Comprehensive characterization of complex linkage structures in a bispecific monoclonal antibody (mAb) using electron activated dissociation (EAD)

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ABSTRACT

Here, a new fragmentation type based on EAD[1,2] was used to confirm the existence of disulfide and trisulfide linkages as well as free thiols in a bispecific mAb in which 2 different heavy chains and 2 identical light chains are linked. The data were acquired using a 10 Hz data-dependent acquisition (DDA) method and interpreted with Byos software (Protein Metrics Inc.). This workflow demonstrates a routine approach for the streamlined characterization of sulfide-linked peptides with a new level of data quality.

INTRODUCTION

The role of disulfide bonds is essential for maintaining tertiary and/or quaternary structures in proteins. Since the overall structure of proteins is related to their function, it is critical for the safety and efficacy of biotherapeutics to ensure that disulfide bond arrangements are as designed and expected. Existence of trisulfides can affect protein folding, structure and stability. In addition, they can further interfere with drug conjugation in case of cysteine-linked protein-drug conjugates. Characterization of linkage arrangements using bottom-up workflows has become increasingly popular due to advances in modern accurate mass spectrometry, novel fragmentation technologies and automated data processing software.[3,4] However, sulfide bonds prevent effective fragmentation around linked cysteine residues when using collision-induced dissociation (CID), which limits the information available for identifying sulfide-bound peptides as well as sulfide patterns. Although alternative fragmentation mechanisms exist, these techniques can suffer from long reaction times, low sensitivity and lack of reproducibility.

MATERIALS AND METHOD

Sample preparation: The bispecific mAb sample was prepared using the AccuMap Low pH Protein digestion kit (Promega) under non-reducing condition.

Chromatography: A total of 3 µL (4 µg) of the Lys-C digest were separated with a Waters ACQUITY CSH C18 column (2.1 × 100 mm, 1.7 µm, 130 Å) using an ExionLC AD system (SCIEX). The mobile phase consisted of water with 0.1% formic acid, while the organic phase was acetonitrile with 0.1% formic acid. A 60 min gradient profile was used at a flow rate of 250 µL/min. The column temperature was maintained at 50°C.

Mass spectrometry: Data were acquired using DDA with the ZenoTOF 7600 system (Figure 1). The electron energy for the alternative fragmentation in the EAD cell was set to a value of 7 eV.

Data processing: Data were processed using the workflow for disulfide analysis in Byos software (Protein Metrics Inc.).

Figure 1. The ZenoTOF 7600 system from SCIEX.

RESULTS & DISCUSSION

Disulfide-bonded peptides

Figure 2 shows an example of typical EAD and CID MS/MS spectra for a regularly sized disulfide-linked peptide with a molecular weight (MW) of 3,536 Da. Both EAD and CID achieved a high MS/MS coverage for this linked peptide (97% for EAD and 90% for CID). Among the dominant fragments observed in the EAD spectrum are the charge states +1 and +2 of the 2 corresponding peptides (green labels of peptide 1 and peptide 2 in Figure 2, top) derived from cleavage of the disulfide bond with EAD. That additional level of information obtained by EAD enables confirmation of the identity of the connected peptides, ruling out potential misassignments, especially in the case of low-abundance scrambled disulfide bonds.

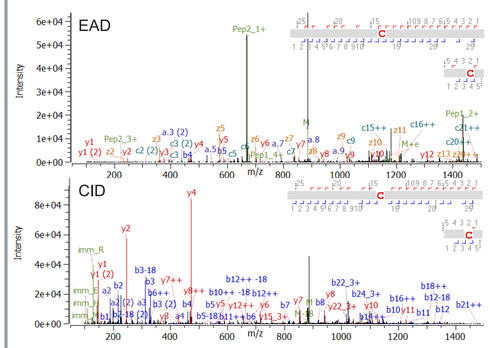

Figure 2. MS/MS spectra for an intrachain disulfide bond from the heavy chain. EAD spectrum (top), CID spectrum (bottom).

Figure 3 demonstrates an example of EAD and CID MS/MS spectra from a large and highly charged disulfide-linked peptide at *m/z* 952.617 with *z* = +8 and a MW of 7,612 Da. While CID is effective in fragmenting the termini of the connected peptides, the technique struggles to effectively dissociate the peptide backbones towards the center of the long sequences (red circles in Figure 3 right, bottom). Adversely, EAD can achieve a much more complete fragment coverage throughout the peptide backbone (Figure 3 right, top). For this disulfide peptide, Zeno EAD provided a 73% fragment coverage while Zeno CID only achieved a 45%, proving the higher fragmentation efficiency of EAD for long peptides compared to CID.

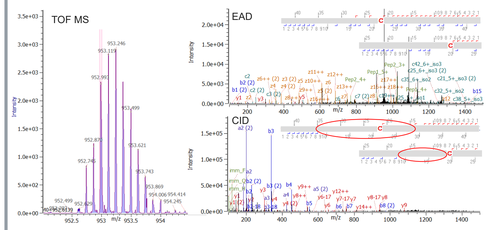

Figure 3. Data for disulfide-linked peptide with *z* = +8. TOF MS data for precursor with *z* = 8 (left hand side). EAD MS/MS and CID MS/MS spectra on the right hand side. EAD spectrum provides a much higher fragment coverage compared to CID spectra.

Identification of free thiols

To accurately identify existing free thiols, N-ethylmaleimide (NEM) is used as a capping agent to protect all existing free cysteines before enzymatic digestion. The capping agent introduces a 125 Da increase of the MW. Here, a low amount of free thiols were detected at different cysteine sites of the bispecific mAb sample. Figure 4 is an example of a peptide containing a free cysteine in the conserved region of the heavy chain of the bispecific mAb. Both Zeno EAD (Figure 4, top) and Zeno CID (Figure 4, bottom) provided highly descriptive spectra with full fragment coverage, which confirm the existence of a free thiol at the cysteine in this peptide. The fragments *c*7, *z*4 in the EAD spectrum and *y*4, *y*5, etc. in the CID spectrum all show a +125 amu increase, which can be attributed to the NEM capping of the free thiol. Besides the high level of sequence confirmation, EAD also offers the ability to differentiate leucine (Leu) from iso-leucine (Ile). In this particular peptide, 2 Leu residues present based on the theoretical sequence. Fragments *z*6-43 and *z*3-43 were detected in the MS/MS spectrum (encircled fragments in Figure 4, top), which verify that these 2 amino acids are both Leu. No evidence for Ile was found for this peptide.

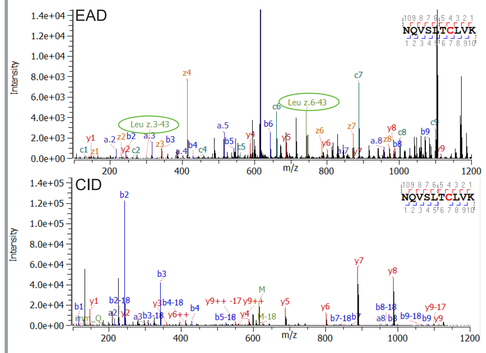

Figure 4. Identification of free thiol in a doubly charged peptide with both, EAD and CID. The free cysteines were capped with NEM. In addition to obtaining a full fragment coverage, EAD provided confirmation of Leu by diagnostic *w*-ions (encircled ions).

Trisulfide-bonded peptides

Trisulfide bonds are formed by the insertion of an additional sulfur into a disulfide bond, causing a shift of the MW by 32 Da. Although it is reported that trisulfides have no effect on antigen binding or on the stability of the therapeutic molecule, they are known to change protein folding and may cause challenges in case of additional bioengineering processes. Therefore, the identification and close monitoring of this post-translational modification is necessary. A high abundance of trisulfides was detected in the studied molecule at the interchain bonds between heavy and light chains. As expected for trisulfide-bonded peptides, a 32 Da increase was observed for the MW of the precursor compared to the disulfide-bonded peptide. Further evidence was found using MS/MS information. Figure 5 shows the comparison of the MS/MS spectra of the disulfide- and trisulfide-containing peptide using CID. As the precursor is triply charged, a 10.67 amu increase was observed in the spectrum of the trisulfide-bonded peptide. Meanwhile the *y*5++ fragments presented a 16 amu shift.

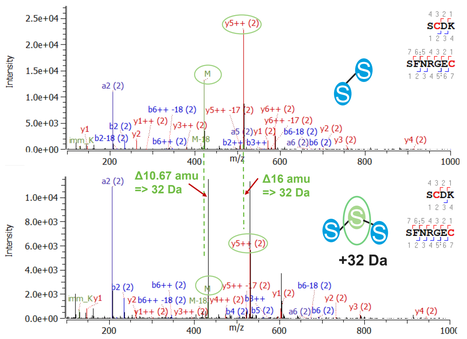

Figure 5. CID MS/MS spectra for an interchain disulfide-linked peptide (top) and the peptide with an interchain trisulfide (bottom). The precursor mass (indicated as M in the spectra) shows an *m/z* difference of 10.67 amu, which corresponds to a mass difference of 32 Da in the case of a triply charged precursor. This difference can be linked to an additional sulfur (trisulfide bond). The corresponding *m/z* shift of 16 amu can also be observed for the doubly-charged the y5-ion.

The EAD spectrum for the trisulfide-containing peptide is shown in Figure 6. As previously discussed, EAD can dissociate disulfide bonds, resulting in signals for the 2 cleaved peptides in the MS/MS spectrum. Furthermore, EAD can dissociate trisulfide bonds, leading to 4 different fragments: peptide 1, peptide 1 + sulfur, peptide 2, peptide 2 + sulfur (Figure 7). While the signals for peptide 1 and peptide 2 in the MS/MS spectrum give further confidence in the identity of both linked peptides, the fragments of the respective peptide with additional sulfur can be used as diagnostic ions to confirm the existence of trisulfide bonds with very high confidence. Neither of these fragments can be obtained by CID.

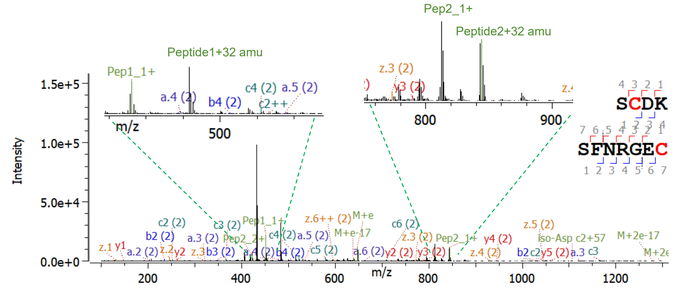

Figure 6. EAD MS/MS spectrum for interchain trisulfide-linked peptide. The peptides shown contain an interchain disulfide bond between heavy chain and light chain. Insets show evidence of both peptides 1 and 2 and each of the peptides with an additional *m/z* shift of 32 amu corresponding to an additional sulfur, which increases the confidence in the correct identification of a trisulfide bond.

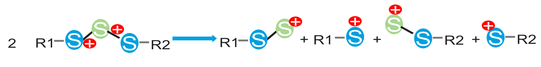

Figure 7. Schematic of the dissociation of a trisulfide-bond with EAD.

CONCLUSION

- The existence of trisulfide bonds was confidently confirmed through signature ions generated by DDA with Zeno EAD: a 1-injection platform method without the need for optimization
- Confident sequence and disulfide linkage confirmation of long disulfide-bonded peptides were achieved with EAD, showing superior fragmentation coverage compared to CID
- Free thiols were detected at low levels with high confidence using EAD, even in case of low-charged precursors
- In addition to studying sulfide arrangements, amino acid isomers can be differentiated with the same Zeno EAD data, enabling high efficiency to obtain a wealth of information on the analyte of interest
- Automatic, state-of-the-art data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using Byos software from Protein Metrics Inc.

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