Charge state dependent top-down characterisation using electron transfer dissociation

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The dissociation of protein ions (5–30 kDa) as a function of charge state has been explored in order to suggest the optimal charge state range for top-down sequencing. Proteins were generated under denaturing conditions and their charge states were modified via ion/ion proton transfer reactions prior to dissociation. Electron transfer dissociation (ETD) data suggested optimal sequence coverage for charge states in the m/z range from 700 to 950 while limited sequence coverage was noted when the precursor m/z was above 1000. Sequence coverage from ETD data was found to be dependent on protein size, with smaller proteins having better sequence coverage. An observed depletion in sequence-related information was mainly attributed to limited instrument (ion trap) performance (m/z range and resolution). For a combined ETD/collision-induced dissociation (CID) approach it is difficult to propose an optimal m/z range since good sequence coverage for CID is at intermediate charge states and the optimal m/z range increases with protein size. When only one charge state can be analysed in a combined ETD/CID approach, a range around 950 m/z is suggested as a starting point. Alternatively, two charge states should be explored, each optimal for either ETD or CID. Overall, these suggestions should be useful to achieve enhanced characterisation of smaller proteins/large protein fragments (generated from denaturing solutions) in minimal analysis times.

Mass spectrometry (MS) has emerged as the most favoured method for rapid 'classification' (identification, characterisation, quantification) of proteins. There are two ways of tackling protein analysis by MS: the bottom-up and the top-down approaches.[1,2] In the bottom-up strategy, proteins are subjected to proteolytic digestion and the resulting peptide mixture is analysed in the gas phase by MS and/or tandem mass spectrometry (MS/MS). On the other hand, the top-down approach uses intact proteins or large protein fragments (sometimes termed the 'middle-down' approach) which are introduced into the gas phase, fragmented and analysed by MS. At the moment, the majority of protein 'classification' is done by the bottom-up strategy.[2–4] However, potential use of the intact proteins rather than proteolytic fragments can reduce the complexity of the analytical space and immediately provide the primary structure (rather than of a fragment) as well as reveal its potential modifications. So far, application of the top-down strategy has mostly focused on single purified proteins.[2–4] It has been suggested that one of the improvements in top-down MS should be the ability to analyse large numbers of proteins when dealing with limited amounts of complex mixtures.[2–4] Therefore, maximum structural information obtained in the shortest measurement time is desirable.

Proteins formed by electrospray ionisation (ESI) produce complex mass spectra with multiple charge states. When dealing with mixtures of proteins, spectra can become even more complex in terms of multiple overlaps between different charge states of different proteins in a narrow mass to charge range. Considering the limited time for MS/MS analysis (e.g. on-line high-throughput experiment) prediction of the charge state that gives the best sequence coverage and provides enough diagnostic information may be vital.

Different charge states of the same peptide or protein can have distinct fragmentation properties and yield different fragmentation product ions.[5,6] Due to the use of denaturing solutions higher charge states tend to be formed by ESI.[7] However, various charge state manipulations, e.g. ion/molecule and ion/ion proton transfer reactions as well as metal cationisation, allow other charge states to be accessed.[5,6,8,9] Charge state dependent behaviour of protein ions has been extensively studied by McLuckey's group, where multiply charged ions were charge manipulated before and after collision-induced dissociation (CID).[5,10–21] These studies have suggested that, in the top-down approach, the dissociation channels are strongly dependent on precursor ion charge state. Low and high charge states show relatively modest sequence coverage while intermediate charge states yield more information. The low charge states favoured cleavage C-terminal to Asp residues as well as losses of small neutral molecules, while the highest charge states favoured cleavages N-terminal to Pro residue. Non-specific amide bond fragmentation was observed at the intermediate charge states.

Electron transfer/capture dissociation (ETD and ECD) is the second most widely used approach to induce protein ion fragmentation.[22,23] It is an alternative and complementary

dissociation technique to CID. The little work that has been reported on ETD/ECD charge state dependent behaviour of protein ions has suggested that as the protein charge state decreases, the number of fragments produced with ETD decreases.[24]

In the present study, we explored the propensities of different protein charge states towards fragmentation. Charge states of several proteins/protein fragments (5–30 kDa) were modified and investigated by MS/MS analysis (mainly ETD) to provide sequence information. General behaviour of the analysed proteins under ETD is described. The resulting charge state dependent ETD and CID information has been used to infer optimal charge state ranges for the top-down sequencing.

EXPERIMENTAL

Analytes and reagents were obtained from Sigma-Aldrich (Dorset, UK) and used without further purification. Protein samples were made up to 5 μM in water/methanol/acetic acid (50:50:1 v/v/v), and introduced into the ESI source by direct infusion at a flow rate of 100 μL/h.

The charge state dependent CID and ETD experiments were performed on a HCT ultra PTM Discovery ion trap system (Bruker Daltonics, Coventry, UK) which incorporates a negative chemical ionisation (CI) source capable of providing reagent anions for both ETD and proton transfer reaction (PTR). The following experimental event sequence was applied. Typically, ions of a single charge state were selected from the electrosprayed ion population and subjected to dissociation (ETD or CID). After dissociation product ions were subjected to PTR in order reduce their charge prior to mass analysis. Lower charge states of precursor ions were formed by employing PTR on the initial electrosprayed ion population. ETD, PTR and CID conditions were optimised for each charge state in order to maximise the dissociation and the appearance of the product ion spectra. The acquisition software was set up in manual MS/MS mode with an acquisition time between 5 and 20 min. Spectra were acquired within a scan range from 200–3000 *m/z* and a scan speed of 800 (*m/z*)/s using averages from 5 spectra.

Obtained mass spectra were deconvoluted and protein sequence assignment was done using BioTools 3.1 (Bruker Daltonik GmbH, Bremen, Germany).

RESULTS AND DISCUSSION

Charge state dependent ETD

Charge state dependent top-down ETD was performed on: tumor necrosis factor-α fragment (2310.7 Da), insulin β chain (3495.9 Da), corticotropin A (4541.1 Da), insulin (5715.5 Da), ubiquitin (8564.8 Da), cytochrome C (12359.9 Da), myoglobine (16951.3 Da) and carbonic anhydrase (29024.3 Da). Proteins were electrosprayed under denaturing conditions that give rise to the higher charge states. In order to access the lower charge states, PTR on the initial electrosprayed ion population was employed. Due to the modest resolution of the mass analyser, PTR were also used to reduce the (high) charge state product ions prior to mass analysis. Since instrument setup allowed only one PTR per spectrum higher/intermediate charge states were subject to PTR post ETD while for the low charge states PTR was used prior to ETD.

Ubiquitin was selected as a representative protein because it is a well-studied standard and provides the opportunity to compare results with those obtained with ECD.[7] Sequence coverage for the $[M+13H]^{13+}$, $[M+9H]^{9+}$ and $[M+6H]^{6+}$ ions of ubiquitin is shown in Fig. 1 to illustrate differences in the fragmentation behaviour. 13+ is the highest charge state obtained and produced ETD fragment ions correspond to 57% sequence coverage. The N-terminus is represented by c-type ions and the C-terminus by z-type ions. Only two c/z ion complementary pairs were observed, probably due to the upper *m/z* limit of the instrument (3000 *m/z*) where post-dissociation PTR placed larger fragments outside of the *m/z* range. On the other hand, smaller proteins (e.g. insulin chain B) fragmented along the whole backbone giving almost all c/z complementary ion pairs (data not shown). At intermediate charge state, $[M+9H]^{9+}$, a total of 48 bond cleavages was observed, resulting in the highest sequence coverage for ubiquitin (64%). $[M+6H]^{6+}$ represents the transition between the intermediate and low charge state groups. Charge state 6+ is the lowest dissociating charge state, characterised with few, low-abundance product ions, mainly associated with cleavages at the N-terminus. Loss of small neutral fragments (e.g. NH_3, CO) was also observed in the spectrum. Observation for charge state 6+ of a few ions derived from fragmentation close to the termini may suggest a compact gas-phase structure with unravelled ends. For ubiquitin 5+ to 3+ no fragment ions were observed. Overall, the behaviour of ubiquitin ions reported here is consistent with ECD data from Williams and co-workers,[7] who observed relatively high ECD fragmentation efficiency for charge states from 13+ to 7+.

Summarised results of charge state dependent top-down ETD for all analysed proteins are depicted in Fig. 2 This small dataset allows several conclusions to be drawn. For smaller proteins (fragments) up to 5000 Da, as the protein charge state decreases, the number of fragments produced decreases accordingly, in accordance with McLuckey's observations on insulin.[24] However, proteins with mass above 5000 Da behave slightly differently. Going from the highest charge state, a slight increase in number of fragments/sequence

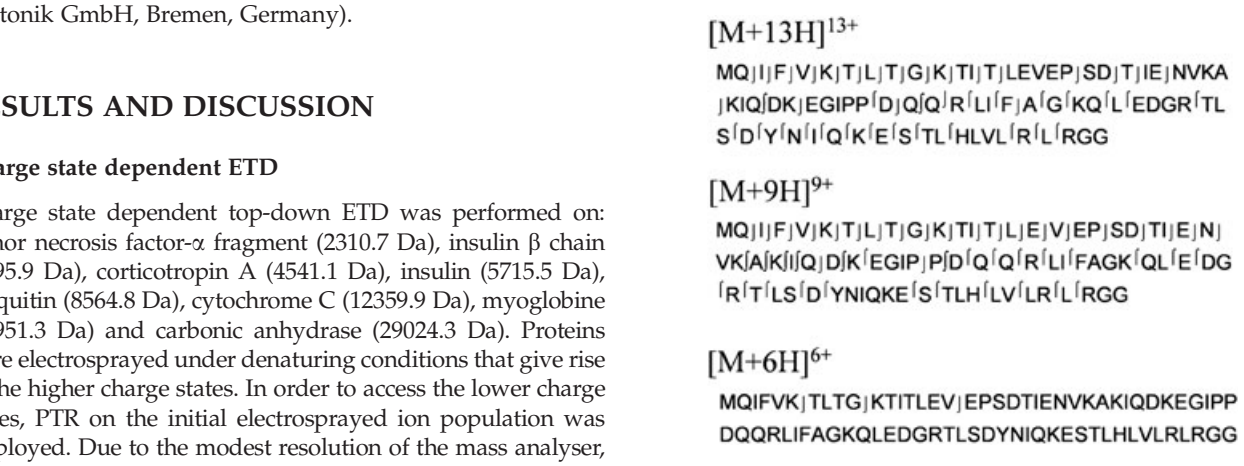

Figure 1. Sequence coverage for charge states 13+, 9+ and 6+ of ubiquitin generated by ETD.

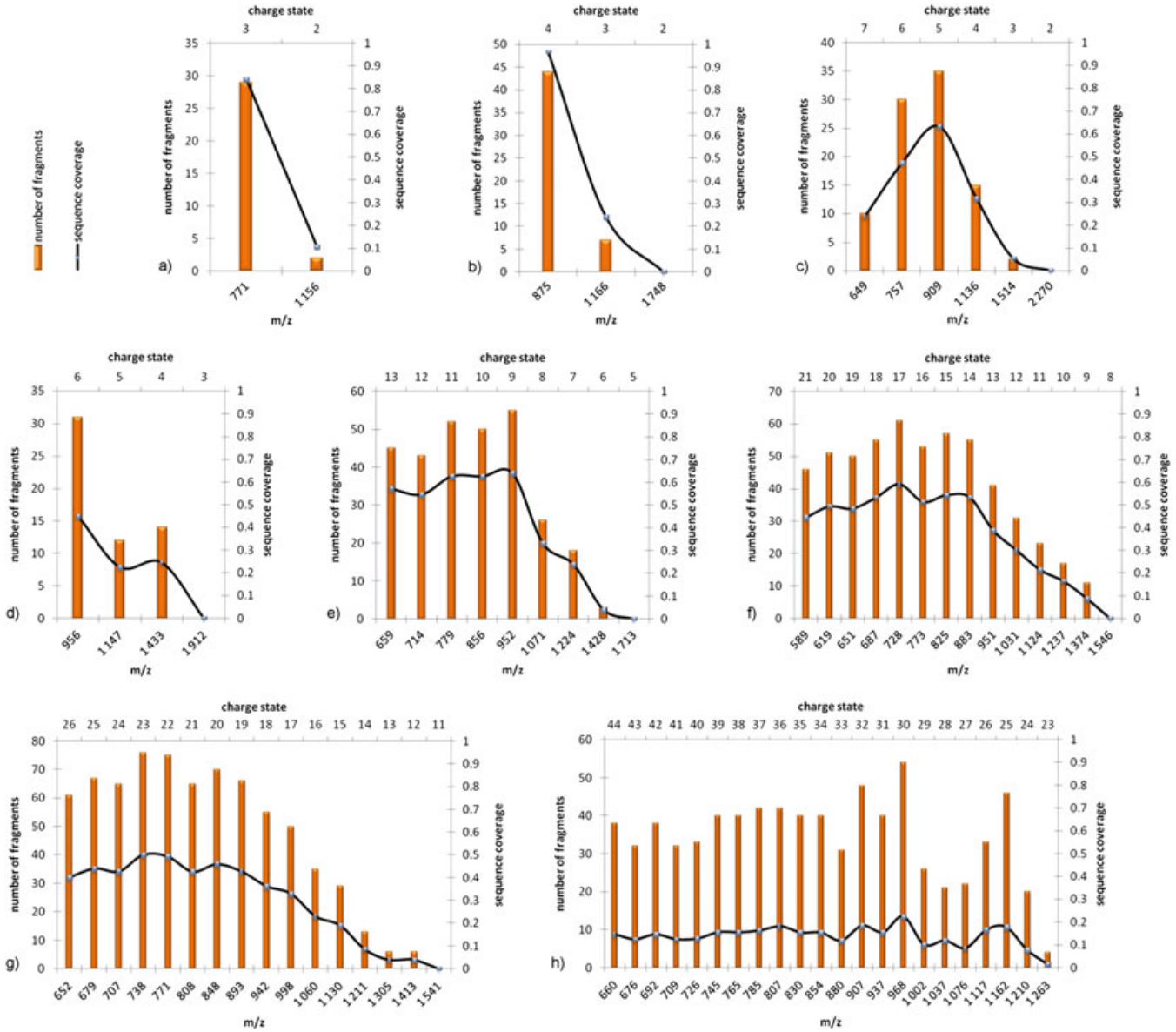

Figure 2. Number of product ions and sequence coverage generated by ETD as a function of precursor charge state: (a) tumor necrosis factor-α fragment (2.3 kDa); (b) insulin β chain (3.5 kDa); (c) corticotropin A (4.5 kDa); (d) insulin (5.7 kDa); (e) ubiquitin (8.5 kDa); (f) cytochrome C (12.4 kDa); (g) myoglobin (17 kDa); and (h) carbonic anhydrase (29 kDa).

coverage is observed. Maximum sequence coverage occurs at transition from high to intermediate charge state range. Intermediate charge states exhibit a sudden decrease of ~10% in sequence coverage and at the transition from intermediate to low charge states dissociation usually becomes highly inefficient. A more general picture is obtained when an *m/z* scale is used (Fig. 2). Good to maximum sequence coverage is obtained at the precursor range of *m/z* 700 to 950 and a sharp coverage decrease happens when the precursor *m/z* is over 1000. It follows for unknown proteins analysed by ETD, a precursor charge state in a range *m/z* 700–900 would likely give the best sequence coverage.

Moving from smaller toward larger proteins a decrease in the sequence coverage was observed. Our data set starts from 90% sequence coverage for e.g. insulin chain B, while for carbonic anhydrase (the largest protein analysed) sequence coverage reduces to 22%. Figure 3 represents sequence

SH⌋H⌋W⌋G⌋Y⌋G⌋K⌋H⌋N⌋GP⌋E⌋H⌋W⌋H⌋K⌋D⌋FPI⌋A⌋N
⌋G⌋E⌋R⌋QSP⌋V⌋D⌋ID⌋T⌋K⌋AVV⌋Q⌋DPA⌋LKPLALV⌋Y⌋
G⌋E⌋ATSRRMVNNGHSFNVEYDDSQDKAVLKDGPLT
GTYRLVQFHFHWGSSDDQGSEHTVDRKKYAAELHL
VHWNTKYGDFGTAAQQPDGLAVVGVFLKVGDANPA
LQKVLDALDSIKTKGKSTDFPNFDPGSLLPNVLDYWT
YPGSLTTPPLLESVTWI⌈VLK⌈EP⌈ISVSSQ⌈QMLK⌈F⌈
RTLN⌈F⌈N⌈AEG⌈E⌈P⌈E⌈LLM⌈LANWRP⌈A⌈QPL⌈K⌈N⌈
R⌈Q⌈V⌈RG⌈FPK

Figure 3. Sequence coverage for carbonic anhydrase charge state 30+ generated by ETD.

coverage for carbonic anhydrase charge state 30+. However, much better coverage (65 %) for the first and last 35 amino acids is obtained. This observation is likely associated with

the upper *m/z* limit of the HCT ion trap instrument (3000 *m/z*). Post-dissociation PTR can place larger fragments outside of the *m/z* range of the instrument.[21] On the other hand, due to the limited resolution (~0.30 u FWHM), multiply charged fragments without PTR cannot be resolved. Thus there are limitations on sequencing the interior region of the larger proteins. Although it has been reported that larger proteins exhibit much stronger electrostatic intramolecular interactions which can lead to intractable structure cleavable only at its ends,[25] we believe that the main reason for limited sequence coverage in the current study is related to instrument performance. In terms of protein identification, good sequence coverage of the N- and C-terminus obtained by top-down ETD has still the advantage over standard bottom-up analysis because ion mass values need only be matched against DNA terminal fragments instead of the whole sequence.[25]

Combination of ETD and CID

The use of alternative ion activation methods can improve analyte identification and enhance confidence in its recognition. ETD and CID are complementary dissociation methods and it is useful to obtain data from both approaches.[21,26]

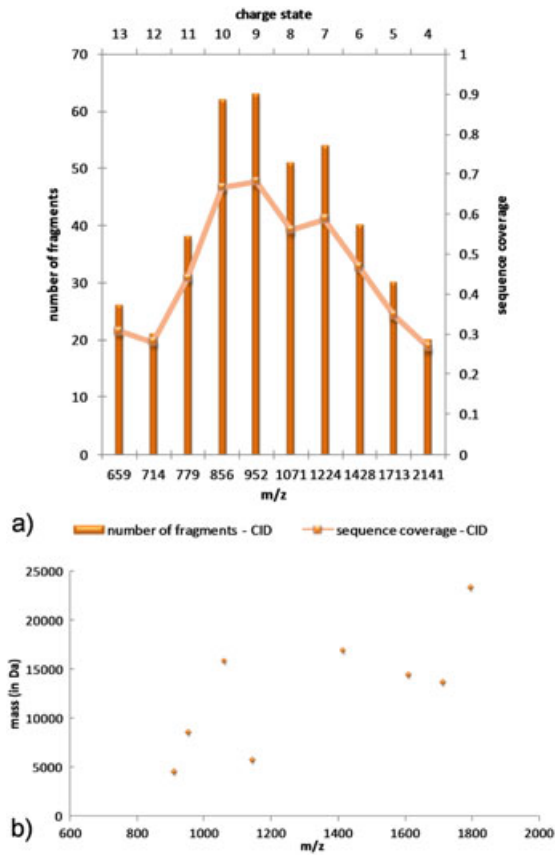

Figure 4. (a) Number of product ions and sequence coverage generated by CID for ubiquitin as a function of precursor charge state. (b) The best fragmenting charge state under CID conditions of a several proteins plotted on *m/z* scale: 4.5 kDa – corticotropin A; 5.7 kDa – bovine insulin;[12] 8.5 kDa – ubiquitin; 13.7 kDa – ribonuclease A;[15] 14.5 kDa – α-synuclein;[20] 15.7 kDa – hemoglobin β-chain;[11] 17 kDa – apomyoglobin;[13] 23.3 kDa – bovine trypsin.[19]

Sequence coverage obtained with charge state dependent top-down CID has a shape of an open down parabolic curve (Fig. 4(a)). Low and high charge states show relatively modest sequence coverage while intermediate charge states provide, in accordance with previous observations, the best sequence coverage.[5,10–21] The best fragmenting (under CID) charge state of several proteins (estimated from literature[11–13,15,19,20]) plotted on the *m/z* scale suggests that as the mass of a protein increases, there is also an increase in *m/z* of the best fragmenting precursor ion (Fig. 4(b)). The plot starts with ~900 *m/z* for the charge state of the smallest protein considered (4.5 kDa). Since for ETD the best sequence coverage can be expected (on the basis of the results reported here) for charge states in a range from 700 to 950 *m/z*, there could be a problem if in a combined ETD/CID approach just one charge state can be analysed (e.g. short measurement time). In that case a good starting point could be a charge state which falls in the upper end of the *m/z* range suggested for ETD approach. Otherwise, two charge states should be considered, each optimal for corresponding dissociating technique applied, i.e. one of the intermediate charge states for CID and one from the range of *m/z* 700 to 950 for ETD.

CONCLUSIONS

The charge state dependent ETD of several protein ions was investigated. Good to maximum sequence coverage is obtained for charge states in the *m/z* range from 700 to 950. Limited sequence coverage was observed when the precursor *m/z* is over 1000. Moving from smaller toward larger proteins a decrease in the sequence coverage was observed and is explained by limited instrument (ion trap) performance (*m/z* range and resolution).

The charge state dependent ETD data were used to suggest an optimal charge state range for the top-down protein sequencing. The range from 700 to 950 *m/z* is suggested for the ETD approach. For the combined ETD/CID approach, when only one charge state can be considered, the range around 950 *m/z* is implied as a good starting point. Otherwise, two charge states should be explored, each optimal for decomposition process (ETD or CID). These general suggestions are based on dissociation behaviour of 5 to 30 kDa peptide/protein ions electrosprayed under denaturing conditions and therefore should be mainly applicable for smaller proteins/large protein fragments in similar conditions and mass range.

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