Deamidation: Differentiation of aspartyl from isoaspartyl products in peptides by electron capture dissociation

JASON J. COURNOYER, JASON L. PITTMAN, VERA B. IVLEVA, ERIC FALLOWS, LUCY WASKELL, CATHERINE E. COSTELLO, AND PETER B. O’CONNOR

1Department of Chemistry, Boston University, Boston, Massachusetts 02215, USA
2Mass Spectrometry Resource, Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118, USA
3University of Michigan, VA Medical Center, Ann Arbor, Michigan 48105

(RECEIVED August 20, 2004; FINAL REVISION October 27, 2004; ACCEPTED October 27, 2004)

Abstract

Deamidation of asparaginyl and isomerization of aspartyl residues in proteins proceed through a succinimide intermediate producing a mixture of aspartyl and isoaspartyl residues. Isoaspartic acid is an isomer of aspartic acid with the Cα incorporated into the backbone, thus increasing the length of the protein backbone by one methylene unit. This post-translation modification is suspected to contribute to the aging of proteins and to protein folding disorders such as Alzheimer’s disease, so that differentiating the two isomers becomes important. This manuscript reports that distinguishing aspartyl from isoaspartyl residues in peptides has been accomplished by electron capture dissociation (ECD) using a Fourier transform mass spectrometer (FTMS). Model peptides with aspartyl residues and their isoaspartyl analogs were examined and unique peaks corresponding to c₇₊58 and z₇−n−57 fragment ions (n, position of Asp; ℓ, total number of amino acids in the peptide) were found only in the spectra of the peptides with isoaspartyl residues. The proposed fragmentation mechanism involves cleavage of the Cα−Cβ backbone bond, therefore splitting the isoaspartyl residue between the two fragments. Also, a complementary feature observed specific to aspartyl residues was the neutral loss of the aspartic acid side chain from the charge reduced species. CAD spectra of the peptides from the same instrument demonstrated the improved method because previously published CAD methods rely on the comparison to the spectra of standards with aspartyl residues. The potential use of the top-down approach to detect and resolve products from the deamidation of asparaginyl and isomerization of aspartyl residues is discussed.

Keywords: Deamidation; protein aging; isoaspartic acid; mass spectrometry; electron capture dissociation

An important in vivo modification of proteins is the deamidation of asparaginyl and isomerization of aspartyl residues, through a common cyclic intermediate, to aspartyl or isoaspartyl residues (Clarke 1987; Radkiewicz et al. 1996; Robinson and Robinson 2001a; Robinson et al. 2001; Fig. 1), wherein the formation of the isoaspartyl residue is believed to be partly responsible for the inactivation, aggregation, and aging of proteins in tissue because the backbone is lengthened by one methylene unit (—CH₂—) (Roher et al. 1993; Kim et al. 1997; Aswad et al. 2000; Shimizu et al. 2000; Robinson and Robinson 2001a; Robinson et al. 2001; Ritz-Timme and Collins 2002; Reissner and Aswad 2003). The conversion from asparaginyl to aspartyl or isoaspartyl residues (monoisotopic masses 114.0429 and 115.0269 Da,
The common, and largely accepted, model for this non-enzymatic, post-translational modification is that it spontaneously occurs under physiological conditions through a succinimide intermediate whose rate is affected by both its amino acid sequence and three-dimensional structure. Asparaginyl residues undergo ammonia loss (deamidation) to form the succinimide intermediate while aspartyl residues experience mass change. The deamidation rate increases 10-fold when replaced with glycine (Robinson and Robinson 2001a). The three-dimensional structure affects both succinimide formation as well as the final products. A dihedral $\psi$ angle of $-120^\circ$ and $\chi$ angle of $120^\circ$ offers the most favorable position (distance of 1.89 Å) for nucleophilic attack of the peptide nitrogen on the $\gamma$-carbonyl to form the succinimide intermediate (Clarke 1987). However, such configurations are uncommon in proteins, suggesting that protein tertiary structure mitigates this modification by placing these residues where succinimide formation is hindered. For example, deamidation of an asparaginyl residue within the $\alpha$-helix of rabbit muscle aldolase experienced a 15-fold slower half-life than that of its linear tetrapeptide model (Robinson and Robinson 2001a). The addition of water opening the succinimide ring to form either an aspartyl or isoaspartyl residue (pathways 1 and 2 of Fig. 1, respectively) depends on which of the amide bonds of the intermediate is hydrolyzed. This choice is partially governed by the three-dimensional structure of the peptide or protein (Clarke 1987; Kossiakoff 1988; Robinson and Robinson 2001a,b; Robinson et al. 2001; Athiner et al. 2002), which effects the accessibility of each of the bonds to attack by water. Typically, >60% of the products resulting from succinimide hydrolysis are isoaspartyl residues (Athiner et al. 2002; Reissner and Aswad 2003).

Upon hydrolysis to the isoaspartyl form, the aspartyl or asparaginyl position in the protein backbone is lengthened by one methylene unit, resulting in a modification that has been correlated with protein inactivation and misfolding and whose physiological importance has been illustrated in experiments involving the repair enzyme L-isoaspartyl-O-methyltransferase (PIMT) (Roher et al. 1993; Kim et al. 1997; Aswad et al. 2000; Shimizu et al. 2000; Ritz-Timme and Collins 2002; Reissner and Aswad 2003). PIMT is a highly conserved enzyme that uses S-adenosyl-L-methionine (AdoMet) as a methyl donor to convert isoaspartyl to aspartyl residues, partially restoring the function of enzymes affected by deamidation or isomerization (Johnson et al. 1987). The importance of the PIMT enzyme and the negative effects of the isoaspartyl modification have been shown in several experiments such as seizures and early death experienced by PIMT knockout mice (Kim et al. 1997) and the extension of life by 30% for Drosophila with overexpression of PIMT (Chavous et al. 2001). This experimental evidence has led researchers to suspect aspartate isomerization as a possible contributor to Alzheimer’s disease since the isoaspartyl modification alters the fundamental structure of the protein backbone and that the highest level of PIMT activity was located in the brain. The cerebral plaque samples of Alzheimer patients have shown evidence of aspartyls isomerized to isoaspartyls at residue positions 1, 7, and 23 of the $\beta$-amyloid peptide, where the isoaspartyl content was the highest at position D$_7$ (75%) (Roher et al. 1993). Other affected proteins include those long-lived proteins important for the structure of tooth, skin, lens of the eye, or the matrix of bone and teeth. The level of PIMT is correlated with the age of the patients, and the cerebral cortex shows the highest level of PIMT activity located in the brain. The cerebral plaque samples of Alzheimer patients have shown evidence of isomerization as a possible contributor to Alzheimer’s disease.
eye, and bone in humans (Ritz-Timme and Collins 2002). Some proteins such as HMAP (high mass methyl-accepting protein), found in the mammalian brain, contain a high percentage of isoaspartyl residues which is suspected to modulate its activity, and may present an example of a beneficial modification (Reissner and Aswad 2003). Whatever role is ultimately attributed to this modification, developing an analytical technique to reliably and easily differentiate aspartyl from isoaspartyl residues is critical to the biological assessment of how certain proteins aggregate, age, and regulate their own activity.

Since the initial publication in 1998 (Zubarev et al. 1998), electron capture dissociation (ECD) has helped to drive mass spectrometry (MS) to the forefront of proteomics and other areas related to the structural analysis of important biological molecules. ECD is used in conjunction with Fourier transform mass spectrometry (FT-ICR-MS or FTMS) (Marshall and Verdun 1990) where multiply charged positive ions ([M+nH]n+, n is number of H+) are trapped in the ion cyclotron resonance (ICR) cell and irradiated with low energy electrons to form odd (OE+) and even (EE+) electron fragment ions (Fig. 2). More recently, a related technique, electron transfer dissociation (ETD), has demonstrated similar fragmentation on the far more ubiquitous ion trap instruments (Syka et al. 2004). Analysis by ECD requires a charge state of at least 2+ for the precursor ion so that a positive charge remains for detection of fragment ions. ECD is unique because it uses free radical chemistry to cleave the peptide backbone (Leymarie et al. 2003) creating c and z· ions as opposed to b and y ions (Fig. 3) that are typical of fragmentation techniques such as CAD (collisionally activated dissociation) (Gauthier et al. 1991; Senko et al. 1994). For ECD, capture of a 0.2 eV electron by the precursor ion produce fragmentation lending to c and z ions while capture of 9.0 eV electrons (hot ECD) produces additional ions such as b, y, a, v, w, and d fragment ions due to ion-electron inelastic collisions (Fig. 3; Kjeldsen et al. 2002). The fragmentation mechanism of ECD (Fig. 2) involves capture of an electron by the positively charged species, thus neutralizing one charge site and producing enough energy to initiate the homolytic cleavage of the N—Cα bond (either c· and z or c and z· fragments) of a peptide in the vicinity of capture. This type of cleavage is useful for peptide and protein sequence analysis (Tsymb et al. 2004) because fragmentation occurs almost without regard to amino acid composition (with the exception of proline) (Leymarie et al. 2003), causing a more uniform cleavage pattern. This type of fragmentation is contrary to standard MS/MS methods such as CAD (Kruger et al. 1999) where collisions with a neutral gas excite the many vibrational modes of the peptide or protein producing spectra dominated by fragments resulting from the cleavages of the most labile bonds (such as the peptide bonds adjacent to proline and aspartic acid) (Gu et al. 2000), making complete sequencing difficult. In addition to sequencing, ECD has been shown to be useful for the analysis of post-translational modifications of peptides and proteins (Kelleher et al. 1999b; Mirgorodskaya et al. 1999; Stensballe et al. 2000; Hakansson et al. 2001; Shi et al. 2001), distinguishing isomeric (Kjeldsen et al. 2003) and enantiomeric structures (Adams et al. 2004) and revealing gas phase protein conformation (Horn et al. 2001; Breuker et al. 2002; Oh et al. 2002). For example, ECD can preserve labile protein modifications such as phosphorylation, which may account for ~30% of the ~300 known modifications (Qian et al. 2003). Also, side-chain cleavage by ECD has been used to help define the amino acid residues (Cooper et al. 2002; Haselmann et al. 2002; Leymarie et al. 2003) in peptides and proteins including differentiat-

![Figure 2](image)

**Figure 2.** ECD fragmentation mechanism. Upon electron capture at the positively charged site, OH bond formation with a carbonyl oxygen initiates the cleavage of the Cα—N bond producing (A) c and z· ions or (B) c· and z ions.
ing between isoleucine and leucine (Kjeldsen et al. 2003). With all these attributes, ECD is a useful technique not only for routine sequencing but also for studying biological molecules with structural ambiguities that have previously been difficult to discern by typical mass spectrometric techniques.

Aspartyl or isoaspartyl residues, resulting from the deamidation of asparaginyl and isomerization of aspartyl residues, are difficult to differentiate by mass spectrometric means because their masses are essentially identical (Schindler et al. 1996; Gonzalez et al. 2000; Lehmann et al. 2000; Luu et al. 2004). Presently, the most efficient way to detect the modification is by immunological methods (Reissner and Aswad 2003), PIMT assays with labeled AdoMet (Roher et al. 1993; Aswad et al. 2000) and Edman degradation (Roher et al. 1993). Other analytical techniques such as HPLC and NMR both have their drawbacks. For example, HPLC can separate the two isoforms (Roher et al. 1993) but they remain indistinguishable from one another, and NMR requires more sample than can realistically be expected from most biological experiments. Several MS methods have been developed to indirectly detect the presence of isoaspartyl residues in peptides based on the relative abundance of fragment ions (Lehmann et al. 2000), modifications to isoaspartyl terminal fragments (Schindler et al. 1996; Gonzalez et al. 2000), and the specific sequence of neutral losses experienced by isoaspartyl residues (Luu et al. 2004). Although useful for their particular studies, these mass spectrometric methods require control samples in order to clearly differentiate between aspartyl and isoaspartyl residues in peptides, and therefore may yield ambiguous results when applied to real biological samples for which there is no control sample. Data presented in this paper demonstrates that direct differentiation of aspartyl from isoaspartyl residues in peptides can be accomplished by ECD. Under ECD conditions, peptides with aspartyl residues underwent neutral loss of the aspartic acid side chains that was not observed in the isoaspartyl analogues. Also, the data yielded the observation of a unique fragmentation pattern that accounts for peaks observed only in the ECD spectra of peptides with isoaspartyl residues that do not appear in the spectra of the peptides with aspartyl residues. These fragments can be used to unambiguously determine the presence of isoaspartyl residues in biological samples without the need for control samples.

**Results**

The peptides BUSM1–4 (Table 1) were analyzed by both ECD (Fig. 4) and CAD (data not shown); BUSM1 and 3 contain aspartyl residues while BUSM2 and 4 are their analogs that contain isoaspartyl residues. All peptides from
Table 1. **Peptides analyzed by ECD**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Monoisotopic mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUSM-1</td>
<td>RAAAGAD$_2$GD$_2$GAGAD$_2$AR</td>
<td>1400.6393</td>
</tr>
<tr>
<td>BUSM-2</td>
<td>RAAGAD$_2$GD$_2$GAGAD$_2$AR</td>
<td>1400.6393</td>
</tr>
<tr>
<td>BUSM-3</td>
<td>RAG$_3$AD$_2$GD$_2$D$_2$:AD$_2$GD$_2$:AD$_2$AG$_2$:AAR</td>
<td>1452.6785</td>
</tr>
<tr>
<td>BUSM-4</td>
<td>RAGAD$_2$GD$_2$:AD$_2$GD$_2$:AGAAR</td>
<td>1444.6291</td>
</tr>
<tr>
<td>BUSM-5</td>
<td>RAAAD$_2$:FAAR</td>
<td>947.4937</td>
</tr>
<tr>
<td>BUSM-6</td>
<td>RAAAD$_2$:FAAR</td>
<td>947.4937</td>
</tr>
</tbody>
</table>

Table 1 were designed to facilitate the detection of all possible fragment ions by incorporating arginine at both the C- and N-termini so that both ECD and CAD analysis would result in a high abundance of both N- (i.e., a, b, c) and C- (i.e., y, z) terminal fragments. The peptides were completely sequenced by ECD at 0.2 eV based on c and z· ions with the radical driven fragmentation mechanism shown in Figure 2 (all c ions down to the Nyquist limit, ~215 Da, were present at 0.2 eV). Figure 3 shows the expected structure of these ions along with additional z, a, v, d, b, and y ions that were observed upon irradiation with 9.0 eV electrons. No x ions were observed. Likewise, the CAD data (BUSM1–3, 23 eV and BUSM4, 17 eV) revealed the complete sequence based on b and y ions. The presence of b and y ions in the 0.2 eV ECD spectra are most likely from collisions with the gas pulse used preceding detection while these ions in the 9.0 eV ECD spectra are from both the

![Figure 4](image-url)

Figure 4. ECD spectra of the six peptides from Table 1 (BUSM1 and 2 at 9 eV; BUSM3–6 at 0.2–0.25 eV). * signifies c$_n$+58 and z$_{i=57}$ for BUSM2, 4 and 6, and † indicates electrical noise.
cooling gas and collisions with high energy electrons. For the ECD data, both c- (odd electron, OE\(^+\)) and z- (even electron, EE\(^+\)) ions were detected in abundances comparable to their corresponding hydrogen-transfer fragment ions, c and z- (Fig. 2), respectively, a trend previously observed for proteins and peptides (Zubarev et al. 2000). The neutral loss of C\(_2\)H\(_4\)O\(_2\), corresponding to a loss of the aspartic acid side chain (Haselmann et al. 2002), from (M+ 2H\(^+\))\(^+\) was observed for all of the peptides containing aspartyl residues and was not observed in the peptides with isoaspartyl residues. Also, the neutral losses of CH\(_5\)N\(_3\) and C\(_4\)H\(_9\)N\(_3\) from the arginine side chains were observed in the ECD spectra of these peptides. Unexpected ions corresponding to c\(_n\)+58 and z\(_{n-57}\) ions (\(n\) is the position of aspartyl/isoaspartyl from the N terminus and \(\ell\) is the total number of amino acids in the peptide) were observed in BUSM2 and 4 but not in BUSM1 and 3, therefore making these unique to the isoaspartyl residues (marked with asterisks for BUSM2 and BUSM6 in Fig. 4). The ion corresponding to z\(_{n-57}\) for BUSM2 was not detected. Although these ions are unresolved from other c and z ions for BUSM3 and 4, making it difficult to interpret the data, their presence is clearly indicated by changes in relative abundances of peaks when spectra of the two peptides are compared. Neither the side chain losses nor the c\(_n\)+58 and z\(_{n-57}\) fragment ions were observed in the spectra from CAD analysis (BUSM1–4).

Analysis by ECD at 0.2 eV of BUSM5 and 6 showed complete coverage based on c and z- ions (Fig. 4). Only the z\(_{n-57}\) ion was observed for the isoaspartyl peptide (BUSM6). Also, the neutral loss of C\(_2\)H\(_4\)O\(_2\) from the aspartic acid side chain was observed for BUSM5 but not for BUSM6.

Discussion

The proposed fragmentation mechanism leading to the loss of C\(_2\)H\(_4\)O\(_2\) (60.0211 Da) from (M+ 2H\(^+\))\(^+\) is shown in the top portion of Figure 5, and the resulting MS peaks are shown below in the shaded regions for Asp (top) and isoAsp (bottom). Upon electron capture by the positively charged arginine side chain, an O—H bond is formed between the neutralized proton (H\(^-\)) and the carbonyl oxygen of the aspartic acid side chain. This bond formation promotes the cleavage of the C\(_\alpha\)—C\(_\beta\) bond of the aspartic acid residue, resulting in the loss of acetic acid and leaving behind what is essentially a glycine residue with a C\(_\alpha\)-radical. This C\(_\alpha\)-radical amino acid has been reported to be the most stable of all C\(_\alpha\)-radical amino acid residues (Easton 1991; Rauk et al. 1997). Its stability is reflected in its substantial abundance in

![Figure 5](image-url)

**Figure 5.** (Top) Proposed fragmentation scheme for the neutral loss of C\(_2\)H\(_4\)O\(_2\) from Asp residues. (Bottom) Comparison of ECD spectra of the three peptides (Asp versions, top row, and isoAsp versions, bottom row) at 0.2 eV. ‡ indicates the loss of CH\(_5\)N\(_3\) from arginine side chain, † indicates loss of NH\(_3\) and CO\(_2\), dotted lines represent remaining residues, and shaded regions indicate —C\(_2\)H\(_4\)O\(_2\) loss peak from Asp.
all of the ECD spectra for peptides with aspartyl residues. This type of cleavage has been previously reported but without a proposed fragmentation mechanism (Haselmann et al. 2002). All three aspartyl versions of the peptides (BUSM1, 3, and 5) experienced the loss of C₃H₄O₂ (bottom portion of Fig. 5, top row), which was not observed in the spectra for isoaspartyl peptides (bottom row of Fig. 5). A peak corresponding to the possible loss of C₃H₄O₂ in the isoaspartyl peptides could be observed in these spectra, but this signal is most likely due to the A+1 isotope, the second isotopic peak (i.e., ¹³C₁) (McLafferty and Turecek 1993), of the peak corresponding to the loss of both CO₂ and NH₃ from these peptides (loss of 61.0164 Da). The A+1 isotope peak should have 50% or greater abundance relative to its adjacent monoisotopic peak; its appearance in the spectra with an abundance appropriate for the A+1 ion argues against the interpretation as the loss of C₃H₄O₂ from the isoaspartyl peptides.

Isoaspartyl residues generated peaks corresponding to cₙ⁺58 and zₙ⁻57 (numbers represent Daltons) that were observed for BUSM2 and not for BUSM1, as indicated in the bottom of Figures 6 and 7. Again, the relevant monoisotopic peaks are highlighted in the mass spectra for the Asp (BUSM1, left) and isoAsp (BUSM2, right) containing peptides along with the theoretical monoisotopic masses of these fragment ions (center). These ions are present in the ECD spectra for the isoAsp peptides at 0.2 eV and with increasing abundance at 9 eV but completely absent at both energies for the Asp peptide. The proposed mechanism for the formation of these ions is shown in the top portion of Figures 6 and 7 illustrating the homolytic cleavage of isoaspartyl residue at the N—Cβ bond. Although the usual Roepstorff nomenclature breaks down for isoaspartic acid, because the fragments result from ECD, the c and z notation is used to describe them. Upon electron capture, the electron neutralizes the protonation site forming an O—H bond with the backbone carbonyl oxygen adjacent to the isoaspartyl residue. The formation of the O—H bond induces an electronic rearrangement in which a double bond between the Cβ and carbon of the reduced carbonyl group is formed (zₙ⁻57). This results in the cleavage of the Cα—Cβ bond, now part of the backbone for isoaspartyl residues, and a complementary radical product (cₙ⁺58).

Although in lower abundance compared to cₙ and zₙ⁻57 ions, the cₙ⁺58 and zₙ⁻57 fragment ions are quite stable, as is reflected in their significant abundances. The zₙ⁻57 ion, similar to one of the products from a McLafferty rearrangement (McLafferty and Turecek 1993; Figs. 6, 7),

Figure 6. (Top) Proposed fragmentation scheme for the formation of cₙ⁺58 ions from ECD of peptides with isoAsp residues. (Bottom) Expanded views of ECD spectra of BUSM1 and BUSM2 at 0.2 and 9.0 eV. # indicates an interfering secondary fragment ion due to loss of NH₃ and CHON from y₁₄ (found in the spectra of both peptides), dotted lines represent remaining residues, and shaded regions indicate the monoisotopic peak of interest.
is stabilized by resonance ($\text{H}_2\text{C} = \text{C}(=\text{O}) = \text{R} \leftrightarrow \text{H}_2\text{C} = \text{C}(=\text{OH}) = \text{R}$). The $c_n + 58$ ion has a C-terminal glycine residue with a C$_n$ radical which is known to be a relatively stable radical position (Easton 1991; Rauk et al. 1997). The one Dalton difference between a fragment containing a glycyl residue and the odd electron $c_n + 58$ fragment is easily resolved on an FTMS, eliminating any possibility of incorrectly assigning one fragment for the other. Also, the complementary $z_n - 57$ fragment ion can provide additional supporting proof to the presence of an isoaspartyl residue.

Peptides BUSM3 and 4 showed the $c_n + 58$ and $z_{\ell-n} - 57$ fragment ions as well but could not be resolved from other $c$ and $z$ ions present in the spectra (data not shown). The presence of the $c_n + 58$ and $z_{\ell-n} - 57$ ions is revealed upon examination of changes in isotopic abundances between the spectra of the two peptides. The $c_n + 58$ and $z_{\ell-n} - 57$ fragments ions for BUSM4 coincided with the $z_{n+1}$ and $c_{\ell-n-1}$ ions, respectively, with both sets differing by 0.0126 Da and were not resolved from each other. Peaks corresponding to both the isoAsp fragments and interfering $c$ and $z$ ions were higher in relative abundance for BUSM4 than for BUSM3 relative to adjacent peaks that were assumed to be of constant abundance in the two peptides ($z_{n+1}$ was used for the $c_{n+58}/z_{n+1}$ overlap and $c_{\ell-n-1}/z_{\ell-n-57}$ overlap). All overlapping regions where the diagnostic isoaspartyl peak should appear showed the same trend except for $c_{\ell+58}$ that experienced interference from $y_{11} - \text{H}_2\text{O}$. Therefore, the isoaspartyl residue must be responsible for this trend since that is the only difference between the peptides.

Analysis of BUSM5 and 6 by ECD at 0.2eV (Fig. 4) showed similar trends to those found for BUSM1–4. Neutral loss of $\text{C}_2\text{H}_4\text{O}_2$ was experienced only by BUSM5 while the $z_{\ell-57}$ peak ($m/z = 506.2727$) was found only for the isoaspartyl peptide but its complementary $c_{\ell+58}$ ion was not detected. The deficiency of the $c_{\ell+58}$ ion in the isoaspartyl spectrum may be due to steric hindrance from the neighboring phenylalanine residue such that the charged C-terminal arginine residue upon electron capture is unable to facilitate the $\text{O} - \text{H}$ bond formation to the isoaspartyl backbone carbonyl because of interference from the bulky phenyl group adjacent to the isoaspartyl residue.

Using the CAD data collected for BUSM1–4 (data not shown), comparisons were made to previous studies that differentiated aspartyl from isoaspartyl residues in peptides. Studies by Lehman et al. (2000), using low-energy CAD and ESI-MS/MS, showed that the $b_n/y_{\ell-n}$ abundance ratio for peptides with aspartyl residues is larger than those of their counterpart peptides with isoaspartyl residues. The $b_n$ ion abundances are thought to be diminished in isoaspartyl peptides due to interference from the carboxylic acid side
chain upon ion formation, which occurs via an oxazolone intermediate (Lehmann and Schlosser 2000), while y ions are considered to be of constant abundance between the two peptides because they are formed by direct cleavage during bombardment with collision gas. The same ratios were calculated using b and y fragment ion abundances from CAD data for BUSM1–4 and shown in Table 2 for the cleavage of Xxx-(Asp/isoAsp) and (Asp/isoAsp)-Xxx bonds (Xxx is adjacent residue). Only two ions (b9/y8 and y5/y11 for BUSM4) showed an increase in their b/y ratio upon substitution of aspartyl with isoaspartyl residues while all the other calculated ratios showed an opposite trend. The data shows no correlation to the trend found in the previous study; however, the authors noted that their results were highly sensitive to instrument parameters, which probably explains the discrepancy. However, some b and y fragments were not detected (too low an abundance or out of mass range) so a full comparison of trends could not be made. These data suggest that, although the side chain is shorter by one methylene unit, the acidic hydrogen of the hydroxyl group of an isoaspartyl residue can still participate in the formation of a five-membered heterocyclic intermediate (oxazolone), resulting from the nucleophilic attack of the hydroxyl oxygen on the carbon of the adjacent carbonyl (oxazolone), which probably explains the discrepancy. However, some b and y fragments were not detected (too low an abundance or out of mass range) so a full comparison of trends could not be made. These data suggest that, although the side chain is shorter by one methylene unit, the acidic hydrogen of the hydroxyl group of an isoaspartyl residue can still participate in the preferential C-terminal bond cleavage attributed to aspartyl residues.

Another method to distinguish aspartyl from isoaspartyl residues using ESI-MS/MS and low-energy CAD used the diagnostic b9+H2O and y9−1+H2O ions found in the spectra of peptides with isoaspartyl residues to distinguish it from the analogous peptides with aspartyl residues (Schindler et al. 1996; Gonzalez et al. 2000). The fragmentation mechanism to support these ions as indicators proposes that formation of a five-membered heterocyclic intermediate (oxazolone), resulting from the nucleophilic attack of the hydroxyl oxygen on the carbon of the adjacent carbonyl group (n−1 position) of an isoaspartyl, is more stable than a six-membered intermediate involving an aspartyl side chain. The b9+n+1H2O and y5+n−1H2O ions resulting from rearrangement should be of higher abundance for isoaspartyl than that of aspartyl because of the relative stability of their intermediates. Upon examination of the CAD data for BUSM2, the three b9+H2O peaks were detected in the isoaspartyl peptide based on increases in abundances (data not shown). However, all three peaks were also detected in BUSM1, albeit showing a significantly lower abundance that could be accounted for in two ways. First, these peaks could correspond to b9+n+1H2O ions for aspartyl residues, which are possible but unlikely. Second, and more likely, these peaks could be the A+1 isotope for the c6, c8, and c13 ions, which occur at low abundances in the CAD spectra. The y5+n−46 ions were not detected for BUSM2 but are suggested by the authors to be only prevalent with tryptic peptides. The b9+H2O ions for BUSM4 and BUSM3 could not be resolved from interfering fragment ions so no conclusions could be drawn for these peptides.

The ESI-qQ-FTMS with ECD capability used in the study makes both ECD and CAD possible in order establish a method to distinguish isoaspartyl from aspartyl residues in model peptides which can be applied to real biological samples. The instrument has the capability for application of the top-down approach (Kelleher et al. 1999a; Tsibin et al. 2004) to protein analysis by selecting and fragmenting selected charge states of the intact protein then isolating fragments in the ICR for subsequent ECD analysis. Proteins with suspected deamidated asparagine residues could be fragmented and the fragments containing the asparagine residues of interest can be isolated for detection (with possible SORI-CAD analysis) in order to determine if there is indeed a 1-Da mass shift from the theoretical value. If the mass shift is present, the fragment can be subjected to ECD analysis to determine if the result of the modification is either an aspartyl or isoaspartyl residue indicated by the diagnostic ions or side chain fragmentation discussed above. Likewise, fragments believed to have isomerized aspartyl residues can also be subjected to ECD by applying the method used in this study. The instrumentation used here could facilitate the determination of the modification, either the deamidation of asparagine or the isomerization of aspartyl residues at the picomole (0.02 µg for a 20-kDa protein) or lower sensitivity levels, without the need for chemical tests such as the PIMT assay and Edman degradation as well as control samples.

Differentiating aspartyl from isoaspartyl residues in digested peptides by ECD could be complicated by the need for multiply charged states and favorably situated basic residues required for the production of c6+58 and z6−57 ions. For example, trypsin, the most common protease used for sequencing proteins, cleaves on the C-terminal sides of arginine and lysine residues and results in mostly tryptic peptides with N-terminal basic residues. If such peptides were suspected to have isoaspartyl residues, ECD analysis would yield only z6−57 ions. Furthermore, efficient protease activity might only produce tryptic peptides of a single charge state that would be worthless for analysis by ECD. Therefore, partial digestion procedures (shorter reactions times,

Table 2. b/y Abundance ratios from CAD data for peptides BUSM1–4

<table>
<thead>
<tr>
<th>Peptide</th>
<th>b9/y8, α/β Asp</th>
<th>α</th>
<th>β</th>
<th>1/β</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAAAGADGDGAGADAR</td>
<td>0.33</td>
<td>1.09</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAAR</td>
<td>0.23</td>
<td>4.45</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>RAAAGADGDGADGAR</td>
<td>2.67</td>
<td>6.47</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAGAR</td>
<td>1.95</td>
<td>3.51</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAGAR</td>
<td>1.68</td>
<td>0.01</td>
<td>261.76</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAGAR</td>
<td>1.75</td>
<td>8.3</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAGAR</td>
<td>1.21</td>
<td>3.29</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>RAGADGDGADGAGAR</td>
<td>1.49</td>
<td>36.23</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>
less enzyme) would have to be developed to produce tryptic peptides that can be multiply charged under ESI conditions and different enzymes could be used, such as chymotrypsin or Glu-C, to yield peptides that meet the needed specifications.

In summary, the ability to distinguish aspartic acid from its isomeric form, isoaspartic acid, in peptides was successfully demonstrated via ECD experiments in which several fragmentation trends were detected that clearly characterize this amino acid modification. Six peptides, three with aspartyl residues and their equivalent peptides with isoaspartyl residues, were subjected to analysis by ECD. The isoaspartyl peptides showed a cleavage pattern corresponding to formation of c_{n}+58 and z_{c−n}−57 ions that were not found in the aspartyl peptides. The proposed mechanism involves cleavage of the C_{α}–C_{β} bond producing two fragments unique to isoaspartyl residues. The isoaspartyl residue is essentially split; the odd electron fragment contains a radical glycine structure while the even electron ion contains the remaining methylene group (C_{β}). The side chain loss of C_{2}H_{4}O_{2} from aspartic acid was found only in the ECD spectra of peptides with aspartyl residues; this observation could also help to distinguish the isomers in peptides and proteins, although the appearance of this fragment is more uncertain than the c_{n}+58 and z_{c−n}−57 ions due to interfering isotopes of fragments from parallel fragmentation channel. The CAD data obtained for peptides 1–4 were used to compare other methods to detect isoaspartyl residues in peptides using low-energy CAD and ESI-MS/MS. The data showed no correlation to the report of the increase in b_{n}/y_{c−n} ratio for aspartyl compared to isoaspartyl residues in peptides, but the authors had suggested this trend may be sequence and instrument dependent. However, the CAD data for BUSM1 and 2 agreed well with several other studies that show the formation of b_{c−1}+H_{2}O is enhanced by the presence of isoaspartic acid in peptides, as opposed to aspartic acid. The advantage of the ECD method developed here relies not on the relative abundance of fragment ions, but on the appearance of specific diagnostic ions, M–C_{2}H_{4}O_{2} for aspartic acid and c_{n}+58/z_{c−n}−57 for isoaspartic acid, which makes determination of the aspartyl versus isoaspartyl residues unambiguous. Furthermore, this method provides the potential for the Asp/isoAsp assignment without synthetic control samples. Using ECD, observation of the two combined fragmentation trends could prove to be a powerful means for efficiently detecting this ubiquitous protein modification via one mass spectrometric experiment.

Materials and methods

Sample preparation

The peptides RAAAGAD_{β}GD_{α}GAGAD_{α}AR (BUSM1), RAAGAD_{β}GD_{α}GD_{α}GD_{α}GAGAD_{α}AR (BUSM2), RAG2_{β}AD_{α}GD_{α}2_{β}D_{α}AD_{α}G2_{β}, D_{α}AG2_{β}GAGAD_{α}AR (BUSM3), and RAGAD_{β}GD_{α}GD_{α}GD_{α}AGAD_{α}AR (BUSM4) were synthesized by AnaSpec (Table 1). The Protein Structure Facility at the University of Michigan, directed by Dr. Henriette Remmer, synthesized the RAAD_FAAR (BUSM5) and RAAD_FAAAR (BUSM6) peptides. All other chemicals were purchased from Sigma-Aldrich. All peptides were dissolved to a final concentration of 1 μM in methanol, water, and acetic acid (49.5:49.5:1, v/v).

Mass spectrometry

Although these experiments can be carried out on any mass spectrometer with ECD capability including commercial instruments from Finnigan, Bruker, or IonSpec, analysis was carried out on a home built qQq-FTMS with a nanospray source and 7T actively shielded magnet (Cryomagnetics). The qQq refers to a set of front-end quadrupoles which have the ability to select, fragment, and accumulate ions which are subsequently transmitted into the FTMS for ECD and detection (Pittman et al. 2004). The front-end quadrupoles were controlled using the program LC2Tune 1.5 (MDS Sciex), and the program IonSpec99 controlled data acquisition in the ion cyclotron-resonance (ICR) cell. A 5-μL aliquot of each peptide solution was loaded into a pulled-glass capillary tip (Valaskovic et al. 1996) (1-μm orifice diameter) pulled in-house with a micropipette puller (Model P-97, Sutter Instruments Co.) although similar capillaries can be purchased (New Objective). For ECD analysis, (M+2H)^{2+} ions were isolated in Q1 and externally accumulated in the Q2 region for accumulation periods ranging from 15 to 100 msec. The collected ions were then transmitted to and trapped in the cylindrical ICR cell and irradiated with electrons emitted from a dispensar cathode (Tsybin et al. 2001, 2004); the cathode heater was held at 1.2 A and the offset voltage applied to the electron gun was selected to produce 0.2 and 9.0 eV electrons while a potential of 9.0 V was applied to the grid. For external Q2 CAD analysis, (M+2H)^{2+} ions were isolated by Q1 and accelerated (23 eV for BUSM1–3 and 17 eV for BUSM4) into Q2 for collision with N_{2} gas. Fragment ions were accumulated for 5–250 msec and transmitted to the ICR cell with subsequent cooling using a N_{2} gas pulse and detection. All data were analyzed without apodization and with two zero-fills and was internally calibrated based on ions (M+2H)^{2+}, (M+2H)^{+}, (M+H)^{+} and their isotopes.

Acknowledgments

We thank Dr. Bogdan Budnik and Raman Mathur for helpful discussions and Dr. Henriette Remmer for synthesizing some of the model peptides. This work was supported in part by Federal funds from the National Center for Research Resources under grant P41-RR10888 (C.E.C.), the National Heart, Lung, and Blood Institute under contract HHSN268200248178C (C.E.C.), a grant from the ACS Petroleum Research Fund (P.B.O.), a Research Collaborative Agreement with MDS Sciex (P.B.O.), and NIH Grant GM35533 (to L.W.).

References


Aswad, D.W., Paranandi, M.V., and Schurter, B.T. 2000. Isoaspartate in pep-

www.proteinscience.org 461


Differentiation of Asp from isoAsp residues by ECD