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Investigation of apparent mass deviations in electrospray ionization tandem mass spectrometry of a benzophenone-labeled peptide

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In a previous study utilizing benzophenone-based topological probes to study conformationally dependent changes in mouse muscle nicotinic acetylcholine receptor (nAChR) topology, electrospray ionization tandem mass spectrometric (ESI-MS/MS) analysis led to a consistent −2.0 Da mass deviation from expected values. In the present study a synthetic peptide, corresponding to nAChR α1 subunit residues 130–139, was photolabeled. MS/MS analysis of this peptide using an ion trap confirmed the previously observed mass deviation, associated only with fragment ions that contain the incorporated benzophenone moiety. Analysis of peak profiles for the photolabeled ions does not indicate the typical 'peak fronting' that produces a mass shift when labile ions are prematurely ejected from the ion trap. Rather, hydrogen/deuterium (H/D) exchange experiments support the hypothesis that a chemical rearrangement involving phenyl migration and ketone formation has formed an unexpected oxidized peptide, with molecular mass 2 Da less than that expected, that is isolated for collision-induced dissociation in the ion trap together with the predicted precursor due to the broad ion isolation window specified. Copyright © 2003 John Wiley & Sons, Ltd.

Photoactivatable probes play an important role in studies of the relationship between protein structure and function. These photo-probes can be exploited to map ligand-binding sites, to analyze the topology of integral membrane proteins, or to identify binding partners in various metabolic processes.[1] Benzophenone (BP) is a preferred photo-probe due to its convenient absorption spectrum, 350–360 nm, which avoids protein damage, its chemical stability relative to diazo esters, aryl azides, and diazirines, and its specificity towards unreactive C–H bonds.[1,2] Once photoactivated, BP often photoincorporates into peptides at the α-carbon position and the BP-labeled peptides can be identified by electrospray ionization (ESI) mass spectrometry[3] due to the characteristic mass shift of 182.1 Da. Collision-induced dissociation (CID)[4,5] of the BP-labeled peptide and tandem mass spectrometry (MS/MS) may then reveal the site of BP attachment if sufficient diagnostic fragment ions are formed during CID.

A recent study from this laboratory has reported the use of BP-based photo-probes in electrophysiology-coordinated photolabeling experiments (manuscript submitted for publication). These photolabeling experiments analyzed the differences in the topological profile for the nicotinic acetylcholine receptor (nAChR) (a ligand gated ion-channel) between the closed, open and desensitized conformations. On-line liquid chromatography/tandem mass spectrometry (LC/MS/MS) with an ion trap was employed in that study to identify the photolabeled peptides and amino acid residues, and to relate differences in labeling pattern to conformational changes in the nAChR. Key segments of the protein were identified; however, interpretation of the CID mass spectra of the BP-labeled peptides was complicated because a series of y ions deviated from the expected molecular masses by approximately −2 Da. These y ions were eventually identified as BP-labeled ions. Unlabeled fragment ions in these mass spectra were observed at their expected masses.

The present study describes experiments to confirm and understand the anomalous masses that were noted during the previous work. For this study, we synthesized a peptide corresponding to a V8 fragment from the nAChR α1 subunit (nAChRα1; detected in the previous study). The peptide was labeled with BP and examined by ESI-MS and MS/MS in the ion trap. For independent confirmation, samples were also submitted to an outside laboratory and were analyzed by ESI using a triple-quadrupole mass spectrometer. Once the masses of the labeled peptides had been confirmed, hydrogen/deuterium (H/D) and carboxy-methyl esterification exchange experiments were performed to discover where and/or how potential hydrogen losses might have occurred, resulting in formation of species −2 Da from those expected.

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EXPERIMENTAL

Sample preparation

The synthetic peptide that corresponds to the nAChRα1 subunit residues 130–139 was synthesized and purified by the California Institute of Technology Biopolymer Synthesis Center. The peptide was dissolved (final concentration 1 mg/mL) in 50 mM ammonium bicarbonate, pH 7.8, 0.9 mg/mL phosphatidylcholine (Avanti Polar Lipids, Inc.) and 750 μM BP (Sigma-Aldrich), in a 500-μL thin-walled PCR Eppendorf tube. The solution was sonicated and incubated at 4°C for 1 h. Photoirradiation (3 min) was performed utilizing a 1000 W Hg/Xe arc-lamp (Oriel) set at 300 W, utilizing a UG-11 filter (Schott) and infrared water filter. The photolabeled peptide was separated from the unlabeled peptide by reversed-phase chromatography on a Velocity HPLC column (Optimize Technologies, OR, USA) fitted with a 2.1 × 100 mm Reliasil cartridge packed with 5 μm C18 particles. A Beckman Gold HPLC system (model 126, 200 μL/min) generated a linear gradient of 0% to 100% solvent B over 30 min (solvent A: 2% acetonitrile, 100 mM acetic acid; solvent B: 90% acetonitrile, 100 mM acetic acid), and the eluate was monitored at 230 nm. Eluted fractions were collected, pooled and dried. The photolabeled peptide eluted as a single peak that was subsequently identified as a singly labeled BP-peptide by direct-infusion ESI-MS.

H/D exchange

Deuterated water-d_2 (99.96% d), acetic acid-d_4 (99.5% d) and acetonitrile-d_3 (99.8% d) were obtained from C/D/N Isotopes (Quebec, Canada). HPLC fractions containing the native and BP-photolabeled peptides were dried in a heated sandbox (75°C). 100–500 μL of a freshly prepared solution (0.8 mL *d*-acetonitrile, 0.8 mL D_2O and 1 μL *d*-acetic acid) were added to each sample. Peptide concentrations are estimated as approximately 2 μM. Samples were analyzed by direct-infusion electrospray after mixing for 2 min in the deuterated solution.[6]

Esterification

A methanolic reagent was prepared by careful addition of 160 μL of acetyl chloride (Fluka) to 1 mL of anhydrous methanol (99.9%, Aldrich) over 5 min. 30–50 μL of this solution were added to dried HPLC fractions of native and BP-labeled peptide. Esterification was allowed to proceed at room temperature for 2 h, and then samples were lyophilized. Esterified samples were reconstituted in an appropriate volume of 1:1 (v/v) 50% acetonitrile/0.1% acetic acid, and examined by direct-infusion electrospray.[3]

Mass spectrometry

Samples were analyzed by ESI-MS using an LCQ Classic ion trap (ThermoFinnigan) in direct infusion mode and/or by on-line micro-LC/MS/MS. For the latter, a Surveyor HPLC pump (ThermoFinnigan) was used for delivery of solvent gradients at 10 μL/min through a 0.3 mm i.d. × 150 mm Magic C18 microcolumn (Michrom BioResources, CA, USA). A tee with a section of 50 μm i.d. fused-silica tubing was used to achieve the 10 μL/min flow rates. The gradient used was 90% solvent A (2% acetonitrile, 100 mM acetic acid) and 10% solvent B (90% acetonitrile, 100 mM acetic acid) to 40% solvent A and 60% solvent B in 35 min. Instrument calibration was performed according to the manufacturer's procedures, using a tuning solution consisting of caffeine, peptide MRFA, and UltraMark. Ion trap settings were AGC ON (default settings), 3 microscans and 200 ms maximum ion inject time. For MS/MS, the ion isolation window was typically 2.5 Th except when instrument mass accuracy was being tested. In the latter case, the ion isolation window was varied from 2 to 8 Th. Mass spectra were acquired in both profile and centroid modes when samples were analyzed by direct-infusion ESI. Only centroid data were acquired during on-line micro-LC/MS/MS. For the latter, data-dependent scanning was used for automatic acquisition of the full-scan MS and MS/MS data. The repeat count was 2, repeat duration was 0.4 min, exclusion duration was 1 min, and exclusion mass width was 3 Th, default charge state was 2, and normalized collision energy was set to 35%. To examine peak shapes, profile data were acquired by isolating the desired ion with an ion isolation window of 2–8 Th with the collision energy set to 0. Theoretical peak profiles were generated using commercial software, Mass Spec Tools™ from ChemSW.

Samples submitted to the Protein/Peptide MicroAnalytical Laboratory (Caltech) for independent confirmation were analyzed by ESI using a triple-quadrupole mass spectrometer (Sciex API 365, Perkin Elmer).

Mass accuracy

To inspect the instrument's mass accuracy, we prepared solutions of the native peptide (0.05–5 μM) in 1:1 (v/v) acetonitrile/0.1% acetic acid and analyzed them by direct-infusion ESI in full-scan MS and MS/MS modes. Specifically for MS/MS, ion isolation window, normalized collision energies, and the number of scans averaged were varied over a broad range (2–8 Th ion isolation width, 0–35% normalized collision energy, 3–10 scans averaged). Masses of precursor and fragment ions were recorded for each set of experimental conditions. There were no deviations greater than 0.2 Da from the expected monoisotopic masses under all tested conditions.

RESULTS AND DISCUSSION

A synthetic peptide (I-1217) with amino acid sequence IIVTHFPFDE, matching nAChRα1 residues 130–139, was synthesized. In previous studies with nAChR expressed in oocytes, nAChRα1 residue H134 of this peptide became photolabeled with benzophenone when the nAChR was in the closed state (manuscript submitted for review).

To mimic the conditions of the previous experiment, the synthetic peptide I-1217 was mixed in a solution of phosphatidylcholine and BP, then irradiated. In an aqueous solution at room temperature, phosphatidylcholine molecules associate to form vesicles that include BP within the lipid bilayer. Thus, I-1217 was expected to associate, at least peripherally, with the lipid vesicles and to have access to BP. Indeed, on-line micro-LC/MS/MS analysis of the photoirradiated peptide solutions indicated the presence of a singly labeled BP-peptide I-1217, detected at *m/z* 1397.7 and

699.3, respectively attributed to singly and doubly protonated BP-labeled ions. Notably, the detected masses are 2 Da less than the values expected from labeling with BP. Native I-1217 was detected at *m/z* 1217.6, corresponding to $[M+H]^{+1}$, and *m/z* 609.3, which represents $[M+2H^{+}]^{+2}$.

MS/MS analysis

CID and MS/MS analysis of ions with *m/z* 1397.7 and 699.3, shown in Figs. 1(B) and 1(D), confirmed that these ions were, respectively, singly and doubly protonated BP-labeled I-1217 based on the amino acid sequence. Also, fragment ions exhibiting masses consistent with the presence of a BP moiety were identified (marked by asterisks in Figs. 1(B) and 1(D)), further locating the BP at the C-terminal Glu residue. CID mass spectra of the unlabeled I-1217 are shown in Figs. 1(A) and 1(C) for comparison. The site of BP attachment noted in this study differs from earlier observations of H134 for the native peptide in nAChRα1. This is presumably caused by a different secondary structure and local environment of this synthetic peptide at the time of photolabeling, versus the native peptide within the nAChR structure.

Also consistent with our observations of the previous study with the BP-labeled nAChRα1, the CID mass spectra of the HPLC-purified BP-labeled I-1217 showed at least two series of fragment ions: a *b* ion series that were observed at their calculated masses (except for b_{10}) and a *y* ion series containing the BP label with masses approximately 2 Da less than expected (Figs. 1(B) and 1(D) and Table 1). Table 1 lists the expected and observed masses of *b* and *y* ions obtained from CID of the doubly protonated BP-labeled peptide, *m/z*

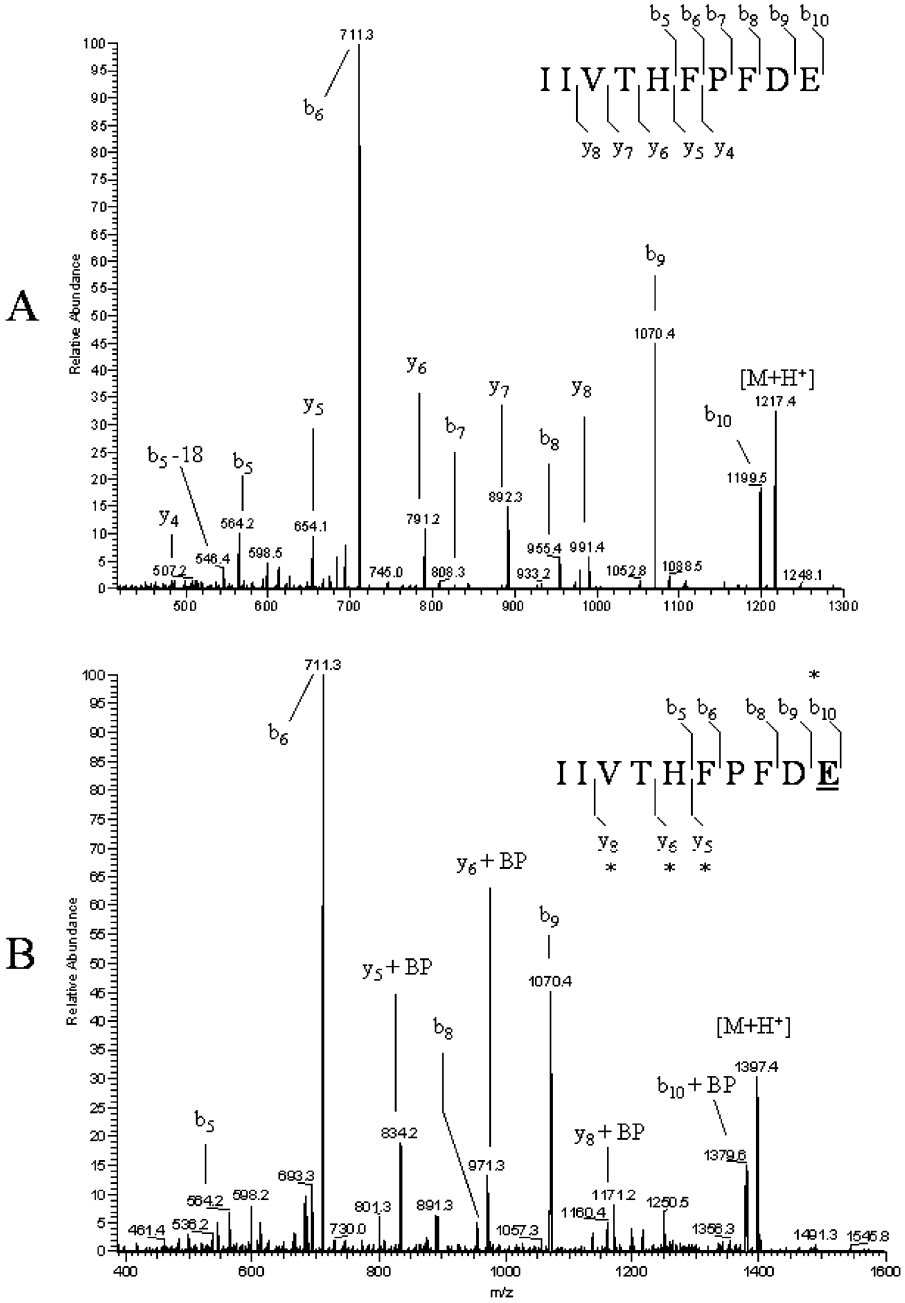

Figure 1. MS/MS mass spectra for (A) the unmodified I-1217 $[M+H\ 1217.6]^{+1}$; (B) the modified I-1217 $[M+H\ 1397.7]^{+1}$; (C) the unmodified I-1217 $[M+2H\ 609.3]^{+2}$; and (D) the modified I-1217 $[M+2H\ 699.3]^{+2}$.

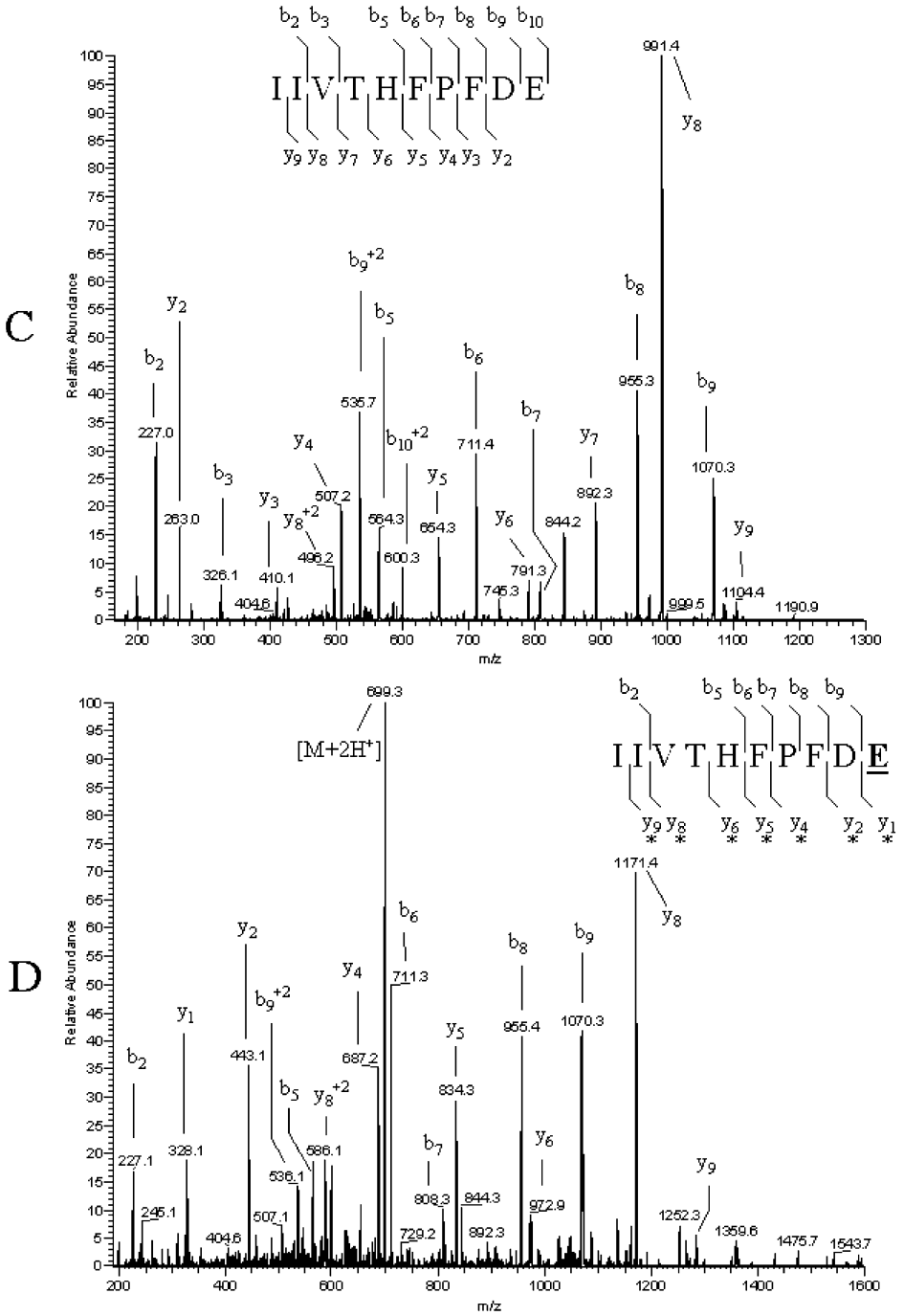

Figure 1. Continued

699.3, and from the doubly protonated unlabeled peptide, *m/z* 609.3. The data in this table are averaged from four experiments. The mean standard deviation of masses for b_1–b_9 ions obtained from CID of the (labeled) ion with *m/z* 699.3 is 0.2 Da, consistent with the *b* ion series obtained from CID of the unmodified peptide with *m/z* 609.6. Because the modification occurs at the C-terminal residue, only b_{10} is labeled with BP while the remaining *b* ions are unmodified. Interestingly, the b_{10} ion, which contains the BP moiety, also exhibits a significant mass deviation (−2.0 Da) from the expected value (Fig. 1(B)). In contrast, the mean standard deviation of masses for the *y* ion series is markedly higher (2.5 Da). Also, because the modification occurs at the C-terminus, every *y* ion in the series contains the BP moiety.

This unexpected observation prompted us to examine the mass accuracy of the LCQ with the unlabeled synthetic peptide under a range of conditions: peptide concentration from 0.05–5 μM, averages from 2–20 scans, ion isolation window 3–8 Th, and normalized collision energies from 0–35%. In all instances deviations were equal to or less than 0.2 Da from the expected monoisotopic masses. These data indicate that the observed mass deviations are not caused by low instrument mass accuracy, and are likely to be associated with the presence of the BP moiety.

Peak shapes and isotopic profiles

We also examined the peak shapes and isotope profiles of the BP-labeled peptides and fragments to test whether

Table 1. Mean standard deviations (SD) from the expected masses for fragment ions resulting from CID MS/MS analysis of (A) $[M+2H]^{+2}$ (*m/z* 609.3) and (B) $[M+2H]^{+2}$ (*m/z* 699.3)

#	*b* (expected)	*b* (observed)	SD	*y* (expected)	*y* (observed)	SD
(A)						
1	114.1	n.o.	n.a.	148.1	n.o.	n.a.
2	227.2	227	0.3	263.1	263	0.2
3	326.2	326.1	0.3	410.2	410.1	0.1
4	427.3	427.3	0.3	507.2	507.2	0.1
5	564.4	564.2	0.2	654.3	654.2	0.2
6	711.4	711.3	0.3	791.3	791.3	0.2
7	808.5	808.5	0.2	892.4	892.3	0.3
8	955.6	955.4	0.2	991.5	991.3	0.3
9	1070.6	1070.6	0.2	1104.5	1104.9	0.8
10	1199.6	n.o.	n.a.	1217.6	n.o.	n.a.
(B)						
1	114.1	n.o.	n.a.	330.2	328.1	2.5
2	227.2	227	0.3	445.2	443.1	2.5
3	326.2	326.1	0.2	592.3	590.7	1.5
4	427.3	427.1	0.3	689.3	687.2	2.5
5	564.4	564.3	0.2	836.4	834.3	2.4
6	711.4	711.4	0.1	973.4	971.4	2.5
7	808.5	808.4	0.1	1074.5	1072.4	2.5
8	955.6	955.5	0.1	1173.6	1171.5	2.5
9	1070.6	1070.5	0.2	1286.6	1284.7	2.1
10	1381.7	n.o.	n.a.	1399.7	n.o	n.a.

centroiding of low-intensity MS data was partly responsible for the anomalous observations. Full-scan MS and CID spectra were acquired in the profile mode with each profile mass spectrum representing an average of 3–10 scans. Figures 2(A)–2(D) show the actual and theoretical isotope profiles of the singly charged (*m/z* 1217.6) and doubly charged (*m/z* 609.3) unlabeled peptide, I-1217. We found good agreement between observed and theoretical isotope profiles as well as between the observed and theoretical masses (0.1 Da). Figures 3(A)–3(D) illustrate the actual and theoretical isotope profiles of the BP-labeled peptide. First, we note that the observed mass, *m/z* 1397.7, is −2 Da from the theoretical mass for the singly protonated peptide labeled with one BP residue (1217.6 + 182.1 = 1399.7). Similarly, the doubly protonated BP-labeled peptide is observed at *m/z* 699.3 instead of the expected *m/z* 700.3. Second, the observed isotope profile for *m/z* 1397.7 (Fig. 3(A)) does not accurately match the theoretical profile. However, it does agree well with a theoretical overlapping profile of *m/z* 1397.7 and 1399.7 (Fig. 3(B)), with a 10% contribution from the latter. Similarly, the peak profile for the doubly charged BP-modified peptide at *m/z* 699.3 (Fig. 3(C)) agrees well with a theoretical profile of *m/z* 699.3 with 10% overlapping contribution from *m/z* 700.3 (Fig. 3(D)).

As shown in Figs. 3 and 4, the peak profiles of the labeled peptide do not indicate 'peak fronting' caused by premature ejection of labile ions from the trap.[7–9] In fact, similar mass deviations were noted when the samples were analyzed with

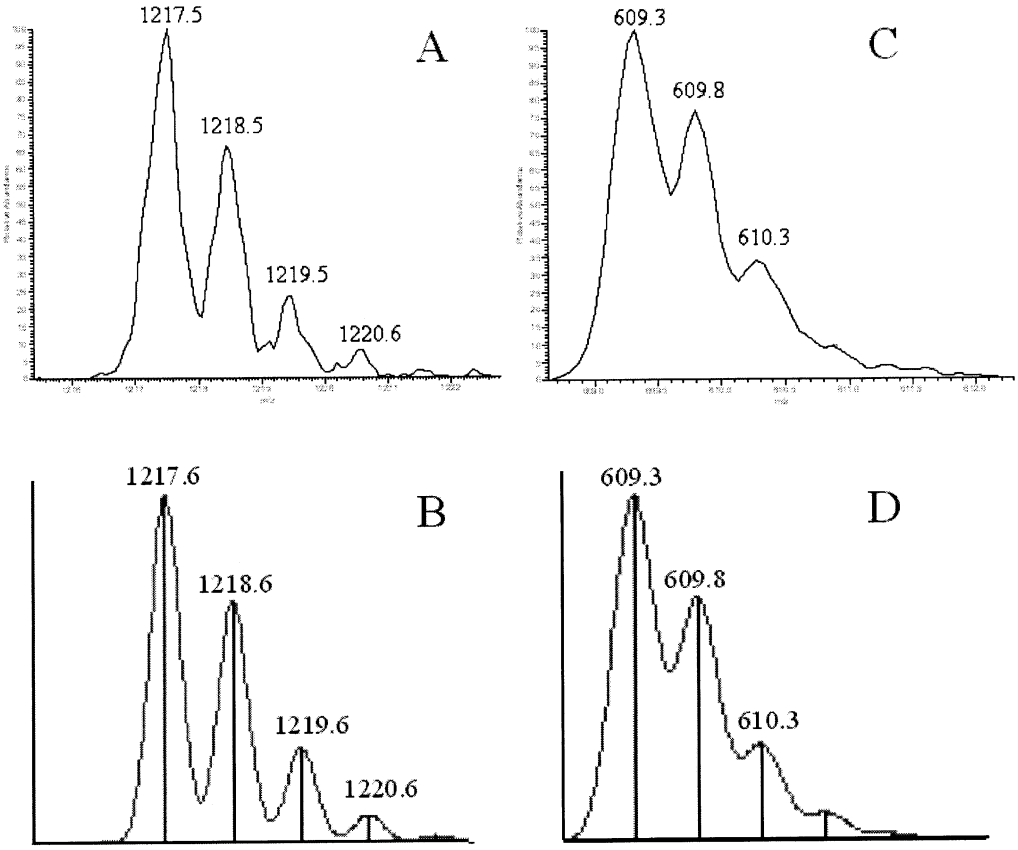

Figure 2. (A) Experimental and (B) theoretical peak profiles for the singly charged ion at *m/z* 1217.6 and (C) experimental and (D) theoretical peak profiles for the doubly charged unmodified I-1217 peptide at *m/z* 609.3.

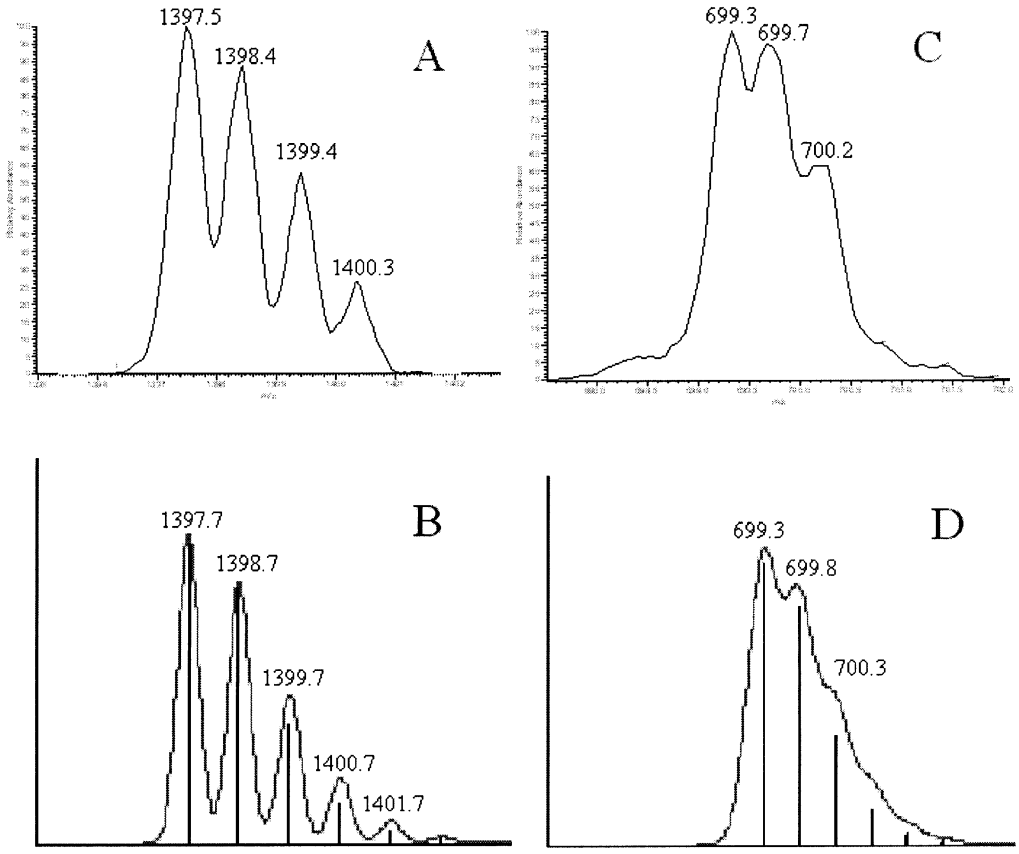

Figure 3. Experimental and theoretical peak profiles of the BP-labeled ions. (A) Experimental peak profile for the BP-labeled I-1217 peptide, *m/z* 1399.7; (B) theoretical peak profile showing overlap of *m/z* 1397.7 (90% contribution) with *m/z* 1399.7 (10% contribution); (C) theoretical peak profile of doubly protonated BP-labeled I-1217 peptide, *m/z* 699.3; and (D) theoretical peak profile showing overlap of *m/z* 699.3 (90%) with 700.3 (10%).

the triple-quadrupole mass spectrometer. Thus it appears that, during CID, two BP-labeled peptide populations differing by 2 Da are isolated in the ion trap (e.g., 90% *m/z* 1397.7 and 10% *m/z* 1399.7) when the ion isolation window is 2.5–3.0 Th. The nearly complete *b* ion series indicates that these species have the same amino acid sequence; however, the observation of the *y* series of ions at −2 Da from the expected masses suggests that two hydrogen atoms may be lost from the first *y* residue for one of these species.

Phenyl rearrangement hypothesis

We first considered whether gas-phase rearrangement reactions in the ion trap (where ion residence times are milliseconds[9] in duration) or in the electrospray ion source (where species may oxidize[10]) caused the apparent losses of H. An instrumental effect is unlikely because the same discrepancy was found with the triple-quadrupole mass spectrometer (where ion residence times are microseconds in duration). Furthermore, literature reports of BP-labeled peptides using ESI-MS for analysis did not favor oxidation chemistry in the electrospray ion source.[11–13]

We hypothesized that a chemical rearrangement was occurring during BP labeling that resulted in the loss of two hydrogen atoms, a reaction favored by the high concentration of BP in the lipid bilayer, as well as the extended photoirradiation time (both necessary conditions for efficient photolabeling). In this reaction, shown in Fig. 4, a second BP abstracts a H atom from the peptide, forming a radical at the β-carbon of the C-terminal Glu residue (rather than the α-carbon). Subsequently, a phenyl moiety from the incorporated BP migrates to the β-carbon, a process that is well documented in radical chemistry.[14] Finally, the hydroxyl loses the second H atom leading to the formation of a ketone. The result of this reaction is loss of two hydrogen atoms (−2 Da) as well as formation of a ketone functional group from an alcohol. This proposed mechanism is consistent with the observations described here and is chemically reasonable, but further work would be required to establish that it is operative here.

H/D exchange analysis

To test the hypothesis of the chemical rearrangement illustrated in Fig. 4, an H/D exchange experiment was performed. If the scheme in Fig. 4 occurs, then the mass of the new molecule will be 2 Da less than expected and the new ketone prevents deuterium exchange at the BP hydroxyl. H/D exchange experiments are often used as MS tools for the study of protein conformations, because the amide hydrogen atoms at peptide linkages (indicators of peptide conformations) exchange on a time scale that can be measured by mass spectrometry.[15] In small peptides devoid of tertiary structure, solvent can access all of the labile hydrogen atoms (e.g., amides and hydroxyls) and readily exchange those atoms for deuterium. Full-scale MS and CID mass spectra

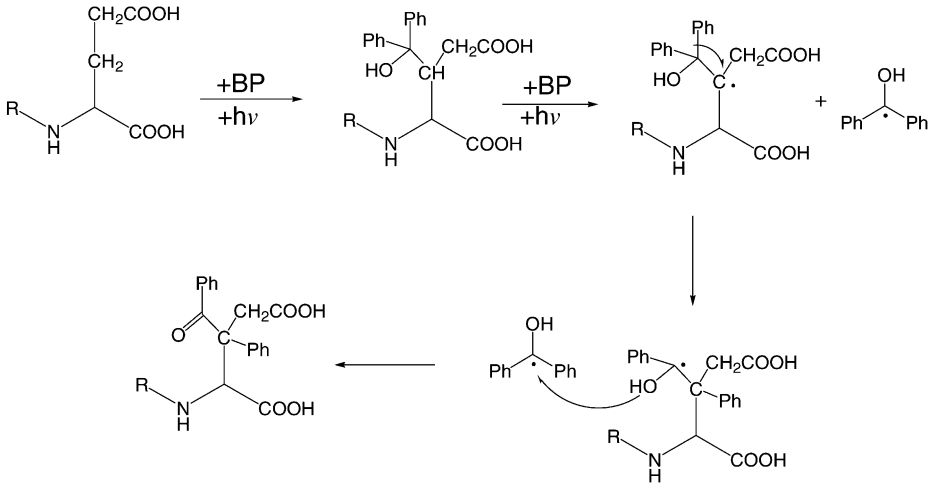

Figure 4. Scheme proposed for chemical rearrangement that produces a BP-modified I-1217 M-2.

were thus acquired for the unlabeled and BP-labeled I-1217 in deuterated solvents. The expected mass (M−2) of each *y* ion and corresponding observed mass are listed in Tables 2(A) and 2(B). Table 2 shows only the *y* ion series for simplicity, although the *b* ion series agreed well with values observed for the *y* ion series. The data of Table 2(A) for the native peptide show good agreement between the expected and observed number of exchanges. For example, y_2 of the unmodified peptide is expected to gain 6 Da upon exchange at the indicated sites (Fig. 5(A)), and indeed we observe the new ion at the expected *m*/*z* 269. Figure 5(B) illustrates the expected sites of exchange for the y_2 ion of the BP-modified peptide. The data of Table 2(B) for the BP-labeled peptide show that (a) observed masses for the *y* ion series are consistent with a modified product that has lost two hydrogen atoms, and (b) the y_1 ion contains four sites of deuterium exchange rather

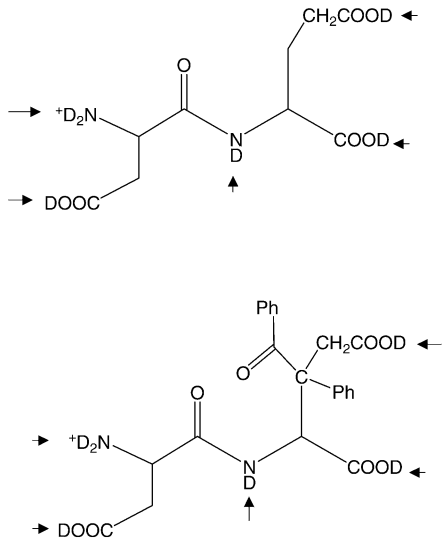

Figure 5. H/D exchange sites (shown by arrows) for the y_2 ion of (A) unmodified I-1217 and (B) BP-modified I-1217 after rearrangement.

Table 2. List of *y* ions detected in H/D exchange experiment for (A) unmodified I-1217 and (B) BP-modified I-1217

y ion	Initial M	M exp	M obs	# of D
(A)				
y_1	148.1	152.1	n.d.	n.d.
y_2	263.1	269.1	269	6
y_3	410.2	417.2	416.9	7
y_4	507.2	514.3	514.2	7
y_5	654.3	662.3	662.2	8
y_6	791.3	801.3	801.3	10
y_7	892.4	903.4	n.d.	n.d.
y_8	991.5	1003.5	1003.3	12
y_9	1104.5	1117.5	1117.3	13
y_{10}	1217.6	1231.6	n.d.	n.d.
(B)				
y_1	328.2	332.2	332.1	4
y_2	443.2	449.2	449.1	6
y_3	590.3	597.3	597.2	7
y_4	687.3	694.3	694.1	7
y_5	834.4	842.4	842.2	8
y_6	971.4	981.4	981.4	10
y_7	1072.5	1083.4	1083.4	11
y_8	1171.6	1183.6	1183.3	12
y_9	1284.6	1297.6	1297.4	13
y_{10}	1397.7	1410.6	n.d.	n.d.

than five (consistent with the loss of the BP-hydroxyl moiety in Fig. 5(B)).

Other evidence in support of the rearrangement hypothesis came from results of esterification experiments (data not shown for brevity). During LC/MS/MS analysis of the methyl-esterified photolabeled peptide, an ion with *m*/*z* 1439.6 was detected, consistent with modification of all three carboxyl groups. MS analysis of the esterified BP-labeled peptide still showed the presence of a peptide at −2 Da from the mass expected from routine BP labeling, in addition to small amounts of the expected product. Thus, the results of this experiment eliminated the carboxyl moieties as potential candidates for H loss.

This study proposes a novel photolabeling mechanism that results in peptide oxidation. The mechanism differs from that reported in the literature[1,2] in that masses of labeled peptides are −2 Da from values expected based on simple addition of a BP moiety to the α-carbon. These findings are significant because they aid in the interpretation of CID data from

low-resolution mass analyzers, such as the ion trap and triple-quadrupole mass spectrometers.

Our group routinely uses a number of similar photo-probes to explore conformational changes of ion channels and has consistently observed this anomalous behavior. However, we did not previously understand the underlying chemistry, because (a) labeled ions are detected at low abundances in our typical experiments, and (b) we suspected that we had isolated at least two species during MS/MS (these long hydrophobic peptides often are not resolved by chromatography and may be co-selected for MS/MS during ion isolation with a 2.5 Th window). Further work is underway to investigate whether other photo-probes display similar behavior.

CONCLUSIONS

In the present study we have photolabeled with benzophenone a synthetic peptide that corresponds to residues 130–139 of nAChRα1. MS/MS analysis of the photolabeled peptide corroborated previously observed mass deviations (−2.0 Da), mainly associated with ions that contained the BP moiety. Analysis of the peak profiles for individual BP-labeled ions ruled out premature ejection of labile ions from the ion trap as the cause of the observed mass shifts. Results of H/D exchange experiments were consistent with a chemical reaction starting with abstraction of an H atom by a BP, a phenyl group migration with loss of another H, and formation of a ketone, resulting in loss of a potential deuterium exchange site at the BP-hydroxyl. In summary, we hypothesize that the observed mass deviation is the result of a chemical rearrangement induced by oxidation by a second BP molecule; we expect that these effects would not be observed if the precursor ion is selected with high resolving capability. The work presented is strong evidence that BP labeling in the liposomes (and the native membrane environment) has resulted in formation of labeled peptides by a mechanism other than that reported in current literature.

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