course study over 24 h in $H_2{}^{16}O$ and $H_2{}^{18}O$. The inset shows the theoretical isotopic distribution of this peptide, which was calculated using the Yergey algorithm [59] as implemented in Isopro 3.0 (IonSource.com). Calmodulin is the primary cellular calcium receptor, which mediates

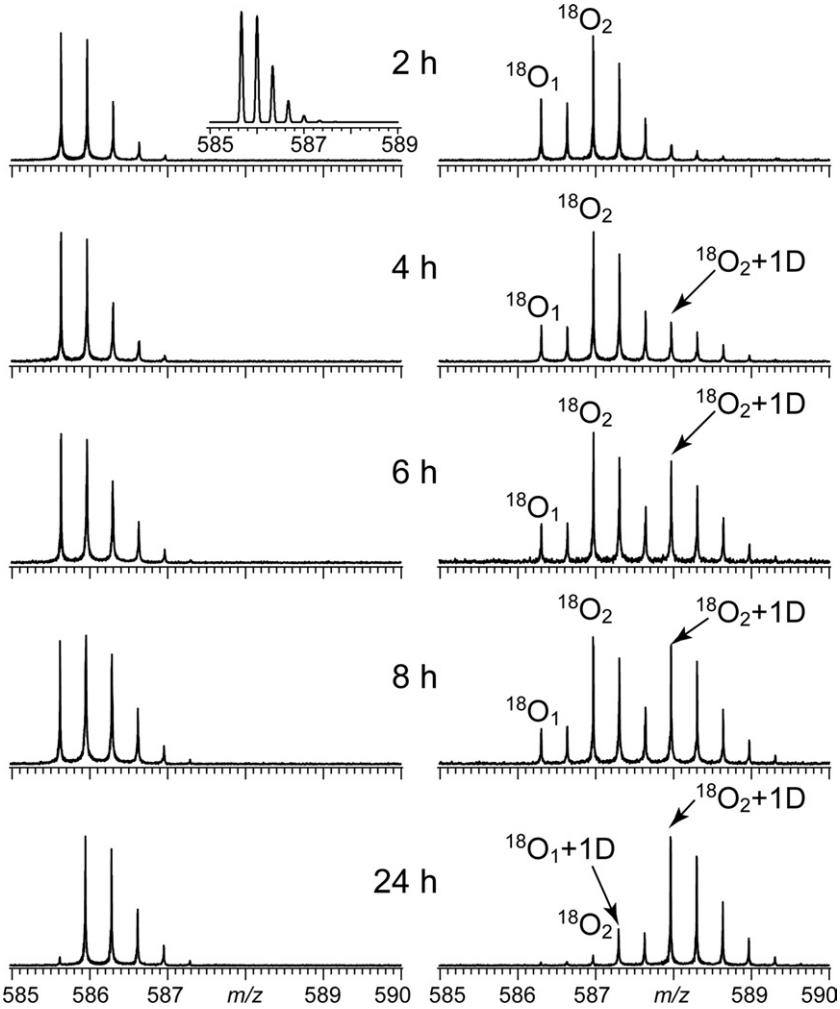

Figure 1. Mass spectra of the triply charged calmodulin peptide (^{91}VFDKDG**NG**YISAAELR$_{106}$) extracted at different times from the tryptic digestion solution in $H_2{}^{16}O$ (left column) and in $H_2{}^{18}O$ (right column). The inset shows the theoretical isotopic distribution of this peptide. The isotopic clusters were assigned in the ^{18}O spectra as $^{18}O_m$ + nD, where m and n indicate the number of ^{18}O incorporations and deamidations, respectively.

calcium concentration and regulates calcium-dependent enzymes [56]. A previous in vitro study found that the Asn97-Gly98 was the greatest contributor to the isoAsp formation in calmodulin [57].

In the control experiment performed in $H_2{}^{16}O$, there was an approximately 1-Da mass shift in the monoisotopic peak from the 2-h spectrum to the 24-h spectrum, indicating the onset of one deamidation in this peptide during the 24-h incubation period at 37 °C. The monoisotopic peak in the 2-h ^{18}O-labeled spectrum showed a mass difference about 2 Da from that in the control spectrum, resulting from one ^{18}O incorporation. The third isotopic peak had the highest intensity in the mass spectrum of this 2-h ^{18}O-labeled peptide, and its mass was shifted by an additional approximately 2 Da, corresponding to a second ^{18}O incorporation. The sixth isotopic peak became significant in the isotopic cluster in the 6-h spectrum, continued to increase in intensity in the 8-h spectrum, and became the most abundant peak in the 24-h spectrum. This peak showed a mass shift of about 7 Da from the monoisotopic peak in the corresponding control spectrum, with the addition of about 4 Da coming from the double ^{18}O substitution, and the remaining nearly 3 Da being the result of one deamidation occurring during the course of the tryptic digestion (Scheme **1**). During the deamidation, the $-NH_2$ group of the Asn residue was substituted by a hydroxyl group (—^{18}OH) via the hydrolysis of the succinimide intermediate in $H_2{}^{18}O$, leading to an increase in the mass of a peptide by 0.984 + 2.0043 = 2.988 Da.

These results show that although deamidation readily occurred during a 24-h tryptic digestion of this easily deamidating calmodulin peptide (91–106), a short trypsin digestion (~4 h) would not introduce detectable deamidations. The first two ^{18}O atoms were incorporated into this peptide's C-terminus, which will be further confirmed by the tandem MS experiment (see following text).

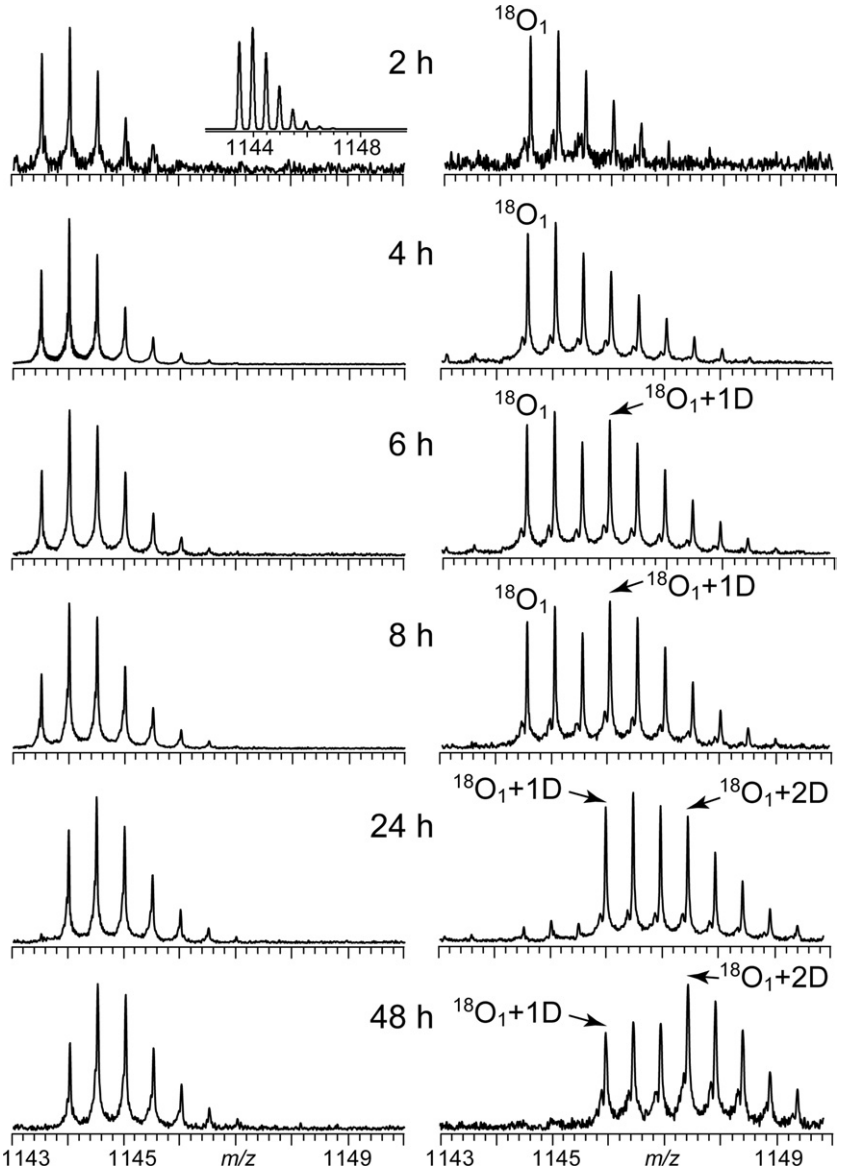

Figure 2. Mass spectra of the doubly charged RNase A peptide (^{67}**NG**Q**TN**C*YQSYSTMSITDC*R_{85}) extracted at different times from the tryptic digestion solution in $H_2{}^{16}O$ (left column) and in $H_2{}^{18}O$ (right column). The inset shows the theoretical isotopic distribution of this peptide. The isotopic clusters were assigned in the ^{18}O spectra in the same way as in Figure 1.

RNase A and Lysozyme Tryptic Peptides ESI FTMS Spectra (^{16}O versus ^{18}O)

Further experiments tested the trypsin digestion time course for tryptic peptides from RNase A (Figure 2) and lysozyme (Figure 3). Once again, the insets show the theoretical isotopic distributions of the corresponding peptides. The results from these time course studies are similar to the calmodulin tryptic peptide results in Figure 1, except that both of these peptides, which are also the most rapidly deamidating peptides from their respective proteins, contain two potential deamidation sites. Again, rapid digestion resulted in no detectable deamidation (<4 h for RNase A; <8 h for lysozyme) in these tryptic peptides. The RNase A tryptic peptide showed little incorporation of a second ^{18}O at the C-terminus, but demonstrated an abundant amount of double deamidation. The lysozyme tryptic peptide showed abundant double ^{18}O substitutions at the C-terminus, but only one deamidation over the 48-h time course.

The RNase A tryptic peptide (^{67}**NG**Q**TN**C*YQSY-STMSITDC*R_{85}) contains the fast deamidating **NG** sequence at the N-terminus, near the exposed and flexible part of the peptide, where C* denotes carbamidomethylated cysteine residue. There is little steric hindrance for the deamidation reaction, which may facilitate the succinimide intermediate formation and the hydrolysis in $H_2{}^{18}O$, thus making the deamidation

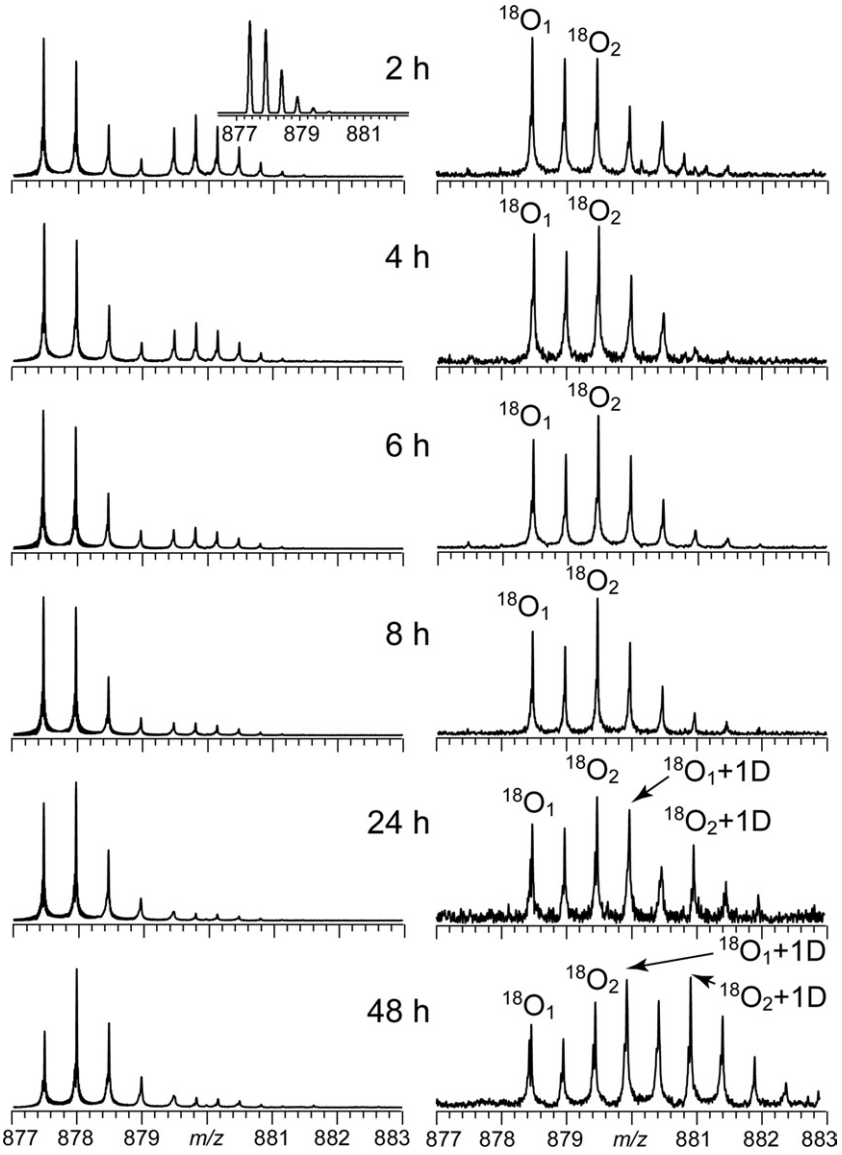

Figure 3. Mass spectra of the doubly charged lysozyme peptide (^{46}**NT**DGSTDYGILQI**NS**R$_{61}$) extracted at different times from the tryptic digestion solution in $H_2{}^{16}O$ (left column) and in $H_2{}^{18}O$ (right column). The inset shows the theoretical isotopic distribution of this peptide. The isotopic clusters were assigned in the ^{18}O spectra in the same way as in Figure 1.

process even faster than the second ^{18}O atom incorporation into the peptide C-terminus. The Thr located at the N-terminal side of the Asn71 residue is also known to accelerate deamidation [12], which may help to explain the second deamidation in this peptide. Moreover, deamidation in one site often causes protein conformational change and accelerates deamidation at a second site [5]. Since the **TN** is located near the N-terminal **NG**, a conformational change at the **NG** site will influence the **TN** local conformation, which may further contribute to the second site deamidation.

The lysozyme tryptic peptide (^{46}**NT**DGSTDYGILQI**NS**R$_{61}$) has the **NT** located at the N-terminus and the **NS** located near the C-terminus. The deamidation rate of this peptide was substantially slower than the calmodulin tryptic peptide and the RNase A tryptic peptide, evident from the control spectra. The one deamidation site of this lysozyme tryptic peptide is likely the Asn residue in the **NS** sequence for two reasons. First, a Ser residue following an Asn residue is known to promote Asn deamidation due to conformational flexibility and its polar side chain, which increases the deamidation rate compared to nonpolar groups [12]. Second, the **NS** is located near the C-terminus, which has little steric hindrance for nucleophilic attack. The assigned **NS** site deamidation also corroborates with the previous study, which found that the deamidation site at Asn59 of the lysozyme peptide Asp48 to Trp62 could be recognized by T cells, with a measured deamidation half-life of about 10 days in PBS buffer (pH 7.5) at 37 °C [1]. Unlike the **NG** and **TN** sites in the RNAse A peptide, the **NT** and **NS** sites in this lysozyme tryptic

peptide are distant from each other so the conformational change induced by deamidation at one site may not have a substantial influence on the local conformation of the other. Finally, in all spectra of this lysozyme peptide, the major isotopic cluster was followed by another triply charged isotopic cluster, which was an unrelated tryptic peptide fragment residues (74–97). However, as evident from the control spectra, this second peptide was much less abundant than the peptide of interest in the 24- and 48-h samples, where appreciable deamidations were observed. Furthermore, in the ^{18}O-labeled spectra of samples taken after at least 6 h of digestion, there was no evidence for a triply charged peptide in the mass range of interest, which would have shown up in between adjacent isotopic peaks of the doubly charged peptide of interest due to its different charge state. Thus, its interference to the quantification of deamidation extent was expected to be minimal and no correction was attempted in the following analysis.

Quantification of the Extent of the ^{18}O Incorporation and Deamidation during the Digestion Time Course

The isotopic distribution in each ^{18}O-labeled peptide spectrum was deconvolved using the least-square fitting method. The initial values were obtained step by step, from the lightest isotopic cluster to the heaviest one. Using the 8-h calmodulin peptide spectrum as an example, the abundance of the $^{18}O_1$ cluster was taken directly from the peak height of the first isotopic peak (A) and then its contribution (A + 2) to the third isotopic peak was calculated based on its theoretical isotopic distribution that, finally, was subtracted from the peak height of the third isotopic peak to give the abundance of the $^{18}O_2$ cluster. This procedure was repeated until the abundances of all isotopic clusters were obtained. These initial abundance values were normalized to give the percentages, which were adjusted iteratively until the resulting sum of all isotopic distributions from the fitting gave the least sum-of-square deviations from the experimental distribution. The final percentages of all isotopic clusters as a function of digestion time were plotted in Figure 4a, b, and c for the calmodulin, RNase A, and lysozyme peptides, respectively. To test the variance of these abundances, triplicate experiments of the ^{18}O-labeled calmodulin tryptic peptide were done using the same method described earlier and the results are plotted in Figure 4d, which correlate well with the results from the single time experiment shown in Figure 4a. After the deconvolution, it was easier to follow the deamidation process taking place during the tryptic digestion in $H_2{}^{18}O$. In general, the results agreed with those from the control experiment, showing that a short (4 h for the calmodulin RNAse A peptides and 8 h for the lysozyme peptide) tryptic digestion would not introduce detectable artificial deamidations.

ECD of ^{18}O-Labeled Calmodulin Tryptic Peptide at Time Points 2 and 24 h

Figure 5 shows the ECD spectra of the 2- and 24-h time point samples from Figure 1. ECD is based on the dissociative recombination of multiply charged polypeptide molecules with low-energy electrons [58]. It cleaves the N—C_α bond non-specifically and generates mostly c and z· ions, although its mechanism is still under debate [27–29].

The 2-h ECD spectrum showed c_3–c_{15} and z_2·–z_{14}· ions (Figure 5a). Neither c_6· + 58 nor z_{10} − 57 ion was observed in this spectrum to indicate the deamidation of Asn97 to isoAsp. Although the [M − 60] fragment ion was observed (Figure 5a, inset), it most likely arose from the side-chain loss of the two pre-existing Asp residues (Asp93 and Asp95), since all c ions that contain the Asn97 residue (c_7 to c_{15} ions) showed no +3-Da mass shift as one would expect if the Asn97 had deamidated in $H_2{}^{18}O$. Furthermore, none of the c ions (particularly the c_{15} ion) showed any mass shift compared with its normal counterpart produced in the control experiment (Supplementary Table 1), whereas all z· ions appeared to contain two adjacent isotopic clusters that were about 2 and 4 Da heavier than their ^{16}O counterparts. These results indicated that the first and second ^{18}O atoms were incorporated into the peptide's C-terminal carboxyl group, and no detectable deamidation occurred during the first 2 h of the tryptic digestion.

The 24-h ECD spectrum showed c_3–c_{15} ions and z_2·–z_{13}· ions (Figure 5b). As in the 2-h ECD spectrum, all z· ions contained two adjacent clusters of isotopic peaks, indicating one and two ^{18}O incorporations at the C-terminus. For z_{10}·–z_{13}· ions, both isotopic clusters were shifted in mass by an additional approximately 3 Da because they all included the Asn97 deamidation site. Although the c_3–c_6 ions showed no mass shifts, the mass of c_7–c_{15} ions increased by about 3 Da when compared to their ^{16}O counterparts, once again indicative of the deamidation that occurred at the Asn97 residue (Supplementary Table 2). Moreover, both complementary diagnostic ions for the Asn97 deamidation to isoAsp, the c_6· + 60 and z_{10} − 59 ions, were observed with nearly 1 ppm mass accuracy, with the 2-Da mass difference comparing with the normal diagnostic ions being the result of one ^{18}OH instead of one ^{16}OH substitution at the deamidation site (Figure 5b, insets). Like all other z· ions, the z_{10} − 59 ion also had two isotopic clusters corresponding to one and two ^{18}O atom incorporations at the C-terminus. The [M($^{18}O_2$ + 1D) − 60] ion corresponding to the Asp side-chain loss was observed, as expected because of the two pre-existing Asp residues in this peptide. The side-chain loss peak of the Asp97 (as the result of Asn97 deamidation) should instead give rise to an [M($^{18}O_2$ + 1D) − 62] ion because of the ^{18}OH substitution at the deamidation site. Although this ion was indeed observed, it might

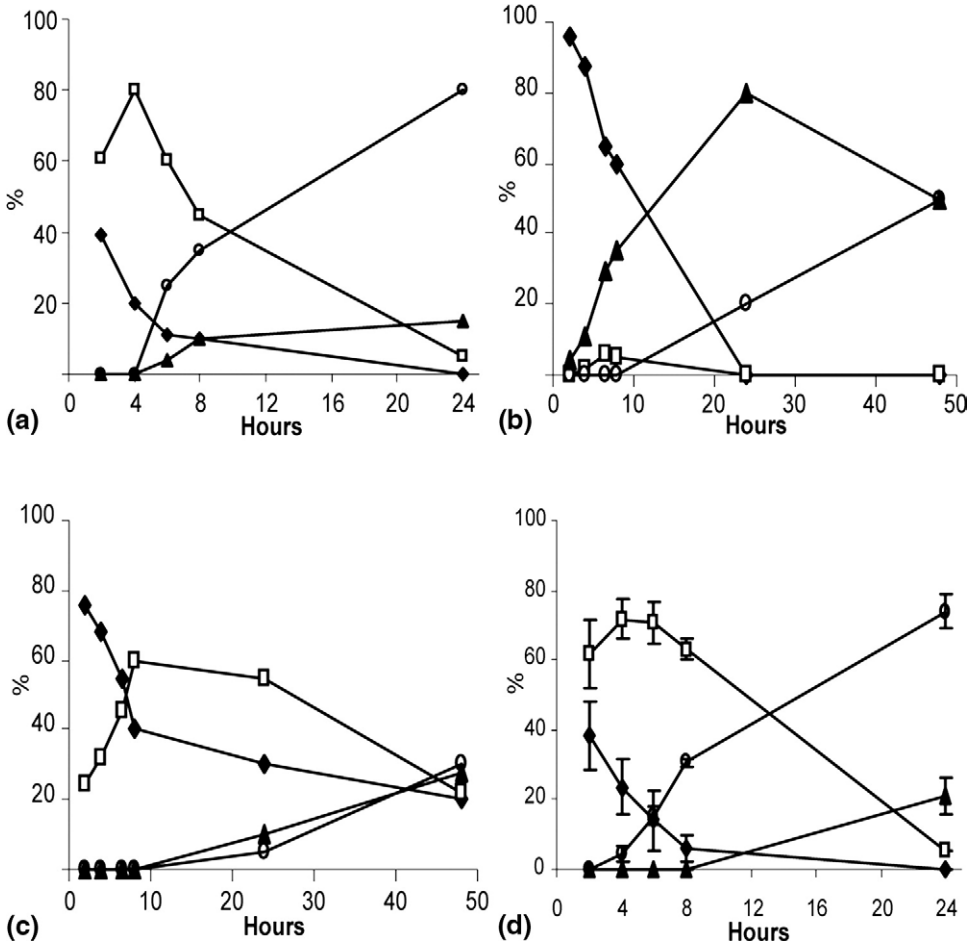

Figure 4. The percentage of each isotopic cluster at different digestion time as calculated using the least-squares method for the ^{18}O-labeled: (**a**) calmodulin tryptic peptide (91–106), (**b**) RNase A tryptic peptide (67–85), (**c**) lysozyme tryptic peptide (46–61), and (**d**) calmodulin tryptic peptide (91–106) triplicate experiments. (**a–d**) –◆–: $^{18}O_1$, -□-: $^{18}O_2$, –▲–: $^{18}O_1$ + 1D, –○–: $^{18}O_2$ + 1D, except in (**b**) $^{18}O_1$ + 2D.

also come from the normal Asp side-chain loss of the singly ^{18}O-labeled molecular ion that consisted of a significant portion of the total molecular ion population (Figure 4a, d); that is, it was actually an [M($^{18}O_1$ + 1D) − 60] ion. The best evidence for the Asp formation from the Asn97 deamidation was perhaps the observation of an isotopic peak at another approximately 2 Da lighter than the [M($^{18}O_1$ + 1D) − 60] peak (Figure 5b, inset, marked by the number sign, #), which could only be the [M($^{18}O_1$ + 1D) − 62] ion, since there was little [M($^{18}O_0$ + 1D)] in the 24-h sample (bottom right spectrum, Figure 1). The detailed peak lists for the ECD experiments are available in the supplementary data (Supplementary Tables 1 and 2).

Influencing Factors

There are several factors that can significantly affect the accuracy of the ^{18}O-labeling experiment. First, formic acid should not be used to halt the digestion reaction [49]. Apparently the acidic environment influenced the ^{18}O incorporation and the ^{18}O-labeling ratio (data not shown). Second, only $H_2{}^{18}O$ should be used in the desalting step. If $H_2{}^{16}O$ was instead used in this step, the ^{18}O atom that had already been incorporated into the tryptic peptides might be exchanged by the ^{16}O atom in the solvent, which would influence the accuracy of the ^{18}O incorporation measurement (data not shown). Centrifugation under vacuum appeared to further accelerate the back exchange with $H_2{}^{16}O$. Finally, excessive desalting should also be avoided. If $H_2{}^{18}O$ was used more than three times to wash out the salts, it could also distort the ^{18}O incorporation ratio (data not shown).

Conclusions

During trypsin digestion, the deamidation rate of the released peptides increases, which may introduce unwanted artificial deamidation that is of no biological relevance. This paper demonstrated the use of

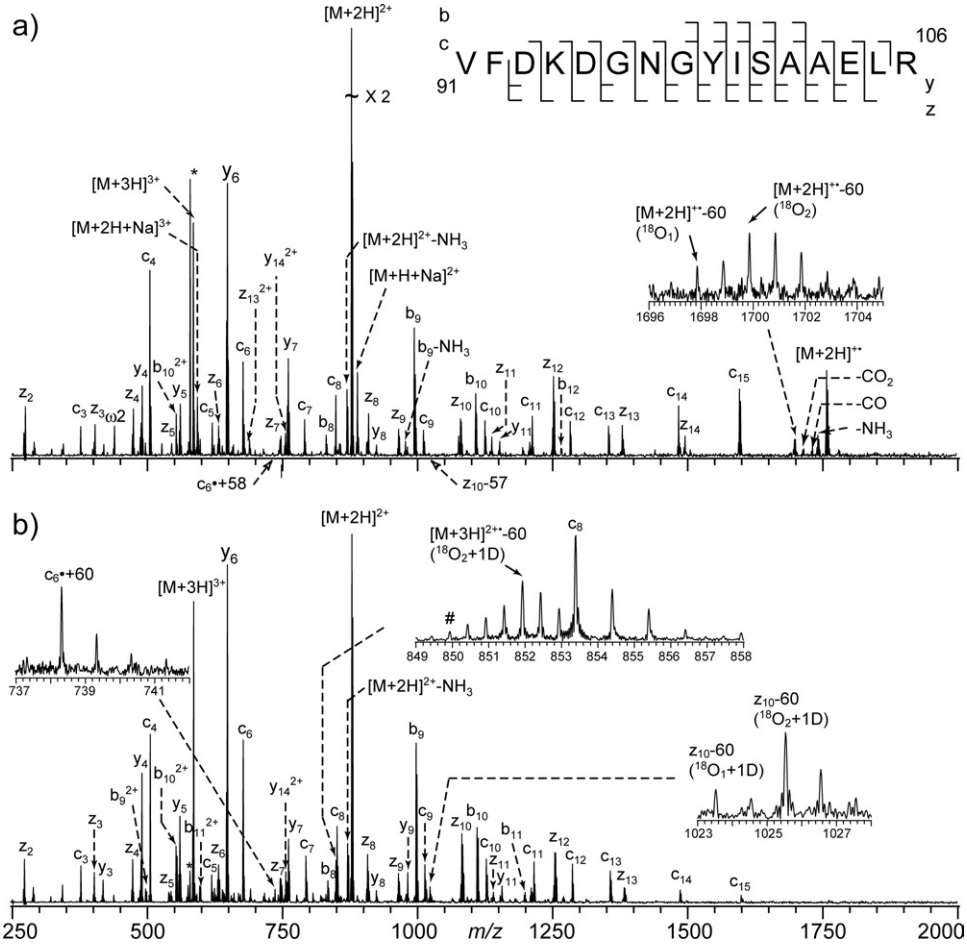

Figure 5. ECD spectra of the triply charged calmodulin tryptic peptide (91–106) labeled in $H_2{}^{18}O$ at different time points: (**a**) 2 h, (**b**) 24 h. The insets of (**a**) show the [M − 60] ion and the cleavage pattern. The insets of (**b**) show the isotopic distributions of the $c_6\cdot$ + 60 (left), [M − 60] (middle), and z_{10} − 59 (right) ions. *: electronic noise, ω2: harmonics.

$H_2{}^{18}O$ as a mass labeling reagent during the trypsin digestion process to distinguish between the deamidation that occurred during sample handling procedures (+3 Da mass increases) and the deamidation that was native to the sample (+1 Da). Tandem mass methods, such as ECD, can further help locate the sites of deamidation and ^{18}O incorporation. The use of ^{18}O, however, generated complex isotopic patterns that must be deconvolved first. In addition, care must be taken so that the isotopic distributions would not be distorted artificially during the digestion, centrifugation, and desalting steps. This study showed that fast trypsin digestion (~4 h) generally would not introduce additional detectable deamidations, even for the most rapidly deamidating peptides studied here. This result should increase the confidence in the quantification of Asn, Asp, and isoAsp residues, when samples need to be digested first to small peptides to facilitate the mass spectrometry analysis. Finally, this ^{18}O-labeling methodology can be easily extended to study the artificial deamidation taking place in other protein sample preparation procedures.

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