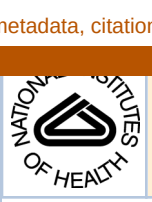

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A Simple Approach to Assign Disulfide Connectivity Using Extracted Ion Chromatograms of Electron Transfer Dissociation Spectra

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Abstract

Increasing interest in production of protein-based pharmaceuticals (biotherapeutics) is accompanied by an increased need for verification of protein folding and correct disulfide bonding. Recombinant protein expression may produce aberrant disulfide bonds and could result in safety concerns or decreased efficacy. Thus, the thorough analysis of disulfide bonding is a necessity for protein therapeutics. The use of ETD facilitates this analysis because disulfide bonds are preferentially cleaved when subjected to ETD. Here, we make use of this well-characterized reaction to assign disulfide bonding networks by coupling the use of extracted ion chromatograms (XICs) of cysteine-containing peptides with ETD analysis to produce an efficient assignment approach for disulfide bonding. This method can be used to assign a disulfide pattern in a *de novo* fashion, to detect disulfide shuffling, and to provide information on heterogeneity, when more than one disulfide bonding pattern is present. The method was applied for assigning the disulfide-bonding network of a recombinant monomer of the HIV envelope protein gp120. It was found that one region of the protein, the V1/V2 loops, had significant heterogeneity in the disulfide bonds.

Introduction

Correct protein conformation is often vital to the protein's activity, and post-translational modifications such as disulfide bonds have substantial roles in maintaining the native fold. Disulfide bonds play a major structural role in proteins by introducing three-dimensional constraints on distant portions of protein. Disulfide bonds have been shown to have a variety of functions in proteins including facilitating viral entry[1], maintaining activity[2–4], and thermostability.[5] Maintaining and verifying correct disulfide bonds becomes increasingly important as more recombinantly-expressed proteins are used in vaccines and therapeutics. Variation between batches could signal a problem with the production, purification, formulation, or storage of the protein therapeutic. Non-native disulfide bonds could potentially mean decreased efficacy or cause adverse reactions to the use of the pharmaceutical.

The assignment of disulfide bonds is difficult because proteins usually contain multiple cysteine residues, and every additional cysteine adds another layer of complexity to the analysis. Mass spectrometry is the preferred technique for disulfide mapping because changes in mass due to formation of a disulfide bond can be detected with common commercial instruments, and fragmentation can be performed to verify the assignment. Collision induced dissociation (CID) is the most common dissociation technique for peptides, and has been previously applied for analysis of disulfide-bonded peptides.[8–12] Several new techniques for disulfide bond analysis have been developed recently, and they

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greatly facilitate disulfide assignment; these methods include electron capture dissociation[13], electron transfer dissociation[14], hydroxyl radical addition with subsequent CID[15], online electrolytic cleavage[16], free radical initiated peptide sequencing with o-TEMPO-Bz-conjugated peptides[17], and direct ultraviolet photodissociation.[18]

Electron capture dissociation (ECD) and electron transfer dissociation (ETD) have been widely implemented in commercially-available instruments. Normal, linear peptides are known to fragment into *c* and *z* ions during ECD and ETD. However, when disulfide-bonded peptides are subjected to ECD and ETD, highly abundant peaks for each linear peptide are observed due to homolytic cleavage of the disulfide bond. Recently, Wu et al. has made use of this unique radical reaction to map disulfide-bonded peptides of monoclonal antibodies and other recombinant proteins.[21–23] However, the approach used by Wu requires tandem mass spectrometry using CID MS^2, ETD MS^2, and MS^3 involving ETD followed by CID on each of the fragments. Subsequent CID fragmentation of each ETD fragment provides more information about that ion but at a detrimental cost to the duty cycle. Other areas for improvement are also apparent for the aforementioned approach; it requires an intense precursor signal due to inefficiencies from alternative fragmentation pathways and it does not flag spectra that may be of interest due to specific ions detected. Commercial instruments are capable of taking thousands of spectra for every LC-MS injection, and identifying which of those spectra are relevant to disulfide bond analysis is a daunting task. It would be beneficial to expedite analysis by having a method that alerts the researcher to spectra of interest so that they may be targeted for further investigation.

Here, we demonstrate a unique and highly efficient approach to disulfide mapping using the homolytic reaction that disulfides undergo during ETD. Instead of searching the thousands of tandem mass spectra typically collected, searching extracted ion chromatograms offers a way to expedite the data analysis. This approach typically reduces the number of spectra searched for a specific peptide to about 1% of the total spectra collected. Making use of extracted ion chromatograms (XICs) of the *m/z* values of cysteine-containing peptides allow a method of searching for disulfide pairs or bonding partners. The method was tested with several model proteins and a recombinant monomer of gp120, the envelope protein on the HIV-1 virus.

Experimental

Materials and Reagents

Albumin from bovine serum (BSA), chicken lysozyme, human apo-transferrin, dithiothreitol (DTT), 4-vinylpyridine (4-VP), iodoacetamide (IAM), acetonitrile, formic acid, acetic acid, Tris-HCl, and Tris base were obtained in high purity from Sigma-Aldrich (St. Louis, MO). The HIV-1 Env 1086.C gp120 was expressed and purified in the laboratory of Dr. Barton F. Haynes, Duke Human Vaccine Research Institute (Duke University, Durham, NC), using the method described elsewhere.[25] Peptide N-glycosidase F (PNGase F) purified from *Flavobacterium meningosepticum* was purchased from New England BioLabs (Ipswich, MA). Sequencing-grade trypsin was obtained from Promega (Madison, WI). Ultrapure water was obtained from a Millipore Direct-Q3 filtration system (Billerica, MA).

Reduction and Alkylation

About 300 μg of lysozyme and BSA were reduced with 5 mM DTT for 1 hour in the dark at room temperature and alkylated with either 8 mM 4-VP or IAM for 1 hour in the dark at room temperature. Tryptic digestion was carried out at 37 °C for 18 hours at a 1:30 enzyme-to-protein ratio (w/w). The digestion was stopped by the addition of 1 μL concentrated formic acid per 50 μL of digestion volume, and the resulting protein digest mixture was analyzed by reversed phase HPLC/ESI-LIT MS and ETD MS/MS.

Non-reduced Protein Digestion

About 300 μg of lysozyme and transferrin and 75 μg of 1086.C gp120 were alkylated with 5 mM IAM in the dark at room temperature to cap free cysteine residues prior to enzymatic digestion. Following alkylation with IAM, 1086.C gp120 was deglycosylated with 2 μL of PNGase F enzyme solution (≥10,000 units/mL) and was incubated at 37 °C for 72 hours. All three proteins were digested with trypsin. In-solution trypsin digestion was carried out at 37 °C for 18 h at a 1:30 enzyme-to-protein ratio (w/w). The digestion was stopped by the addition of 1 μL concentrated formic acid per 50 μL of digestion volume and the resulting protein digest mixture was analyzed by reversed phase HPLC/ESI-FTICR MS and HPLC/ESI-LIT-ETD MS/MS.

LC/ESI-FTICR MS Analysis

The tryptic digests were subjected to reversed phase-high performance liquid chromatography (RP-HPLC, Dionex, Sunnyvale, CA) coupled with a hybrid linear ion trap Fourier transform-ion cyclotron resonance (LTQ FTICR) mass spectrometer equipped with a 7 T actively shielded magnet (Thermo Scientific Corp, San Jose, CA). The mobile phase A was 99.9% water with 0.1% formic acid. Mobile phase B was 99.9% acetonitrile with 0.1% formic acid. Approximately 5 μL of the sample was injected and separated on a C18 PepMap 300 column (150 mm × 300 μm i.d., 5 μM, 300 Å; LC Packings, Sunnyvale, CA) at a flow rate of 5 μL/min. The tryptic peptides were eluted using the following gradient, which was modified from the method described in the literature.[26] The mobile phase initially contained 2% B and the level of B increased linearly to 40% over 30 min, then ramped to 90% B over 20 min. The column was re-equilibrated after holding at 90% B for 10 min.

The samples were infused into the electrospray ion source, and the hybrid LTQ FTICR data acquisition was performed in a data-dependent scanning mode. Briefly, the MS^1 spectra were recorded in an FT MS scan and the six most abundant peptide ions in the MS scan were sequentially selected for CID performed in the LTQ mass analyzer, with 35% normalized collision energy and a 3 min dynamic exclusion window.

Electrospray ionization was achieved with a spray voltage of ~3.0 kV. Nitrogen was used as a nebulizing gas and set at a pressure of 10 psi. The capillary temperature was maintained at 200 °C. All data were acquired in the positive-ion mode and analyzed using Xcalibur 2.0 software (Thermo Scientific Corp, San Jose, CA).

LC/ESI-LIT-ETD MS/MS Analysis

The tryptic digests were subjected to reversed phase-high performance liquid chromatography (RP-HPLC, Waters Acquity, Milford, MA) coupled with a dual linear ion trap (LTQ Velos) mass spectrometer equipped with electron transfer dissociation (Thermo Scientific Corp, San Jose, CA). The mobile phase A was 99.9% water with 0.1% formic acid. Mobile phase B was 99.9% acetonitrile with 0.1% formic acid. Approximately 5 μL of the sample was injected and separated on an Aquasil C18 column (150 mm × 300 μm i.d., 5 μM, 300 Å; Thermo Scientific, San Jose, CA) at a flow rate of 5 μL/min. The tryptic peptides were eluted using the following gradient. The mobile phase initially contained 3% B and the level of B increased linearly to 50% over 40 min, then ramped to 80% B over 20 min. The column was re-equilibrated after holding at 80% B for 10 min.

The samples were infused into the electrospray ion source, and the LTQ Velos data acquisition was performed in a data-dependent scanning mode. Briefly, the MS^1 spectra were recorded and the three most abundant ions in the MS scan were sequentially selected for ETD performed in the LTQ Velos mass analyzer with a two minute dynamic exclusion

window. A reaction time of 100 ms was used and the fluoranthene signal was optimized to approximately 1–6×10^7 counts prior to instrument use.

Electrospray ionization was achieved with a spray voltage of ~3.0 kV. Nitrogen was used as a nebulizing gas and set at a pressure of 10 psi. The capillary temperature was maintained at 250 °C. All data were acquired in the positive-ion mode and analyzed using Xcalibur 2.1 software (ThermoElectron Corp, San Jose, CA).

Results and Discussion

Method Overview

A pictorial representation of the method used to assign disulfide bonds of dipeptides is shown in Figure 1. Briefly, ETD data of proteolyzed proteins is acquired in a data-dependent fashion throughout the chromatographic run, and these data are searched by plotting extracted ion chromatograms (XICs) for each cysteine-containing peptide in the protein. Because disulfide linked peptides fragment efficiently across their S-S bond in ETD, plotting the XICs of the individual peptides, as shown in Figure 1, readily identifies the tandem mass spectra where each of the cysteine-containing peptides are present. The method we describe here involves interrogating each of these peaks and assigning them in order to assign the complete disulfide bonding in any given protein. The key advantage of this approach, over other competing approaches, is that the masses of the disulfide-linked peptides do not have to be known, or calculated, in advance. Instead, only the masses of the cysteine-containing peptides must be known, and the disulfide bonding pattern can be deduced, without interrogating the data file for precursor ions of a given mass. Additionally, because each peak in the XIC is interrogated, all the disulfide heterogeneity in a protein can be accounted for in an expedient manner.

When these cysteine-containing peptides are disulfide bonded to just one other peptide, the dipeptides are identified as pairs of peaks in the XIC plots. As an example, the top two XICs in Figure 1 are for the peptides GCR and CELAAAMK. These two XICs each have a peak at 25.13 minutes. The peak corresponds to the ETD data for this disulfide-linked pair. Figure 1 shows six examples of XICs for six different peptides, and three disulfide pairs are assigned. One can note that in addition to the disulfide pairs, other peaks appear in the XICs. Each of these peaks are also interrogated, and their identity is assigned as either a disulfide linked multipeptide (where more than two peptide chains are connected), an internally linked, disulfide bonded peptide, a peptide capped with alkylating reagent, or an interference, as described in Figure 2. Each of these special cases is elaborated upon later.

In addition to using the XIC data to assign the disulfide-bonded peptides, each assignment can be quickly validated in two additional ways: by validating masses of the assigned precursor ions and verifying that CID data supports the assignment. To determine the precursor ion mass, either high resolution data can be obtained or the charge state of the low resolution ETD spectrum can be deduced, so that the precursor's mass can be calculated. To determine the charge state from the ETD spectrum, the data is queried to identify charge-reduced precursor ions. An example ETD spectrum, inset in Figure 1, shows the charged-reduced precursor ions in ETD data of disulfide-linked peptides are generally quite abundant. In this case, the charge reduced precursors at *m/z* 758.8 and 1518 can be used, along with the precursor ion, at *m/z* 505.9, to infer the charge state of the precursor is three. Using this information, the mass of the precursor ion can be calculated to be 1518, and this mass is equal to the sum of the two peptides assigned to be disulfide linked in this spectrum, thereby validating the assignment. For additional certainty, CID spectra can be acquired on the same peptide, and the CID data can be interrogated to verify that the peak is correctly assigned.

Special Cases

Case 1: Assigning Multipeptide Species—Disulfide linked peptides with three or more peptide chains are assignable with minor modifications to the above method. In these cases, generally two of the three peptide chains are detected individually as cleaved peptides in the XICs, but the masses of these two species do not add to the mass of the precursor ion. In a disulfide linked tripeptide, at least one peptide must contain two or more cysteine residues, and this peptide usually remains attached to one of its partner peptides during ETD. These tripeptide disulfides can be readily assigned because when the masses of the peptides with a single cysteine, which are detectable in the XICs, are subtracted from the precursor mass, the resulting mass perfectly matches that of the tryptic peptide with two cysteine residues. So the identity of each of the three peptides is determined.

Figure 3 shows ETD data for two tripeptides from transferrin that could be readily assigned using this strategy. Figure 3A was identified as an important ETD spectrum because two peptides, at *m/z* 1057 and 685, each had an intense peak in their XIC at 36.51 minutes. Yet, the ETD spectrum at this retention time (Figure 3A) clearly did not correspond to these peptides being linked as a dipeptide. Rather, the mass of the precursor indicated that an additional peptide of 1703 Da was present. Therefore, the tripeptide, as shown in the figure, was assigned, and ions corresponding to the β+γ ion and α+β ion were identified, further supporting the assignment. The α+β ion is detected at *m/z* values 1908 and 1273 in the 2+ and 3+ charge states, respectively, and the β+γ ion is detected at *m/z* 1194 in the 2+ charge state. Figure 3B shows a second example of ETD data from a tripeptide that was assigned in the same manner.

Case 2: Assigning Free Cysteine—Any protein may contain free cysteine residues, which do not participate in disulfide bonding. To characterize these non-linked cysteine residues using our XIC/ETD approach, all readily observable peaks present in the XICs that are not assigned as disulfide linked peptides are further evaluated. For these peaks, when the precursor ion mass is equivalent to the peptide mass plus the mass of the capping reagent, (for example, plus 57 Da when IAM is used), the peak is assignable as an alkylated peptide, originating from a protein with a free cysteine in it.

The above-described approach requires that the peptide/alkylating reagent bond is cleaved each time the alkylated peptide is subjected to ETD. Therefore, to identify an ideal alkylating reagent for this work, two commonly used alkylating reagents, iodoacetamide and 4-vinylpyridine, were tested extensively, and ETD data for alkylated peptides are shown in Figure 4. Of these two choices, IAM was identified as an ideal capping reagent. When peptides alkylated with iodoacetamide were subjected to ETD, the peptide mass marker ions were among the most intense peaks in the spectrum, and the only peaks with a higher abundance were precursor, charge-reduced precursor, and neutral loss ions (Figure 4A and B). In contrast, the peptides alkylated with 4-vinylpyridine did not show a loss of the alkylating reagent to reveal the peptide mass marker ions of *m/z* 1268.7 and 936.5 in Figure 4C and D respectively. Therefore, 4-vinylpyridine is not a useful reagent, when the goal is to identify capped cysteine residues by evaluating the XICs of cysteine-containing peptides.

Case 3: Internally linked disulfides—The peaks in the XICs may also be due to a single tryptic peptide with two cysteine residues, where those two cysteine residues are connected to each other. The XICs can be used to preliminarily identify the internally linked peptides, because in those cases, the peak in the XIC must correspond to a peptide with two cysteine residues in it; it must not have a “partner”, a peak in any other XIC with the same retention time; and the XIC peak must correspond to the mass of the precursor or one of the charge-reduced species. This case is highlighted in Figure 2.

Case 4: Interferences—Not every peak in the XICs generated by this method can be assigned to a disulfide linked or capped cysteine-containing peptide. The peaks may also be due to interferences such as linear peptide precursors, charge-reduced precursors, and fragment *c* or *z* ions. These peaks are quickly evaluated and can be disregarded when they do not fulfill any of the criteria specified above; namely, they do not have a "partner," an ion in another XIC that has an identical retention time, and they are not assignable as internally linked peptides or peptides capped with an alkylating reagent.

Using the above outlined approach, the disulfide bonding in two model proteins, transferrin and lysozyme, was assigned, and in each case the XIC/ETD method provided assignment information that was consistent with the known disulfide bonding pattern of the proteins. The assignments made for transferrin are presented in Table 1 and lysozyme assignments are provided in Supplemental Table 1. All cysteine-containing peptides were detected in their accepted disulfide map with the exception of one from transferrin. This peptide (shown in red in Table 1) consists of three intrachain disulfide bonds and was not detected.

The aforementioned method was also used to investigate the disulfide-bonding patterns of a recombinant monomer of the HIV-1 envelope protein 1086.C gp120. This protein has 18 cysteine residues on 16 tryptic peptides. The protein sequence is available in Supplemental Figure 1. To do a complete *de novo* analysis of the disulfide bonding of this protein using traditional approaches where the potential precursor ions of all the possible disulfide linked pairs are calculated and searched, 426 precursor masses would need to be searched for, each in multiple charge states. In practice, more than a thousand precursor ions would have to be individually searched for in the MS^1 data. Instead, our method simply uses the 16 XIC plots for each tryptic peptide, and infers the disulfide bonding from these plots.

Two of the XICs from the gp120 data are shown in Figure 5, along with the assignments for the peaks. For these two chromatograms, three different types of species are present: a dipeptide disulfide is detected, along with two different multipeptide disulfides. Finally, several spectra that correspond to interferences were also observed. These assignments were based on the approach outlined in Figure 2. After preliminary assignment of each peak in the XIC (as dipeptide disulfide, multipeptide disulfide, interference, etc.), each assignment was confirmed with high resolution MS data along with CID data.

The aggregate results from the disulfide mapping of the gp120 protein are shown in Table 2. These data point to several interesting observations. First, substantial heterogeneity is observed in the first and second variable regions, the V1 and V2 regions, of the protein, which includes the cysteine residues on the 2^{nd} –5^{th} tryptic peptides. Yet, outside that part of the protein sequence, all other cysteine residues proved to be exceedingly faithful to their respective partners and were found only bonded to one partner peptide. Additionally, no alkylation of peptides was detected. Aside from the heterogeneity in the V1/V2 region, all the disulfide assignments are consistent with those published by Leonard *et al.* in the early 1990's on a different gp120, where Edman sequencing was used to assign the peptides.[27] While heterogeneity in the V1/V2 region was not reported in the earlier work, it has been reported by us on a similar protein, an oligomeric gp140.[10]

The XIC/ETD method presented here was not able to identify all disulfide assignments presented in Table 2. One dipeptide, TFDGTGPCR – CNDK, could not be assigned in the ETD data. Searching the high resolution data revealed the cause: The peptide is small, hydrophilic, and has a short retention time. It co-eluted with several other small peptides and unidentified species such that the signal intensity of this species was probably negatively impacted. CID data was acquired on this species, but due to the longer reaction time of ETD, this peak was not selected for fragmentation. Additional efforts in chromatography and/or a

re-design of the ETD data-dependent scanning method could alleviate or mitigate this problem in future analyses. This disulfide assignment was made by confirming the mass of the disulfide-bonded peptide in the high resolution FTICR-MS data and by assigning the CID data associated with this peak.

Conclusion

We developed a new method to map disulfide bonds using extracted ion chromatograms of ETD tandem mass spectra and implemented it to assign the disulfides of a recombinant HIV-1 envelope protein, 1086.C gp120. Before using this method to analyze gp120, the disulfides of two model proteins, lysozyme and transferrin, were assigned to validate the method. These model proteins have a vast array of different disulfide connectivities including intrapeptide disulfides, dipeptides, and tripeptides. Assignments were in agreement with the accepted disulfide linkages reported in Uniprot. Free cysteine residues were mimicked by purposely reducing and alkylating proteins.

This method was then used to map the disulfide bonds in recombinant HIV-1 Env, 1086.C gp120. The method was able to assign the bonding partners of 16 of the 18 cysteine residues and showed that select cysteine residues had several different bonding configurations. While several different structures resulted from analysis of the peptides in the V1/V2 loops, all other disulfide bonds in the protein proved to be exceedingly homogenous and only bonded with their expected partner peptide. The only disulfide peptide pair not assigned was detected but eluted with several other species and was not selected for ETD due to decreased signal.

The method outlined here has proven to be an expedient way of assigning the disulfide bonds of both model proteins and the various structures formed in a recombinant HIV-1 envelope protein. While the method still requires the logical capacity of a human to sort out some of the more complex cases, implementation into a computer program to automate the analysis of disulfide bonds is feasible. With or without automation, the method described herein, combining ETD and XIC’s, is a large step forward for assigning disulfide bonds in any protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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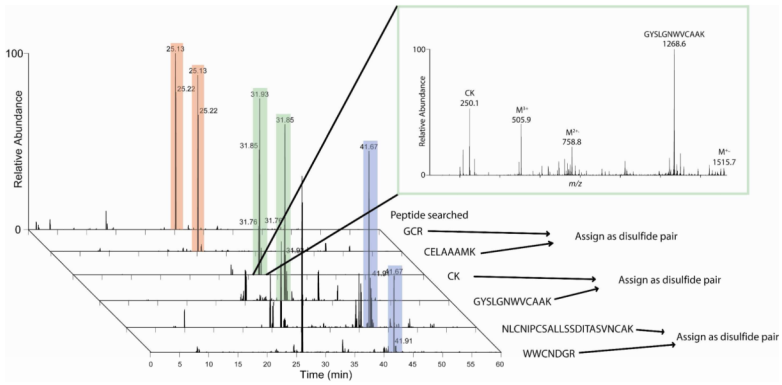

Figure 1.

Assigning peptides with one disulfide bond. Extracted ion chromatograms are constructed to show where peptide mass marker ions of cysteine-containing peptides elute. Co-eluting peptides are assigned as disulfide pairs after further scrutiny, as described in the text. The inset shows an example mass spectrum of the peptides GYSLGNWVCAAK and CK that were disulfide bonded and dissociated in ETD. Data from lysozyme are shown.

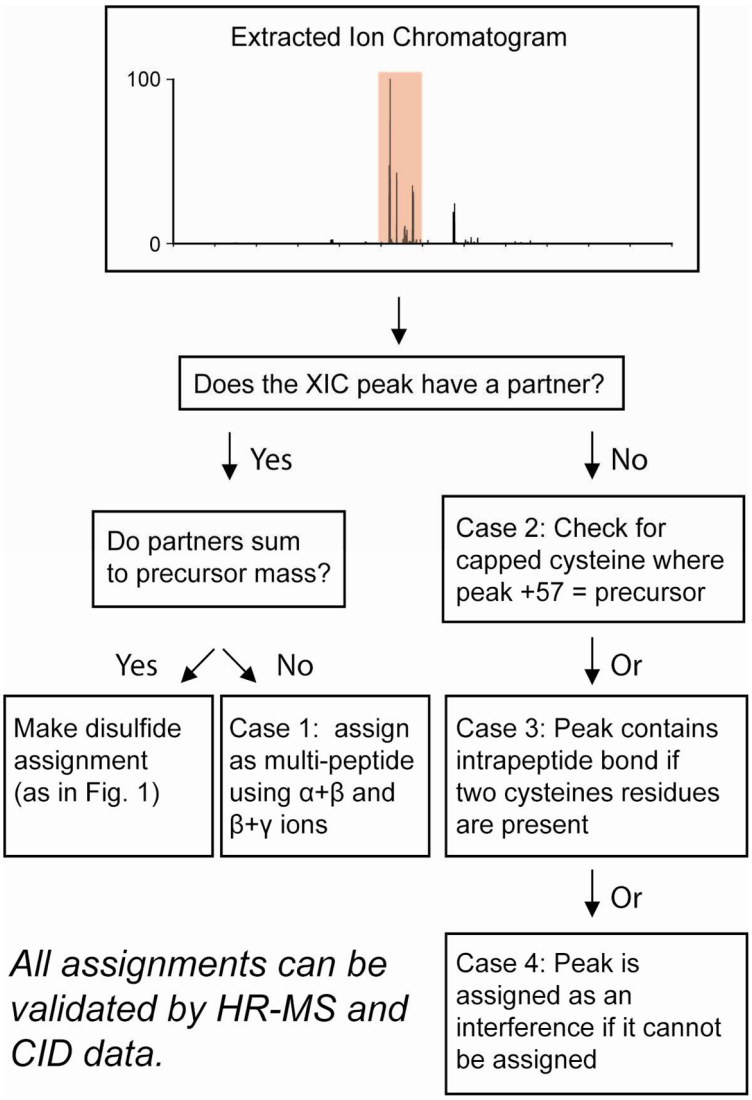

Figure 2.
A representative workflow of the method. An XIC is produced for a cysteine-containing peptide and interrogated with the questions in the flowchart until it is assigned.

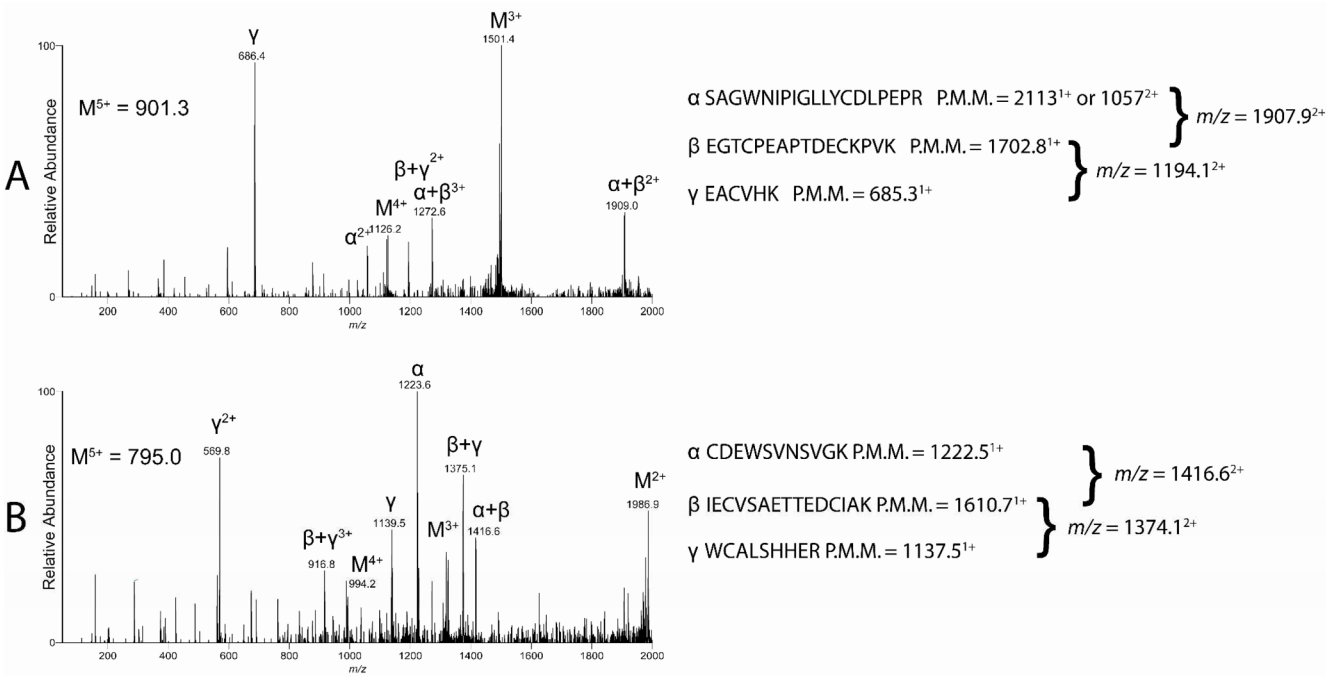

Figure 3.

ETD spectra of disulfide-bonded peptides with three linear peptides from human transferrin are shown. The most abundant ions are charge reduced precursors and combinations of α, α +β, β+γ, and γ peptides which allows the investigator to confirm the β peptide.

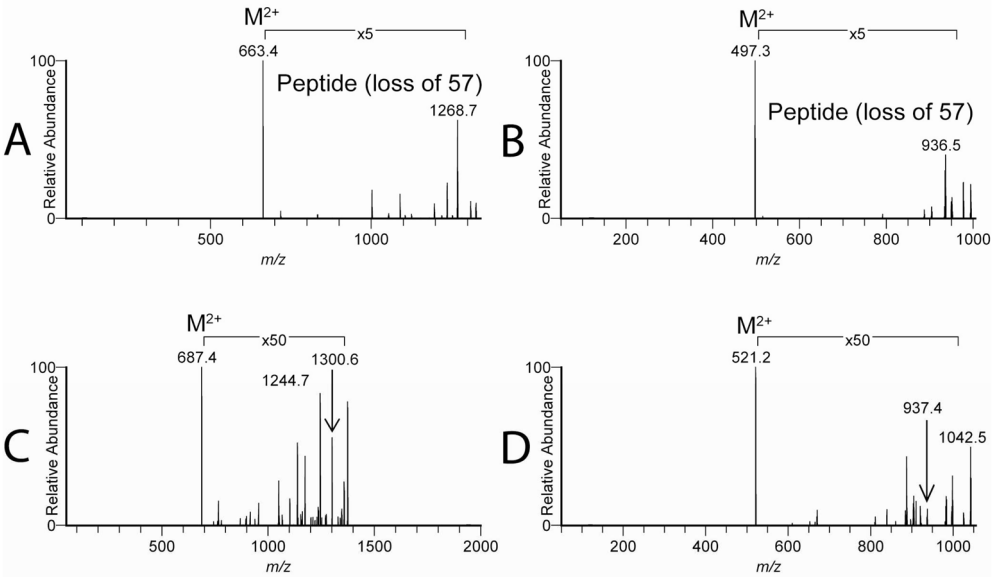

Figure 4.

Results from ETD on peptides from lysozyme that were reduced and alkylated. Peptides alkylated with iodoacetamide (A and B) show a prominent loss of 57, corresponding to loss of the alkylating reagent. When the same peptides were alkylated with 4-vinylpyridine (C and D), the peptide mass marker was not detected after ETD. A and C are peptide GYSLGNWVCAAK with the bare peptide expected at 1268.6 *m/z*. B and D are peptide WWCNDGR with the bare peptide expected at 936.4 *m/z*.

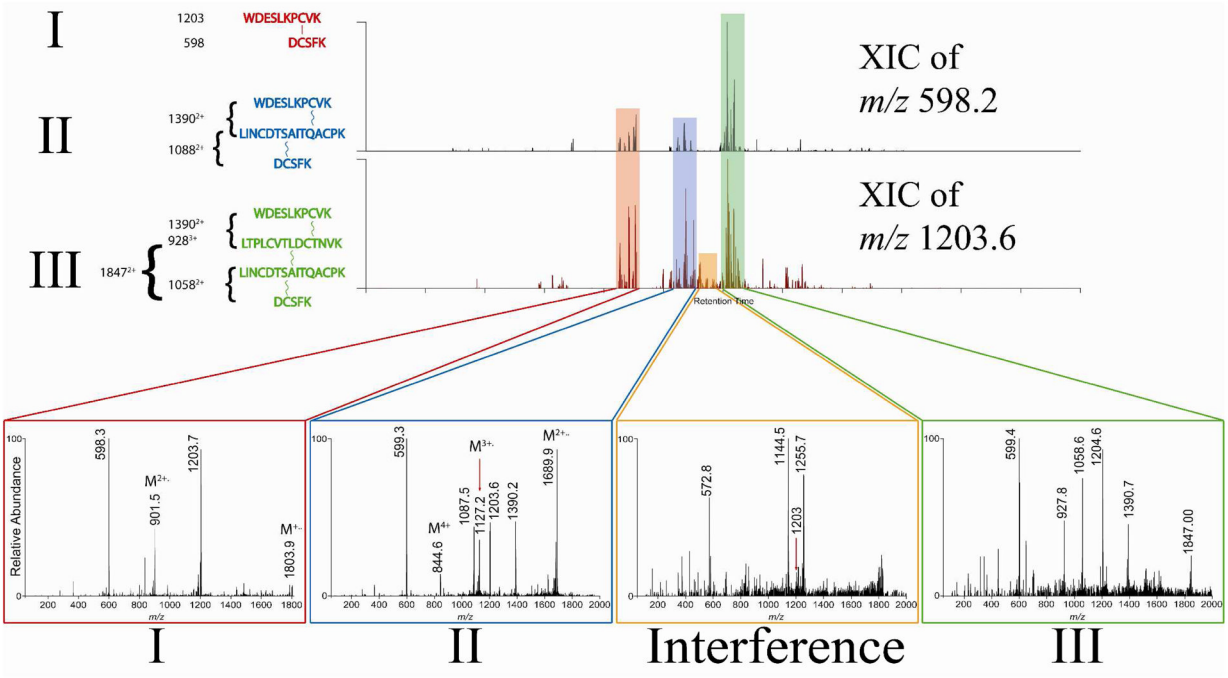

Figure 5.

The different forms detected from the V1/V2 region of 1086.C gp120 can be seen when viewing the XICs for *m/z* 598 and 1203 (top and bottom, respectively). An interfering species is seen with *m/z* 1203 from 28 to 30 minutes. It was the result of a neutral loss from a charge reduced precursor from a different peptide.

Table 1

Disulfide assignments for transferrin from ETD-XIC data.

Peptide	Peptide (XIC)* *m/z* Theor.	Peptide (XIC)* *m/z* Exptl.	Dipeptide** *m/z* Theor.	Dipeptide** *m/z* Exptl.
WCAVSEHEATK	1259.6	1259.6		
ASYLDCIR	939.5	939.5		
CQSFR	639.3	639.3		
SVIPSDGPSVACK	1357.7	1357.7		
SCHTGLGR	829.4	829.4		
CLK	362.2	362.3		
SAGWNIPIGLLYCDLPEPR	1057.0^{2+}	1057.1^{2+}	1272.3^{3+}	1272.2^{3+}
EGTCPEAPTDECKPVK	NA	NA		
EACVHK	685.3	685.4	1194.0^{2+}	1194.0^{2+}
AVANFFSGSCAPCADGTDFPQLC-QLCPGCGCSTLNQYFGYSGAFK	NA	NA		
DQYELLCLDNTR	1481.7	1481.7		
DCHLAQVPSHTVVAR	1631.8	1632.0		
WCALSHHER	1137.5	1137.6	1374.1^{2+}	1374.1^{2+}
IECVSAETTEDCIAK	NA	NA		
CDEWSVNSVGK	1222.5	1222.5	1417.1^{2+}	1417.2^{2+}
CGLVPVLAENYD†K	1419.7	1419.7	1418.7^{2+}	1418.7^{2+}
CSTSSLLEACTFR	NA	NA		
INHCR	641.3	641.4	1029.0^{2+}	1029.0^{2+}
SDNCEDTPEAGYFAVAVVK	1007.5^{2+}	1007.5^{2+}		
DDTVCLAK	863.4	863.4		
SCHTAVGR	829.4	829.5		
CLVEK	590.3	590.3		

Peptide	Peptide (XIC)* *m/z* Theor.	Peptide (XIC)* *m/z* Exptl.	Dipeptide** *m/z* Theor.	Dipeptide** *m/z* Exptl.
FDEFFSEGCAPGSK	1519.6	1519.6	1556.2^{2+}	1556.2^{2+}
LCMGSGLNLCEPNNK	NA	NA		
DSSLCK	651.3	651.3	1121.5^{2+}	1121.6^{2+}
DYELLCLDGTR	1296.6	1296.6		
KPVEEYANCHLAR	1528.8	1528.8		
QQQHLFGSD† VTDCSGNFCLFR	1201.0^{2+}	1201.0^{2+}		

ETD-XIC data were used to make assignments by aligning expected peptide ETD product ions. Assignments were checked against accepted disulfide bonds in Uniprot (Accession P02787). One cysteine-containing peptide from transferrin was not assigned (in red) with the method.

*Peptide product ion *m/z* values of single, linear peptides.

**Peptide product ion *m/z* values of dipeptides as a result of a single, linear peptide loss from a tripeptide.

†Residues deglycosylated by PNGase F.

Table 2

Disulfide assignments for gp120 from ETD-XIC and FTICR-MS data.

Peptide	ETD XIC *m/z*	Theoretical *m/z*	Experimental *m/z*	Mass error (ppm)	Charge state
EVHNVWATCHACVPTDPNPQEMVL	1294^{2+}	910.9277	910.9290	1	4+
TTLFCASDAK	1055.5	1214.2343	1214.2387	4	3+
		1820.8476	1820.8466	<1	2+
WDESLKPCVK	1203.6	844.6492	844.6497	<1	4+
LINCDTSAITQACPK	NA	1125.8630	1125.8674	4	3+
DCSFK	598.2				
WDESLKPCVK	1203.6	816.2292	816.2305	2	6+
LINCDTSAITQACPK	NA	979.2735	979.2782	5	5+
LTPLCVTLDCTNVK	NA	1223.8399	1223.8452	4	4+
DCSFK	598.2				
LTPLCVTLDCTNVK	1518.8	759.3893	759.3886	<1	2+
		1517.7708	1517.7747	3	1+
LINCDTSAITQACPK	1576.8	788.3795	788.3796	<1	2+
		1575.7511	1575.7585	5	1+
VSFDPIPLHYCAPAGFAILK	1080^{2+}	861.6933	861.6937	<1	4+
DVSTVQCTHGIK	1286.6	1148.5884	1148.5928	4	3+
		1722.3787	1722.4146	21	2+
TFDGTGPCR	NA	715.2959	715.2997	5	2+
CNDK	NA				
TIIVHLDESVNIVCTRPNDNTR	1255^{2+}	730.9594	730.9596	<1	5+
QAHCNIDESK	1143.5	913.4472	913.4494	2	4+
		1217.5937	1217.5992	5	3+
TIIVHLDESVNIVCTRPNDNTR	1255^{2+}	727.5541	727.5548	1	5+
Q*AHCNIDESK	1126.5	909.1906	909.1925	2	4+

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Peptide	ETD XIC *m/z*	Theoretical *m/z*	Experimental *m/z*	Mass error (ppm)	Charge state
		1211.9182	1211.9228	4	3+
AIYAPPIEGEITCNSDITGLLLLR	1286^{2+}	799.6604	799.6627	3	4+
SFNCR	625.3	1065.8779	1065.8833	5	3+
		1598.3130	1598.3229	6	2+
GEFFYCDTSDLFDTYR	1018^{2+}	797.0988	797.1004	2	4+
SSDGTITLQCK	1151.5	1062.4625	1062.4672	4	3+
		1593.1898	1593.1987	6	2+

ETD spectra were used for assignments and HR-MS and CID spectra, for validation. Asterisks represent pyroglutamate. No ETD data was acquired for the disulfide bonded peptide in red, but it was assigned using HR-MS and CID data.