Leveraging intrachain disulfide bonds for improved sequence coverage in electron capture dissociation (ECD) middle-down analysis

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ABSTRACT

Middle-down sequencing of NIST mAb subunits of LC, Fd', Fc/2 and HC with intrachain disulfide bonds reduced and nonreduced were analyzed using ECD fragmentation. Middle-down sequencing of the subunits with nonreduced intrachain disulfide bonds significantly improved the fragment identification between the two linked disulfide bonds. Overall sequence coverage was improved by combining the fragment data of the subunit with reduced and nonreduced intrachain disulfide bonds.

Sequence coverage obtained from Fd' subunit between the two intact intrachain disulfide bonds

Sequence coverage obtained from Fd' subunit between the two reduced intrachain disulfide bonds

INTRODUCTION

Top-down and middle-down mass spectrometry approaches, in which intact proteins or large subunits are directly fragmented in the mass spectrometer have gained more interest in recent years[1-3]. One challenge of such analysis is obtaining sequence of the middle portion of the protein, where the diagnostic fragments are particularly vulnerable for secondary and tertiary fragmentation yielding shorter terminal fragments together with uninformative internal fragments[4].

In this work, we performed partial disulfide bond reduction of NIST mAb subunits to retain the intrachain disulfide bonds. Preserved disulfide bonds protect larger fragments from secondary fragmentation in ECD and significantly improve sequence coverage in the region between the two disulfide bridges. When combined with data from complete reduction of disulfide bonds, increase in sequence coverage was observed.

MATERIALS AND METHODS

Sample preparation

For preparation of partially reduced IdeS subunit samples, 1 µg/µL NIST mAb was digested with IdeS then reduced in 20mM TCEP and alkylated in 35 mM Iodoacetamide using iodoacetamide following a standard protocol. For the NIST mAb heavy chain subunits, the reduction was performed in 100mM DTT. For completely reduced samples an extra denaturing step in 5M guanidine was applied prior to the reduction step.

HPLC

Separation was performed on a Shimadzu LC-30AD chromatographic system with a Waters BEH C4 column (2.1 mm × 50 mm, 1.7 µm particles, 300 Å pore) at 60 ° C using 0.1% formic acid in water as mobile phase (A) and 0.1% formic acid in acetonitrile as mobile phase (B). The gradient started with 10% B for 2 minutes, then 10-30% B from 2-4 minutes, finally 30-40% B from 4-7 minutes. The flow rate was set at 300 µL/min.

MS/MS

Middle-down data was acquired on a SCIEX ZenoTOF 7600 using EAD fragmentation with TOF mass range set to 200-2000 Da. The most intense precursor charge state was selected as the precursor for MS/MS: 20+/30+, 18+/25+, 20+/28+ and 39+/54+ for intrachain disulfide bond nonreduced/reduced subunits of Fc/2, LC, Fd' and HC. ECD parameters were optimized and set at KE 0 eV, electron beam current 1500 nA, reaction time 5 ms and ECD RF 150. ETC was set at 100% for IdeS subunits and 50% for HC. Source temperature was set at 400ºC with GS1 and GS2 both at 50 psi. Ion spray voltage was set at 0 V for the first 2 minutes then increased to 4500 V for the rest of the data acquisition.

Data Processing

Data were processed using the BioTools function in a non-commercial research version of PeakView software from SCIEX. Fragment mass tolerance was set at 10 ppm. Only c' and z• fragment ions were included in the search to minimize false positives.

RESULTS

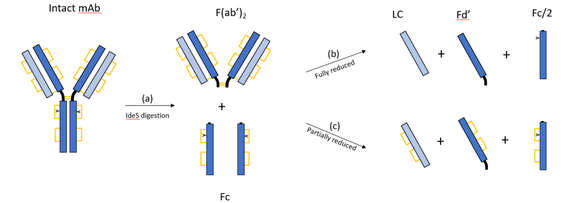

Figure 1. Intact mAb sample, following (a) IdeS digestion (b) complete reduction with all inter and intra chain disulfide bonds reduced (c) partial reduction with only interchain disulfide bonds reduced but intrachain disulfide bonds intact. The yellow lines indicate the disulfide linkages.

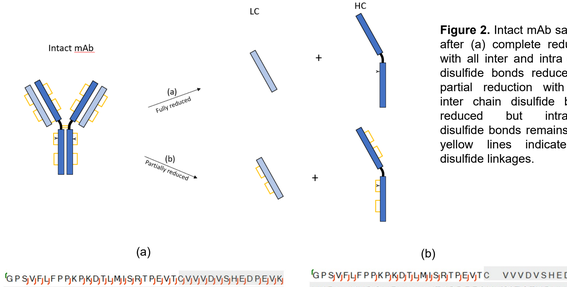

Figure 2. Intact mAb sample, after (a) complete reduction with all inter and intra chain disulfide bonds reduced (b) partial reduction with only inter chain disulfide bonds reduced but intrachain disulfide bonds remains. The yellow lines indicate the disulfide linkages.

Figure 3. Sequence coverage map of intrachain disulfide bond reduced (a) and nonreduced (b) Fc/2_G0F subunit of NIST mAb. The fragment identification of intrachain reduced and nonreduced subunits were complementary, the overall sequence coverage was improved by combining the sequence coverage from intrachain disulfide bond reduced and nonreduced data. The sequence coverage obtained was 65.1%, 34% and 75.1% for reduced, nonreduced and combined Fc/2_G0F, respectively. The shaded regions indicate amino acid sequence enclosed by intrachain disulfide bonds. Red and green lines represent c' and z• ions, respectively.

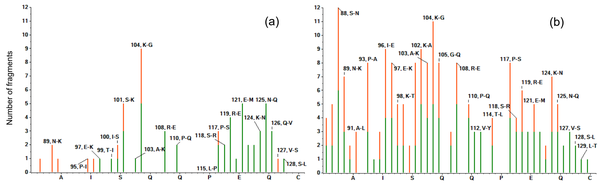

Figure 4. Fragments identified between C[85] to C[131] for intrachain disulfide bond reduced (a) and nonreduced (b) Fc/2_G0F subunit of NIST mAb. The number of fragments identified in this region are 57 and 196 for fully reduced and partially reduced Fc/2_G0F, respectively. Red and green lines represent c' and z• ions, respectively.

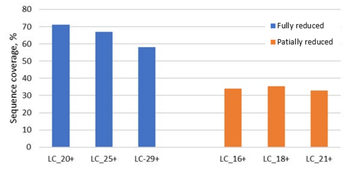

Figure 5. Sequence coverage obtained from three different charge states of the LC subunit of intrachain disulfide bond reduced (blue) and nonreduced (orange) LC. For fully reduced LC, increase of sequence coverage was observed with lower charged precursor. For partially reduced LC, similar sequence coverage was obtained from all three precursor charge states. The total sequence coverage obtained from all three precursor charge states are 85.8% for fully reduced LC and 38.7% for partially reduced LC with a combined sequence coverage of 92%.

Figure 6. Sequence coverage of intrachain disulfide bond reduced (top) and nonreduced (bottom) HC subunit of NIST mAb. The sequence coverage obtained was 30.1%, 21.9% and 40.8% for reduced, nonreduced and combined HC, respectively. The shaded regions indicate amino acid sequence enclosed by intrachain disulfide bonds. Red and green lines represent c' and z• ions, respectively.

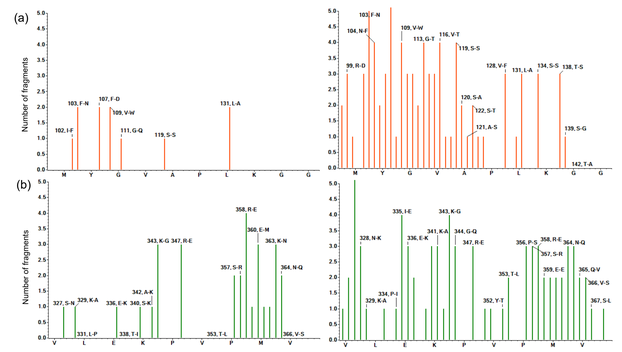

Figure 7. Fragments identified between C[97] to C[147] (a) and C[324] to C[370] (b) from fully reduced (left) and partially reduced (right) HC subunit of NIST mAb. Red and green lines are c' and z• ions, respectively.

CONCLUSIONS

Intrachain disulfide linkage significantly improves the fragment identification in the region between two disulfide bridges of the NIST mAb subunits in middle-down mass spectrometry using ECD fragmentation. Improved total sequence coverage was obtained by combining the data from fully and partially reduced subunits.

REFERENCES

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