Electrochemistry-Assisted Top-Down Characterization of Disulfide-Containing Proteins

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Abstract

Covalent disulfide bond linkage in a protein represents an important challenge for mass spectrometry (MS)-based top-down protein structure analysis as it reduces the backbone cleavage efficiency for MS/MS dissociation. This study presents a strategy for solving this critical issue via integrating electrochemistry (EC) online with top-down MS approach. In this approach, proteins undergo electrolytic reduction in an electrochemical cell to break disulfide bonds and then online ionized into gaseous ions for analysis by electron-capture dissociation (ECD) and collision-induced dissociation (CID). The electrochemical reduction of proteins allows to remove disulfide bond constraints and also leads to increased charge numbers of the resulting protein ions. As a result, sequence coverage was significantly enhanced, as exemplified by β-lactoglobulin A (24 vs. 73 backbone cleavages before and after electrolytic reduction, respectively) and lysozyme (5 vs. 66 backbone cleavages before and after electrolytic reduction, respectively). This methodology is fast and does not need chemical reductants, which would have an important impact in high-throughput proteomics research.

Top-down mass spectrometry (MS)[1–10] has unparalleled strength in protein analysis, which can be used to rapidly identify protein identity[1, 5, 11] and to provide extensive molecular connectivity information including labile post-translational modifications (PTMs)[12]. Despite these striking advantages, it has some challenging issues. For instance, in the case of large proteins, the fragments resulting from electron-capture dissociation (ECD)[13] may not be separated from each other due to non-covalent intramolecular interactions of the residues.[12] Demonstrated solutions for this issue include prefolding-dissociation (PFD)[14] and activated-ion ECD (AI-ECD)[15] which unfold proteins and break the intramolecular interactions prior to ECD. Likewise, covalent disulfide bond linkage in a protein represents another challenge for top-down protein analysis. Disulfide bond is one of the most common PTMs (~19% proteins contain multiple disulfide bonds[16]), which is vital for maintaining the protein structure stability.[17] However, the dissociation efficiency of the protein backbone by electron-based ion dissociation is greatly reduced in the presence of disulfide bonds as the latter would be preferentially broken,[11, 18] which is exemplified by no fragment ions[18] observed for ECD of ribonuclease A ions and a poor sequence coverage for the disulfide-bond protected regions of antibody IgGs[19]. Thus top-down protein analysis with high sequence coverage is limited to those without disulfide bonds or those with relatively simple disulfide linkages like insulin.[18] For proteins containing complicated disulfide bonds such as

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ASSOCIATED CONTENT

Supporting Information. Experimental apparatus, procedure description and additional supporting data. This material is available free of charge via the Internet at http://pubs.acs.org.

lysozyme,[20] chemical reduction of disulfide bonds using reducing reagents like dithiothreitol (DTT) are often performed prior to top-down analysis. Such a chemical reduction is usually not carried out online with MS analysis of proteins/peptides. The offline reduction therefore could limit the applications of top-down approach to high throughput analysis or rapid identification of protein isoforms.[1] Furthermore, the chemical reduction takes minutes to hours, and the removal of the excess amount of reductants is also time-consuming.[21] To tackle this critical problem, our strategy in this study is to couple electrochemistry (EC) with top-down MS, in which proteins are rapidly reduced electrochemically followed with online MS and tandem MS analysis. Remarkably, detectable protein backbone cleavages was significantly enhanced by 3–13 folds with the assistance of electrochemical reduction that removes the protein disulfide bond constraints, as revealed by the ECD and collision induced dissociation (CID) data of two chosen proteins, β-lactoglobulin A and lysozyme.

The experimental apparatus of this methodology consists of a thin-layer μ-PrepCell™ electrochemical flow cell (Antec BV, Zoeterwoude, Netherlands) online coupled with a Bruker 12 Tesla SolariX FTICR-MS (Bruker Daltonics, Bremen, Germany) using desorption electrospray ionization (DESI)[22] as an interface. In our laboratory, DESI has been shown to be suitable for transfer/ionize electrolyzed compounds from solution to the gas phase for MS detection.[23–26] Detailed apparatus and experimental conditions of this study are described in the Supporting Information. β-lactoglobulin A containing two disulfide bridges (Cys^{66}–Cys^{160} and Cys^{106}–Cys^{119}) and one free Cys^{121} (refer to Figure 1c for its sequence)[27] was first chosen as an example for this study. Figure 1a illustrates the DESI-MS spectrum acquired when a solution of 15 μM β-lactoglobulin A in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the electrochemical cell with no potential applied to the cell, the multiply charged ions of intact β-lactoglobulin A with a charge state distribution (CSD) of +10~+18 with the most intense peak at +15 were detected. When the +16 protein ion (m/z 1148.0) was selected for ECD, only 17 c ions were observed in the acquired spectrum, arising from the cleavage of the free protein N-terminal where there is no disulfide bond (the major c ions are labeled in the ECD MS/MS spectrum of Figure 1b and all detected fragment ions are marked in Figure 1c). There was no detected fragment ion resulting from broken backbone bonds that are protected by either or both of the disulfide bonds Cys^{66}–Cys^{160} and Cys^{106}–Cys^{119}. This result can be accounted for by the aforementioned reason that the cleavage of protein backbone bonds by ECD in the presence of disulfide bonds has low efficiency,[11, 18, 28] and the observation of fragment ions from those disulfide bond-protected regions requires the cleavage of both backbone and the disulfide bonds. Likewise, in the case of CID of the +16 ion, it is difficult to produce protein fragment ions via backbone cleavages of the residues enclosed by two disulfide bonds because CID in the positive ion mode is known to be unable to effectively cleave disulfide bonds.[14, 18, 29] As a result, few fragment ions b_{16}, b_{17}, b_{18}, b_{19}, b_{20}, b_{21}, b_{22}, b_{23}, b_{24}, b_{25}, and b_{26} were generated from N-terminal cleavages, as illustrated in Figures 1d and e.

By contrast, when a −1.2 V potential was applied to the electrochemical cell to trigger online disulfide bond reduction, the CSD of protein ions was shifted to higher charges with the appearance of a new population of +19~+22 (Figure 1f), indicating a conformational change occurring to the protein due to the reduction.[30–32] This phenomenon is in agreement with the early ESI report that increased charges of the protein ions occurred after the cleavage of disulfide bonds[31] and our recent observation[25]. Indeed, the reduction of disulfide bonds in a protein causes the unfolding of the protein, thus allowing the protein to have a greater capacity to accommodate a larger number of charges on its surface.[30–32] Thus the newly produced +19 ion was chosen for ECD analysis, under the same experimental conditions used for ECD of +16 unreduced protein ion as described above. As shown in the ECD MS/MS spectrum (Figure 1g) and the labeled sequence map (Figure 1h), 40 c ions and 26 z ions

were generated. In addition, 23 b and 12 y ions were observed in the CID of +19 ion, as displayed in Figures 1i and j. These results represent a significant increase the backbone cleavage after electrolytic reduction (totally 73 backbone cleavages from the combined ECD and CID data) in comparison to that before reduction (totally 24 backbone cleavages from the combined ECD and CID data). This change, the approximately 3 fold enhancement in observable backbone cleavages, is ascribed to electrochemical assistance in removing S-S constraints. It might be also contributed to the resulting increased charge states as a result of disulfide bond reduction.[6, 33] Although the number of ECD cleavages could be further improved by pre-activation of the precursor ions,[14] this was not performed because the purpose in this study is to investigate the effect of electrochemical reduction.

We further examined chicken lysozyme, a 14 kDa protein having 129 amino acid residues and containing four disulfide bonds (Cys^6–Cys^{127}, Cys^{30}–Cys^{115}, Cys^{64}–Cys^{80}, and Cys^{76}–Cys^{94}). The latter two disulfide bonds are intertwined and further enclosed by the former two bonds (Figures 2c). Figure 2a shows the DESI-MS spectrum acquired when a solution of 15 μM lysozyme in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the electrochemical cell, the intact lysozyme exhibits the CSD of +7~+10 with the most abundant peak located at +9. Interestingly, no fragments were observed upon CID of +10 ion of m/z 1430.5 (Figures 2b and c). This result is not surprising as the protein has 4 cross-linked disulfide bonds that protect most of the protein sequence. Again, ECD of +10 ion (m/z 1430.5) gives rise to few fragment ions of c_2, c_3, c_4, c_{12}, and z_8 with low abundances, as displayed in Figures 2d and e. This is in agreement with the previous report about the ECD of intact lysozyme ions.[20] In this case, although the backbone dissociation efficiency is very low, the cleavage of one disulfide bond (Cys^6–Cys^{127}) by ECD occurred, evidenced by the formation of c_{12} and z_8. When a −1.5 V potential was applied to the cell to reduce the protein, the CSD of the protein ions was broadened and shifted to the higher charge states (+7~+16, Figure 2f). It appears that the electrochemical reduction can serve as a stimulant to trigger protein conformational change by reducing S-S linkages to eliminate the protein tertiary structure, which can be monitored online with DESI-MS detection. As shown in the CID mass spectrum of +15 ion (m/z 954.7) of the reduced lysozyme (Figure 2g) and the labeled backbone cleavage sites (Figure 2h), b_7, b_9, b_{18}, b_{27}, b_{28}, b_{29}, b_{30}, b_{31}, b_{39}, y_6, y_{10}, y_{24}, y_{25}, y_{26}, y_{28}, y_{30}, y_{31}, y_{32}, y_{33}, y_{37}, y_{40} and y_{41} were generated after electrolytic reduction of lysozyme, which is in stark contrast with the CID of intact protein ion (Figure 2b). In addition, much more backbone cleavages took place with ECD. As shown in Figures 2i and j, 36 c ions and 14 z ions were observed upon ECD of the newly produced +15 ion (this ECD result is similar to ECD of chemically reduced mononitrated lysozyme which shows a total of 59 fragment ions[20]). Thus, it can be seen that, with electrochemical assistance to remove disulfide bond linkages, the sequence information for lysozyme was remarkably enhanced. Based on the combined CID and ECD data, totally 5 and 66 unique backbone cleavages were resulted before and after electrolysis, respectively). Other methodologies have been reported for enhancing the top-down dissociation efficiency, including CID of low charged lysozyme ions,[11] which showed a maximum of 13 backbone cleavages. In contrast, our method is more effective.

It is evident that substantially increased backbone cleavage by ECD and CID (3–13 fold increments as shown with the two chosen proteins in this study) occurs to the electrolytic reduced protein in comparison to intact protein, producing many more structurally informative fragment ions. The electrolytic reduction for the disulfide bonds is fast and takes place instantaneously when the reduction potential is applied. Equally importantly, it is a clean and "green" approach as it does not involve the use of toxic chemical reductants. Comparison to DTT reduction was also made, using lysozyme as an example. The protein did not show any reduction at pH of 3 with DTT in 100 fold excess amount even when the lysozyme was incubated with DTT for overnight (Figure S-2b, Supporting Information). At

pH 8, the protein can be reduction and the complete reduction takes ca. 90 min (see Figures S-2c to S-2f, Supporting Information). The pH of the sample solution needs to be adjusted to be acidic prior to MS analysis. The reduction of lysozyme by DTT for 90 min (Figure S-2f, Supporting Information) does show higher reduction efficiency than the electrolytic reduction shown in Figure 2f (as indicated by the higher relative abundances of newly produced protein ion peaks in Figure S-2f). However, the electrolytic reduction efficiency could be significantly improved, using a different electrode (Figure S-3, Supporting Information). Overall, the online electrochemistry employed is well suited for the top-down protein analysis, which would be particularly valuable in high-throughput proteomics applications. It is also an addition to the study of proteins by combined electrochemistry and mass spectrometry.[34, 35]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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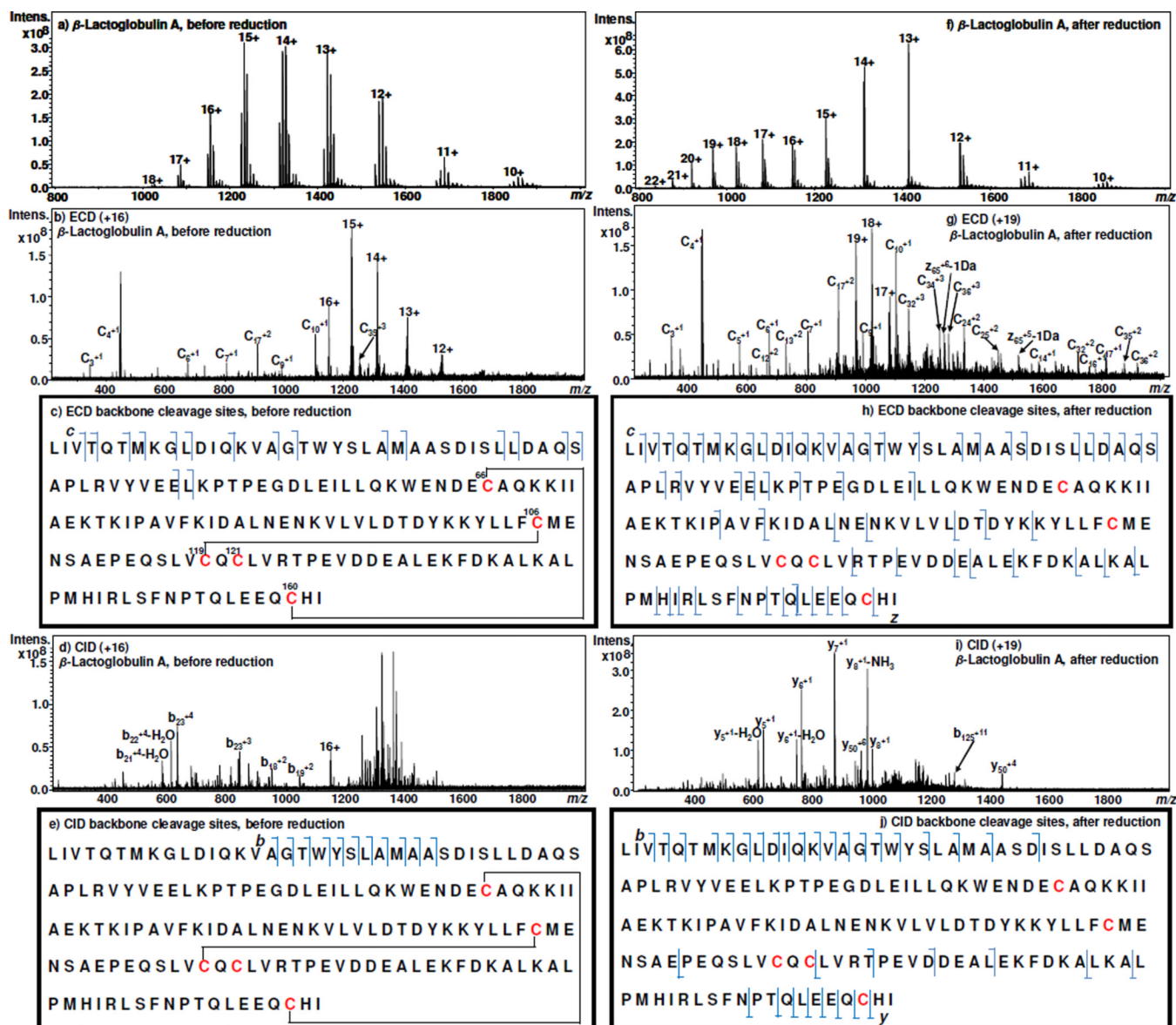35. Permentier HP, Bruins AP. J. Am. Soc. Mass Spectrom. 2004; 15:1707–1716. [PubMed: 15589748]

Figure 1.
DESI-MS spectra acquired when a solution of 15 μM β-lactoglobulin A in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f) −1.2 V; ECD MS/MS spectra of (b) +16 ion of the intact β-lactoglobulin A (*m/z* 1148.0) and (g) +19 ion of the reduced β-lactoglobulin A (*m/z* 967.1); The marked ECD backbone cleavage sites of (c) β-lactoglobulin A and (h) reduced β-lactoglobulin A; CID MS/MS spectra of (d) +16 ion of the intact β-lactoglobulin A (*m/z* 1148.0) and (i) +19 ion of the reduced β-lactoglobulin A (*m/z* 967.1); The marked CID backbone cleavage sites of (e) β-lactoglobulin A and (j) reduced β-lactoglobulin A.
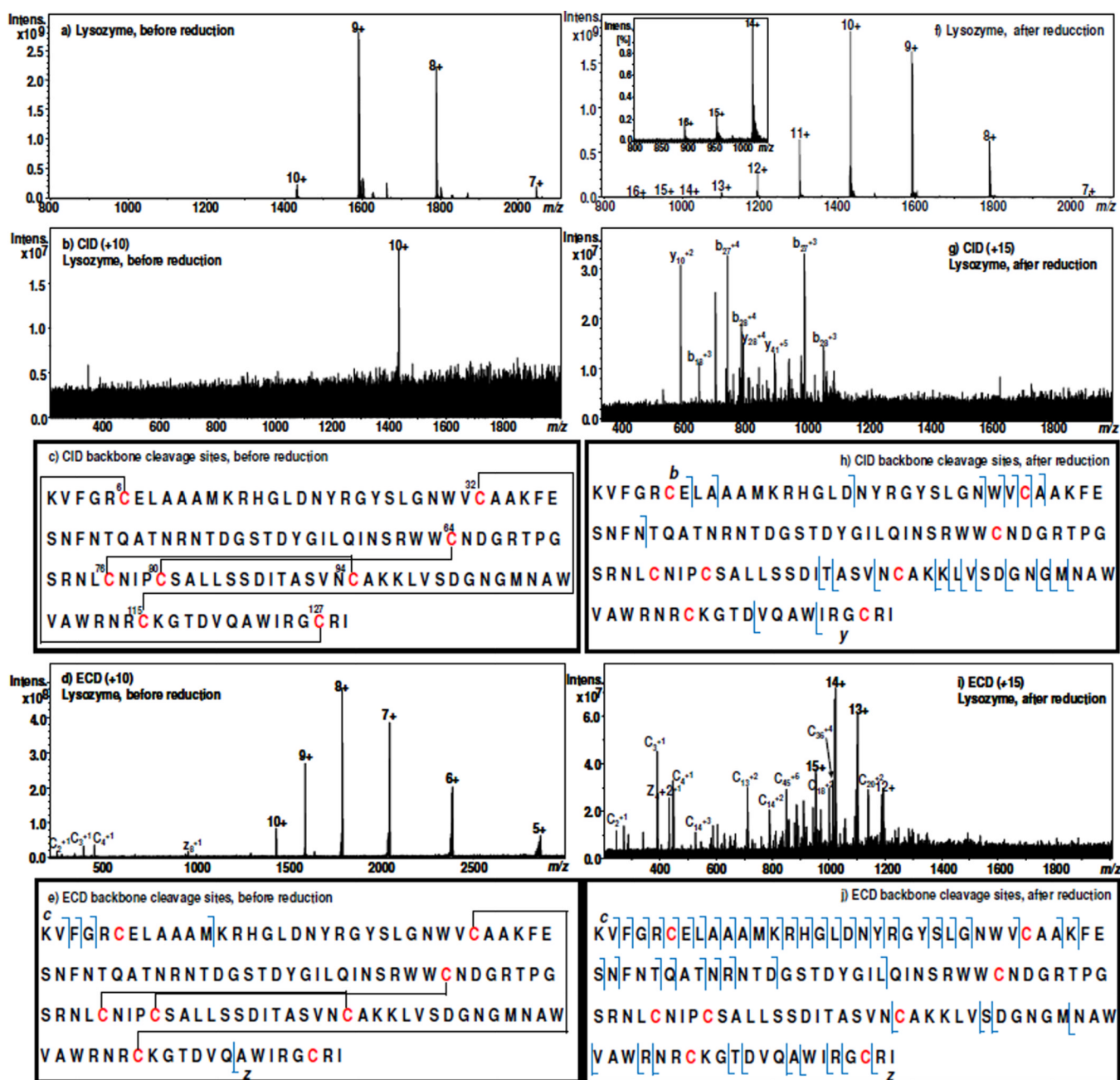
Figure 2.
DESI-MS spectra acquired when a solution of 15 μM lysozyme in methanol/water (1:1 by volume) containing 0.5% formic acid flowed through the thin-layer electrochemical cell with an applied potential of (a) 0.0 V and (f) −1.5 V (the zoomed-in mass range for +14~+16 for the reduced lysozyme is shown in the inset); CID MS/MS spectra of (b) +10 ion of the intact lysozyme (*m/z* 1430.5) and (g) +15 ion of the reduced lysozyme (*m/z* 954.7); The marked CID backbone cleavage sites of (c) lysozyme and (h) reduced lysozyme; ECD MS/ MS spectra of (d) +10 ion of the intact lysozyme (*m/z* 1430.5) and (i) +15 ion of the reduced lysozyme (*m/z* 954.7); The marked ECD backbone cleavage sites of (e) lysozyme and (j) reduced lysozyme.