Characteristic fragment ions associated with dansyl cadaverine and biotin cadaverine adducts on glutamine

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Abstract

Glutamine residues susceptible to transglutaminase-catalyzed crosslinking can be identified by incorporation of dansyl cadaverine or biotin cadaverine. Bacterial transglutaminase and human transglutaminase 2 were used to modify residues in beta-casein with dansyl cadaverine. Bacterial transglutaminase was used to modify residues in human butyrylcholinesterase with biotin cadaverine. Tryptic peptides were analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer. Modified residues were identified in Protein Prospector searches of mass spectrometry data. The MS/MS spectra from modified casein included intense peaks at 336.2, 402.2, and 447.2 for fragments of dansyl cadaverine adducts on glutamine. The MS/MS spectra from modified butyrylcholinesterase included intense peaks at 329.2, 395.2, and 440.2 for fragments of biotin cadaverine adducts on glutamine. No evidence for transglutaminase-catalyzed adducts on glutamic acid, aspartic acid, or asparagine was found. Consistent with expectation, it was concluded that bacterial transglutaminase and human transglutaminase 2 specifically modify glutamine. The characteristic ions associated with dansyl cadaverine and biotin cadaverine adducts on glutamine are useful markers for modified peptides.

Keywords

mass spectrometry; Protein Prospector; transglutaminase; dansyl cadaverine; biotin cadaverine; casein; butyrylcholinesterase

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1. Introduction

This paper is dedicated to Professor Laszlo Lorand, my (OL) advisor when I was a graduate student in his laboratory at Northwestern University in Evanston, IL. Fifty years after leaving his laboratory I have come back to transglutaminase. My path back started with finding that exposure of proteins to organophosphorus pesticides results in crosslinked proteins. The crosslink induced by organophosphorus pesticides is an isopeptide bond between the epsilon group of lysine and the carboxyl side chain of glutamic acid or between lysine and aspartic acid [1; 2]. Transglutaminase can also catalyze isopeptide crosslinks between proteins. The difference is that the transglutaminase-generated isopeptide bond is between the epsilon group of lysine and the gamma glutamyl group of glutamine. The work of L. Lorand, J.E. Folk, and their followers has defined transglutaminase-induced isopeptide crosslinks [3; 4; 5; 6]. Professor Lorand was honored for his research achievements by election to the National Academy of Science.

Lorand et al [6] introduced monodansyl cadaverine as a fluorescent substrate for transglutaminase type enzymes. Incorporation of dansyl cadaverine into proteins has been used to measure the activity of guinea pig liver transglutaminase, fibrinoligase, and lobster muscle transpeptidase [7; 8]. Other applications have included identification of transglutaminase sites in tau, a component of neurofibrillary tangles in Alzheimer’s disease [9] and of fibronectin, a component of extracellular matrix [10]. A biotinylated probe designed as an analog of dansyl cadaverine was used to identify transglutaminase substrates in the brains of Alzheimer disease and Huntington disease patients [11]; results supported the idea that the crosslinking activity of transglutaminase participates in the formation of protein aggregates. An analog of dansyl cadaverine was incorporated by bacterial transglutaminase into the constant region of human IgG1 antibody as a therapeutic for ovarian cancer [12].

Applications that require information on the residues involved in transglutaminase activity may use LC-MS/MS to identify the modified residues [10]. Interpretation of the MS/MS spectra is complicated by the presence of intense unassigned ions in the low mass range. In the present report we identified these ions as characteristic fragments from dansyl cadaverine modified glutamine or biotin cadaverine modified glutamine. The presence of these characteristic ions adds assurance to the interpretation that a particular peptide carries a dansyl cadaverine or biotin cadaverine adduct.

2. Materials and Methods

2.1. Materials.

Recombinant human tissue transglutaminase produced in *Escherichia coli*, Zedira cat # T002 1 μg/μL, UniProt Accession number P21980. Recombinant microbial (Bacterial) transglutaminase from *Streptomyces mobaraensis* produced in *E. coli*, Zedira cat # T001 4.5 mg/mL, 139 u/mL UniProt Accession number P81453. Beta-casein from bovine milk, Sigma cat # C6905 UniProt Accession P02666. Human butyrylcholinesterase purified from frozen Cohn fraction IV-4 as described [13] UniProt Accession number P06276. Monodansyl cadaverine N-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide.
2.2. Transglutaminase-catalyzed incorporation of dansyl cadaverine into beta-casein

Beta-casein solutions at a concentration of 1 mg/mL in 0.5 mL of 20 mM imidazole buffer pH 7.5 containing 2.5 mM CaCl₂, 1 mM dithiothreitol, and 1 mM dansyl cadaverine were treated with 2 μL of human transglutaminase 2 (1 μg/μL) at 37°C for 17 h in a humidified chamber. A 1 mg/mL beta-casein solution in 0.5 mL of 20 mM imidazole pH 7.5 was treated with 2 μL of bacterial transglutaminase (4.5 μg/μL) at 37°C for 17 h in a humidified chamber. Control 0.5 mL samples were treated with 25 μL of DMSO in place of 25 μL of dansyl cadaverine dissolved in DMSO. The volume of each reaction was reduced to 100 μL in a vacuum centrifuge before adding 2 mL of 6 M urea to each tube to denature the protein. Beta-casein was not reduced and alkylated because beta-casein has no cysteine and no disulfide bonds.

2.3. Removal of excess dansyl cadaverine, urea, and DMSO from beta-casein on PD-10 desalting columns.

PD-10 desalting columns were equilibrated with 25 mL of 20 mM ammonium bicarbonate pH 8 before application of 1.5 mL beta-casein sample. After the sample had entered the column, 1 mL aliquots of 20 mM ammonium bicarbonate were applied and 1 mL fractions were collected by gravity flow. Absorbance at 280 nm, measured in the Gilford spectrophotometer, showed that wash fractions 2, 3, and 4 contained protein, and wash fractions 6-14 contained dansyl cadaverine. It was estimated that 80% of the protein was recovered in the desalting step (400 μg). The desalted protein volumes of 3 mL were reduced to 0.1 mL in a vacuum centrifuge (4 μg/μL).

2.4. Bacterial transglutaminase-catalyzed incorporation of biotin cadaverine into human butyrylcholinesterase.

Human butyrylcholinesterase (1 mg/mL) in 0.5 mL of 20 mM imidazole pH 7.5 was incubated with 0.4 mM biotin cadaverine and 2 μL of bacterial transglutaminase (4.5 μg/μL) at 37°C in a humidified chamber overnight. Disulfide bonds were reduced with 0.4 mM Tris(2-carboxyethyl)phosphine hydrochloride for 30 min at 60°C followed by alkylation with 50 mM iodoacetamide. A control sample was treated identically but included no transglutaminase. Excess biotin cadaverine and reagents were removed by dialysis in DispoDialyzers against 4 x 4 L of 20 mM ammonium bicarbonate pH 8 at 4°C. After dialysis the butyrylcholinesterase concentration was 0.7 mg/mL.

2.5. Trypsin digestion

Desalted, concentrated beta-casein samples (20 μL of 2 μg/μL in 20 mM ammonium bicarbonate) were digested with 2 μg trypsin at 37°C for 14 h. Dialyzed
butyrylcholinesterase samples (100 μL of 0.7 μg/μL in 20 mM ammonium bicarbonate) were
digested with 3 μg of trypsin at 37°C for 14 h. Trypsin was inactivated by addition of 1%
formic acid pH 2. Samples were centrifuged at 14,000 rpm for 20 min before 20 μL was
transferred to autosampler vials for mass spectral analysis.

2.6. LC-MS/MS on the Orbitrap Fusion Lumos mass spectrometer.

Trypsin-digested samples were analyzed on the Orbitrap Fusion Lumos mass spectrometer
(Thermo Fisher), a high resolution instrument. A Thermo RSLC Ultimate 3000 ultra-high
pressure liquid chromatography system (Thermo Scientific) was coupled to the Orbitrap
Fusion Lumos via an Acclaim PepMap 100 C18 trap column (75 μm x 2 cm, Thermo
Scientific) and a Thermo Easy-Spray PepMap RSLC C18 separation column (75 μm x 50
cm, Thermo Scientific). A 2 μL sample (about 2 μg peptides) was loaded onto the trap
column, washed with 100% solvent A (0.1% formic acid in water) for 10 minutes at 2 μL/
min, shuttled onto the separation column, and eluted with a biphasic, linear gradient of 5 to
50% solvent B (0.1% formic acid in 80% acetonitrile) over 30 minutes followed by 50 to
100% solvent B over 40 minutes, at a flow rate of 0.3 μL/min. Parent ion mass spectra were
collected in the Orbitrap detector (resolution of 120,000), in positive ion mode, with a
charge state of 2-6, over a mass range of 350 to 1800 m/z. Mass tolerance was 10 ppm, data
collection for a given mass was excluded after the first acquisition for 30 seconds. Maximum
injection time was 100 msec, ion transfer tube temperature was 275°C, ion spray voltage
was 1900 volts, and automatic gain control was 400,000. Fragment ion spectra were taken
using data dependent acquisition, isolation was in the quadrupole, and the detector was the
Orbitrap (resolution 30,000). Fragmentation was by high energy collision-induced
fragmentation at 35% normalized collision energy, maximum injection time was 60 msec,
automatic gain control was 50,000, the scan range was auto normal.

2.7. Search for dansyl cadaverine modified peptides

Mass spectrometry *.raw files were converted to *.mgf files using MSConvert v 3.0 from
Proteo Wizard. Data were loaded onto Batch-Tag Web in Protein Prospector to search for
dansyl cadaverine modified peptides. The search parameters included 1) User Protein– the
complete amino acid sequence of beta-casein (P02666). 2) Digest trypsin; or Digest No
Enzyme. 3) Constant modifications carbamidomethyl (C). 4) Variable modifications
Oxidation (M). 5) Parent Tolerance 20 ppm. 6) Frag Tolerance 30 ppm. 7) Instrument ESI-
Q-high-res. 8) User defined variable modifications: Mod 1 Label dansyl cadaverine;
Specificity Q, Mod 1 Elem Comp C17H22N2O2S. 9) Mass modifications: Range 318 to 319
Da. 10) check mark in the Q box.

Two tryptic peptides of beta-casein are very long. Residues 64-112 with 49 amino acids
contain 5Q; residues 129-184 with 56 amino acids contain 6Q. Adducts on Q in these
peptides were identified by designating the search parameter Digest No Enzyme. This
yielded short peptides in charge states +2 and +3 where adducts were more easily identified.

2.8. Search for biotin cadaverine modified peptides

Mass spectrometry *.raw files were converted to *.mgf files using MSConvert v 3.0 from
Proteo Wizard. Data were loaded onto Batch-Tag Web in Protein Prospector to search for
biotin cadaverine modified peptides. The search parameters included 1) User Protein– the complete amino acid sequence of human butyrylcholinesterase (P06276). 2) Digest trypsin. 3) Constant modifications carbamidomethyl (C). 4) Variable modifications Oxidation (M). 5) Parent Tolerance 20 ppm. 6) Frag Tolerance 30 ppm. 7) Instrument ESI-Q-high-res. 8) User defined variable modifications: Mod 1 Label biotin cadaverine; Specificity Q. Mod 1 Elem Comp C15H25N3O2S. 9) Mass modifications: Range 310 to 312 Da. 10) check mark in the Q box.

3. Results and Discussion

3.1. Dansyl cadaverine adducts on glutamine

It is accepted that dansyl cadaverine makes a covalent bond with the side chain of glutamine [8; 9] in a reaction catalyzed by transglutaminase. What is new in our report is the recognition of characteristic ions in MS/MS spectra that originate from dansyl cadaverine adducts on glutamine. When we incubated bovine beta-casein (P02666) with dansyl cadaverine in the presence of human transglutaminase 2, and analyzed the tryptic peptides by LC-MS/MS we found 10 peptides that had acquired an added mass of 318.14 by incorporating dansyl cadaverine. The 318.14 Da added mass is the mass of dansyl cadaverine (335.14 Da) minus the mass of the amine (17 Da) that is lost upon adduct formation with glutamine. The dansyl cadaverine adducts were on glutamine 53, 69, 71, 94, 138, 182, 190, 197, 203, and 209 of beta-casein. Bacterial transglutaminase yielded the same glutamine adducts. The MS/MS spectrum for the adduct on Q53 of beta-casein is shown in Figure 1. The peptide contains 5 glutamines. The parent ion mass of 767.3395 MH^+ vested (mono) means that only one residue is modified. The mass of the b2 ion excludes Q49 as the adduct site. The masses of y3 to y10 ions exclude Q54, Q55 and Q61 as the adduct site. Direct evidence that the adduct is on Q53 is provided by the internal fragments at 558.24 and 792.32 m/z and by the a8 ion at 1295.59 m/z.

The most intense ion in Figure 1 is at 402.18 m/z and is not part of the peptide sequence. This ion derives from the 447.21 fragment by loss of CO and NH₃. The 402.18 and 447.21 fragments are analogous to the immonium ion minus NH₃ and α-amino-caprolactam fragments found from lysine and modified-lysine containing peptides (see Scheme 1 and Figure 2 in Fenaille et al.) [14]. The proposed structures of the 402.18 and ions are in Figure 2. These structures account for the total mass and composition of these ions. In addition the structures satisfy the requirement for 4 bonds to each carbon atom, and a net positive charge. The structure of the dehydroglutamine portion of the 447.21 ion is speculative. An alternative structure would substitute an acylium ion for dehydroglutamine. The 5-membered ring portion of the 402.18 ion is also speculative; it was selected because it accounts for the molecular composition and is chemically feasible.

The 336.17 ion is consistent with protonated dansyl cadaverine. Additional dansyl cadaverine fragment ions sometimes appear at 234 and 170 m/z [15], though they occur less consistently than the boxed ions in Figure 1.

Bacterial transglutaminase and human transglutaminase 2 modified the same beta-casein peptides on the same glutamnes. All 10 dansyl cadaverine-modified tryptic peptides carried...
an added mass of 318.14 Da on the parent ion. Their MS/MS spectra had ions at 336.17, 402.18 and 447.21 m/z. These characteristic ions support the interpretation that a peptide is modified by dansyl cadaverine and that the modification is on Q. The intensity of the 402.18 ion was high in charge states +2 and +3 peptides and decreased to background levels in charge state +6 peptides.

### 3.2. Biotin cadaverine adduct on glutamine

Biotin cadaverine is a substrate for transglutaminase [16]. Human butyrylcholinesterase (P06276) incubated with biotin cadaverine and bacterial transglutaminase acquired an added mass of 311.16 m/z on glutamines 75, 204, 251, 344, 379, 408, and 588. The 311.16 Da added mass is the mass of biotin cadaverine (328.16 Da) minus the mass of the amine (17 Da) that is lost upon adduct formation with glutamine. The MS/MS spectrum for the adduct on Q588 of human butyrylcholinesterase is shown in Figure 3. The parent ion mass of 519.2578 m/z MH$^{+3}$ (mono) is consistent with modification of one residue. The location of the modification on Q588 is defined by the b2 ion, whose mass of 554.28 adds 311.16 to the mass of unmodified NQ (243.12).

The most intense ion in Figure 3 is at 395.21 m/z and is not part of the peptide sequence. This fragment derives from the 440.23 fragment by loss of CO and NH$_3$. The 395.21 and 440.23 fragments are analogous to the immonium ion minus NH$_3$ and α-amino-caprolactam fragments found for lysine and modified lysine containing peptides, respectively [14]. Proposed structures for biotin cadaverine fragments are shown in Figure 4. The dehydroglutamine portion of the 440.2 ion parallels the structure of the 447.2 ion from dansyl cadaverine in Figure 2. A 5-membered ring is proposed in both the 395.2 ion derived from biotin cadaverine and in the 402.2 ion from dansyl cadaverine. The 329.2 Da mass is consistent with protonated biotin cadaverine, while the mass at 227.1 Da is consistent with protonated biotin.

The 395.2 ion was consistently present in all 9 MS/MS spectra of biotin cadaverine adducts on Q. The 440.2 ion was present in 6 spectra, the 329.2 ion in 7 spectra, and the 227.1 ion in 2 spectra. When present, these characteristic fragment ions confirm the interpretation that a peptide is modified by biotin cadaverine on Q.

### 3.3. Search for dansyl cadaverine adducts on glutamic acid, aspartic acid, and asparagine

We have recently found that the organophosphates are capable of mediating the formation of isopeptide crosslinks between lysine and glutamic acid or lysine and aspartic acid in proteins [Schopfer & Lockridge (2018); Schopfer & Lockridge (2019)]. This finding raised two questions: 1) Could isopeptide bond formation between lysine and glutamic acid or lysine and aspartic acid also be mediated by transglutaminase? And 2) Could isopeptide bond formation between lysine and glutamine be definitively discriminated from isopeptide bond formation between lysine and glutamic acid or lysine and aspartic acid?

When the search parameters in the Protein Prospector/Batch-Tag algorithm were adjusted to find dansyl cadaverine adducts on E or D in data from transglutaminase treated samples, Batch-Tag reported such adducts. However, these assignments were false, despite the fact
that the data were high resolution and the Batch-Tag tolerance parameters for the parent ion (20 ppm) and fragment ions (30 ppm) were stringent. Manual evaluation of the MS/MS spectra led to an understanding of how to recognize a false assignment. The first clue was an incorrect added mass. Dansyl cadaverine adducts on glutamic acid (E) should add 317.2 Da, but adducts on glutamine (Q) should add 318.2 Da. The difference in added mass is explained by loss of water (−18 Da) for adducts on glutamic acid (or aspartic acid), but loss of ammonia (−17 Da) for adducts on glutamine. When Batch-Tag reported an added mass of 318.2 on E or D, it flagged a false assignment. Further examination showed that the hypothetical adduct on E or D, was always in a peptide that included a Q. Masses in the MS/MS spectrum were a good fit for an adduct on Q, but did not support an adduct on E or D. The characteristic fragment ions for dansyl cadaverine on Q did not discriminate between E and Q because the product from the reaction of dansyl cadaverine with Q is identical with the product from the reaction of dansyl cadaverine with E.

False assignment of dansyl cadaverine adducts on aspartic acid and asparagine were readily identified. Theoretical dansyl cadaverine adducts would have an added mass of 317.1 m/z on aspartic acid or 318.1 m/z on asparagine and would have characteristic ions at 336.2, 388.2 and 433.2 m/z. Peptides assigned by Batch-Tag as having an adduct on D always included a Q residue and were reported to have an added mass of 318.1, rather than the expected added mass of 317.1. The MS/MS spectrum included the ions characteristic of dansyl cadaverine on Q (402.2 and 447.2) and did not have the predicted characteristic masses at 388.2 and 433.2 for adducts on D or N.

In conclusion, we found no evidence that transglutaminase catalyzes the incorporation of dansyl cadaverine on glutamic or aspartic acid or asparagine. In the course of this study, we identified characteristic ions for dansyl cadaverine modification of Q that lend support to the assignment of dansyl cadaverine adducts on glutamine. Our results are consistent with the long-held position, first described by Dr. Lorand [6; 7; 8], that transglutaminase specifically modifies glutamine (Q).

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Abbreviations:

- **LC-MS/MS**: liquid chromatography tandem mass spectrometry
- **MS/MS**: masses produced by fragmentation of a parent ion in the mass spectrometer

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Highlights

- Ions at 447, 402 and 336 are characteristic of dansyl cadaverine adducts on Q
- Ions at 440, 395 and 329 are characteristic of biotin cadaverine adducts on Q
- How to recognize false assignment of adducts on E and D
- No transglutaminase catalyzed adducts on E and D were found.
Figure 1.
Human transglutaminase 2-catalyzed incorporation of dansyl cadaverine onto Q53 of bovine beta casein (P02666). The added mass from dansyl cadaverine is 318.14 m/z. Boxed ions at 336.17, 402.18, and 447.21 m/z derive from the dansyl cadaverine adduct on glutamine (Q). The mass of the parent ion in charge state +3 \([\text{MH}]^+\) is 767.3395 m/z.
Figure 2.
Proposed structures of the characteristic ions associated with collision-induced fragmentation of the dansyl cadaverine adduct on glutamine. The dehydroglutamine portion of the 447.2 m/z ion is represented as a 6-membered ring because the ring structure accounts for the observed composition. The 5-membered ring in the 402.2 m/z ion is consistent with the indicated molecular composition. Both ring structures are consistent with structures reported for ε-amine modified lysine [14]. The positively charged dansyl cadaverine ion at 336.2 m/z dissociates from the adduct in the mass spectrometer.
Figure 3.
Bacterial transglutaminase-catalyzed incorporation of biotin cadaverine onto Q588 of human butyrylcholinesterase (P06276). The added mass from biotin cadaverine is 311.16 m/z. Boxed ions at 227.08, 329.22, 395.21, and 440.23 derive from the biotin cadaverine adduct on glutamine (Q). The mass of the parent ion in charge state +3 [MH]$^+3$ is 519.2578 m/z.
Figure 4.
Proposed structures of the characteristic ions associated with collision-induced fragmentation of the biotin cadaverine adduct on glutamine. A fragment at 227.1 m/z for biotin without cadaverine is also present in some MS/MS spectra.