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Identification of Formaldehyde-induced modifications in Diphtheria Toxoid

Introduction

The active pharmaceutical ingredient in diphtheria vaccines is diphtheria toxoid which is prepared by treatment of diphtheria toxin with formaldehyde and glycine. This detoxification process results in covalent modifications while maintaining immunogenicity. Detoxification is critical for both the safety and effectiveness of the vaccine. We studied these modifications by a combination of mass spectrometric techniques.

Methods

The workflow is shown in figure 1.

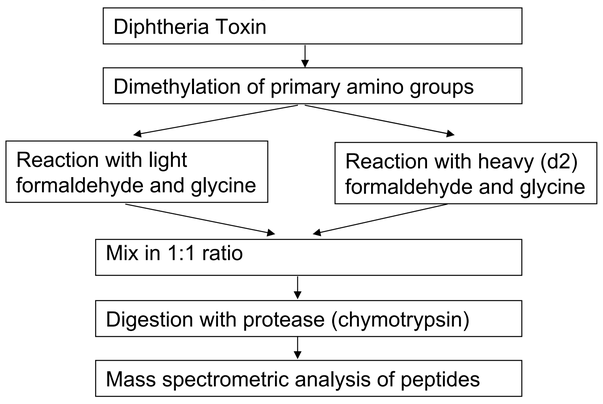

Figure 1. Workflow for the identification of formaldehyde-induced modifications.

- Reaction with formaldehyde and $NaCNBH_3$ to prevent the formation of inter- and intramolecular crosslinks [1].
- Reaction with formaldehyde (CH_2O or CD_2O) and glycine.
- Analysis of digests by nanoscale LC/MS [2] (LTQ Orbitrap XL, Bruker HCT Ultra).
- Allocation of mass spectral doublets by MsXelerator® software (MsMetrix, The Netherlands).
- Targeted CID, multistage CID and ETD fragmentation for sequence information.

Results

PROBLEM: Direct identification of chemical adducts in diphtheria toxoid by proteolytic digestion and LC-MS/MS analysis is complicated because most peptides are not modified. SOLUTION: The use of formaldehyde (CH_2O) and deuterium-labeled formaldehyde (CD_2O) allowed the unambiguous recognition of the modified peptides by their unique isotope pattern (Figure 2). In total, 126 modified peptides with one to eight incorporated formaldehyde molecules were identified by their unique mass spectral doublet.

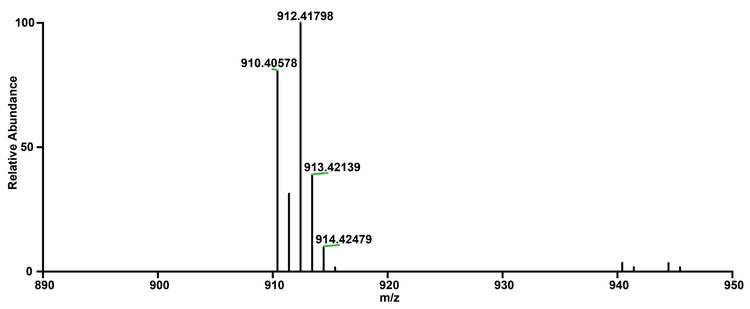

Figure 2. Deconvoluted (MH+) mass spectral doublet of a reaction product with one incorporated formaldehyde molecule.

From a previous study with synthetic peptides [3] we elucidated the structures of formaldehyde-glycine adducts. Arginine residues react with two formaldehyde molecules and one glycine molecule resulting in a net addition of $C_4H_5NO_2$ (M=99.032 Da). Tyrosine residues and, to a lesser extent asparagine, glutamine, histidine and tryptophan, residues react with one formaldehyde molecule and one glycine molecule to an adduct with a net addition of $C_3H_5NO_2$ (M=87.032Da).

High mass accuracy measurements (<5ppm) enabled us to predict a large number of peptides with chemical adducts, based on MS1 data. Peptides were fragmented by (multistage) CID and ETD to localize the sites of modifications.

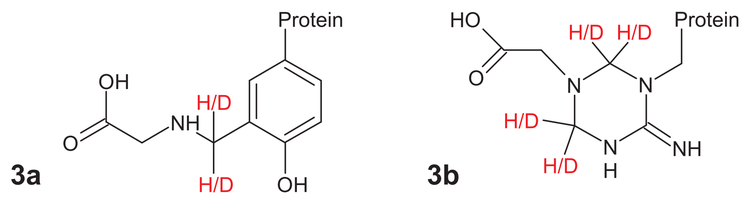

Figure 3. Proposed structures of a tyrosine residue (3a) and an arginine residue (3b) with formaldehyde-glycine adducts. The hydrogen or deuterium atoms in red originate from formaldehyde.

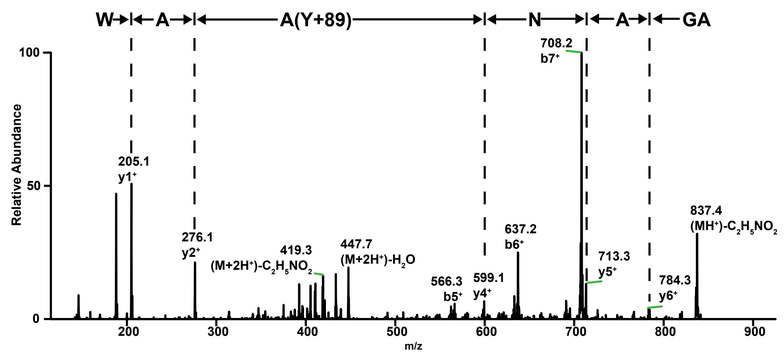

Figure 4a. CID MS/MS spectrum of peptide AGANYAAW with a formaldehyde-glycine adduct.

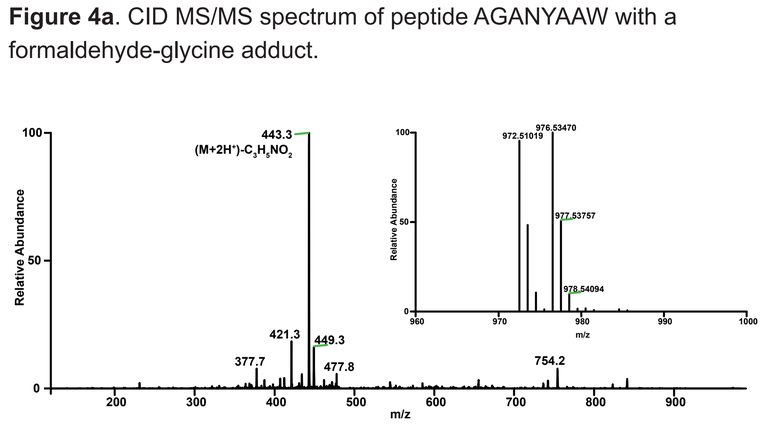

Figure 4b. CID MS/MS spectrum of precursor ion 486.76, low mass partner of doublet 486.76-488.77 (z=2).

PROBLEM: In some cases **neutral losses prevent assignment of modifications** (figure 4b). The spectrum shows a predominant neutral losses of 43.5 at z=2. Based on accurate mass measurements the suggested peptide is GDGASRVVL, but the location of the adduct could not be assigned. SOLUTION: Fragments were selected for **multistage fragmentation**. Neutral losses 37.5 (z=2) and 25 (z=3) Da were triggered for the 75 Da ($C_3H_5NO_2$) fragment loss, and 43.5/44.5 (z=2) 29/29.7 (z=3) were triggered for the 87/89 Da ($C_4H_5NO_2$) fragment loss.

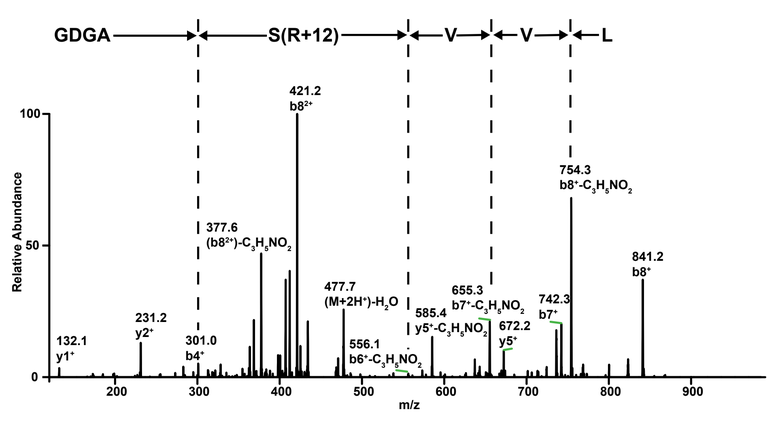

Figure 5. Multistage CID fragmentation spectrum of precursor ion 486.76 (z=2). The spectrum shows a complex mixture of fragment ions with complete +99 Da adducts and fragment ions with +12 Da mass tags remained after loss of an 87 Da neutral fragment. The adduct could be assigned to the arginine residue.

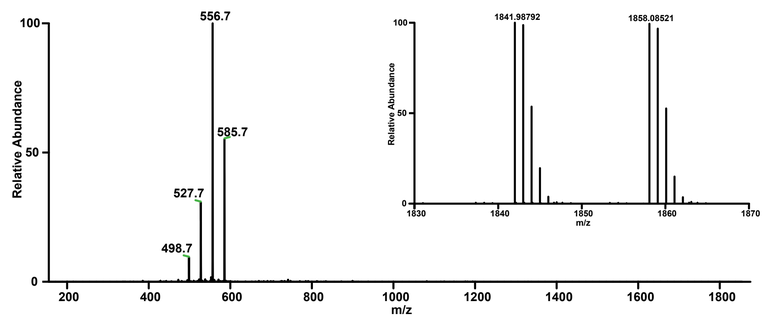

Figure 6. CID MS/MS spectrum of precursor ion 614.67, low mass partner of doublet 614.67-620.03 (z=3).

PROBLEM: The spectrum in figure 6 shows a pattern of neutral losses of 29 Da. Based on accurate mass measurements the suggested peptide is IKRFGDGASRVVL, but adducts could not be assigned to specific residues. Selecting one of the neutral loss fragments for multistage activation lead to disappearance of that specific neutral loss peak, but **no backbone fragmentation** occurred. SOLUTION: To assign formaldehyde-glycine adducts **ETD fragmentation** was performed with the same nanoscale LC system but now coupled to a Bruker HCT Ultra mass spectrometer with an ETD source.

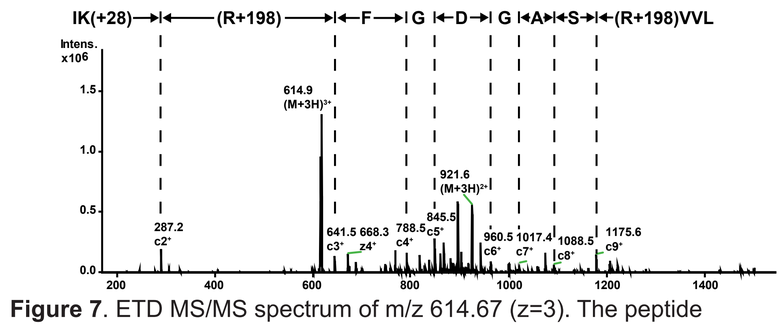

Figure 7. ETD MS/MS spectrum of m/z 614.67 (z=3). The peptide could be identified as IKRFGDGASRVVL with two formaldehyde-glycine adducts at both arginine residues.

ETD fragmentation enabled the localization of the modification sites. Formaldehyde-glycine adducts were detected on tyrosine, arginine and to a lesser extent, on asparagine residues.
We identified 58 of the 126 modified peptides and assigned the location of the formaldehyde-glycine adducts. For 51 modified peptides the sequence could be predicted by the accurate masses, sequences of 17 peptides are not yet identified.

Table 1. Modified peptides derived from toxicity related NAD+ and receptor binding sites.

MH+	Formaldehyde-glycine adducts	Assigned peptide sequence	Fragment
NAD+ binding site			
1416.7118	1	HGT(K+28)PG(Y+87)VDSIQ	21-32
1503.7438	2	HGT(K+28)PG(Y+174)VDSIQ	21-32
1451.6285	1	DAAG(Y+87)SVDNENPL	61-73
1538.6605	2	DAAG(Y+174)SVDNENPL	61-73
1542.6707	1	AEGSSSVE(Y+87)INNW	141-153
1629.7027	2	AEGSSSVE(Y+174)INNW	141-153
Receptor binding site			
1889.0491	1	G(Y+87)Q(K+28)TVDHT(K+28)VNS(K+28)L	513-527
1976.0811	2	G(Y+174)Q(K+28)TVDHT(K+28)VNS(K+28)L	513-527

Conclusion

By combining accurate mass, the number of formaldehyde-glycine modifications and CID, multistage and ETD fragmentation spectra, 85% of the mass spectral doublets were identified. Modified peptides derived from the catalytic domain and the receptor domain were found. Probably modifications in these functional sites contribute to the detoxification of the diphtheria toxin.

References

1: Jentoft et al, Methods Enzymol. (1983). 91, 570–579
2. Meiring et al, J.Pep.Sci. (2002) 25, 557-568
3: Metz et al, J. Biol. Chem. (2004) 279, 6235–6243

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