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Top-down mass spectrometry of intact membrane protein complexes reveals oligomeric state and sequence information in a single experiment

Albert Konijnenberg,[1] Ludovic Bannwarth,[2] Duygu Yilmaz,[3] Armağan Koçer,[3] Catherine Venien-Bryan,[2] and Frank Sobott[1,4]*

[1]Department of Chemistry, Biomolecular & Analytical Mass Spectrometry group, University of Antwerp, Antwerp, Belgium
[2]Bioinformatique et BioPhysique, Institut de Minéralogie, de Physique des Matériaux et de Cosmochimie (IMPMC), Université Pierre et Marie Curie, Paris, France
[3]Department of Neuroscience, University of Groningen, Groningen, The Netherlands
[4]UA-VITO Centre for Proteomics, University of Antwerp, Antwerp, Belgium

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Abstract: Here we study the intact stoichiometry and top-down fragmentation behavior of three integral membrane proteins which were natively reconstituted into detergent micelles: the mechano-sensitive ion channel of large conductance (MscL), the Kirbac potassium channel and the p7 viroporin from the hepatitis C virus. By releasing the proteins under nondenaturing conditions inside the mass spectrometer, we obtained their oligomeric sizes. Increasing the ion activation (collision energy) causes unfolding and subsequent ejection of a highly charged monomer from the membrane protein complexes. Further increase of the ion activation then causes collision-induced dissociation (CID) of the ejected monomers, with fragments observed which were predominantly found to stem from membrane-embedded regions. These experiments show how in a single experiment, we can probe the relation between higher-order structure and protein sequence, by combining the native MS data with fragmentation obtained from top-down MS.

Keywords: mass spectrometry; ion channels; top-down sequencing; integral membrane proteins; collision-induced dissociation

Introduction

The development of electrospray ionization (ESI)[1] and matrix-assisted laser desorption ionization (MALDI)[2] has led to an explosion of mass spectrometry (MS)-based proteomics approaches. Central to these approaches is the detection and/or quantification of proteins under denaturing conditions, usually after enzymatic digestion, by fragmenting them to obtain sequence-specific information. At the same time the development of a miniaturized version of Fenn's electrospray (nano-ESI) by Wilm and Mann has made it possible to transfer proteins and protein complexes intact and in their folded state into the gas-phase.[3,4] Especially in combination with ion mobility, native mass spectrometry is rapidly becoming an invaluable tool for structural biology, as it yields not only information on mass, ligand binding and oligomeric size, but also allows for separation of copopulated conformations of a single protein.[5–9]

In recent years, native mass spectrometry has been extended to the study of integral membrane proteins (IMP) in the gas-phase. The realization that

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*Correspondence to: Frank Sobott, Groenenborgerlaan 171, B-2020 Antwerpen. E-mail: frank.sobott@uantwerpen.be

micelles—the formation of which is to a large extent hydrophobically driven—could survive transfer to the ultimate hydrophobic environment,[10] the vacuum of the mass spectrometer, enabled studies on detergent micelles with IMPs inside.[11] The apparent protective capabilities of suitable detergent micelles were found to be sufficient in most cases to observe oligomeric membrane protein complexes in their native global fold.[12,13] More recent developments in the field show how native MS can reveal lipid binding,[14] probe conformational dynamics of membrane proteins,[15,16] and even assist with finding the ideal crystallization conditions for X-ray diffraction.[17]

One of the strengths of mass spectrometry is its ability to provide information ranging from sequence information of proteins (e.g., mutations, truncations, post-translational modifications) to small molecule binding and the global folding or oligomerization state of complexes, without averaging over all different species which may be present in a sample.[18–20] Bottom-up approaches, where a protein is digested in solution prior to analysis by MS, are very effective in sequencing globular and membrane proteins with suitable enzymatic cleavage sites, based on peptide fragmentation and subsequent comparison to a database of theoretical fragments. Sequence coverage for IMPs however that consist of extensive membrane spanning regions can be poor with bottom-up approaches. Infrequent prevalence of cleavage sites in membrane spanning regions makes sequencing difficult, due to large precursor peptides.[21]

Another approach that is currently drawing renewed interest is the sequencing of proteins using top-down methods, for example with the aim to characterize individual protein isoforms. Top-down approaches use collision-induced dissociation (CID) of intact proteins—often denatured—to produce fragments, yielding sequence information. Sequencing by CID can even be performed starting from native proteins and protein complexes, as reported previously.[22–24] In CID, ions are accelerated by a voltage offset ("collision energy") and undergo multiple, energetic collisions with an inert gas (e.g., argon). The activation of the ions, their internal energy, is controlled via the kinetic energy of the ions and the frequency of collisions with the inert gas. Low acceleration voltages and high gas-pressures reduce the internal energy of the ion ("cooling"), whereas lower gas-pressures and high acceleration voltages allow the ion to accelerate more between collisions, resulting in an energy transfer that augments the internal energy of the ions ("heating").[25] As CID is a rather slow, multi-step process, the internal energy of the ions increases slowly until dissociation occurs. As such, CID is an ergodic (quasi-thermal) process, which preferentially leads to fragmentation of the weakest interaction in the protein or complex—usually a noncovalent bond.

When using top-down approaches, time-consuming steps such as enzymatic digestion which are commonly used in bottom-up approaches, are no longer necessary. Apart from (partial) primary sequence data which can be used to investigate the identity of an unknown protein, top-down sequencing can also provide information on the full complement of post-translational modifications present.[26] The interest for top-down MS has been instigated by the results of the human genome project which led to the realization that we have fewer genes than initially anticipated.[27] As a consequence, the complexity of our biological machinery must stem, amongst others, from protein sequence variations.[28] Recently the term "proteoform" has been coined to encompass such variations of primary structure, and linked with top-down MS.[29]

IMPs have previously been studied using top-down MS, but so far always in combination with a denaturing LC/MS setup.[18,30–32] Membrane proteins were solubilized using organic solvents, leading to loss of their oligomeric state and unfolding of the protein.[19] Using this approach, the use of detergents can be largely circumvented and the denaturing conditions for the protein make top-down fragmentation an efficient tool for probing sequential heterogeneity and post-translational modifications. Although yielding information on proteoforms, such approaches do not maintain the link to their higher-order structure, which adds yet another level of complexity allowing for an even greater diversity in our biological machinery.[33] Notable examples which underline this relationship are phosphorylation of a kinase to regulate its activity,[34] and the alternative splicing of the FAS receptor which either generates a membrane bound form that promotes apoptosis, or a soluble isoform that prevents programmed cell death.[35]

Here we show how—by combining native MS with top-down CID fragmentation—a single experiment can reveal both the higher-order structure (oligomeric distribution of a membrane protein) as well as proteoforms (accurate mass and amino acid sequence) of a membrane protein. By increasing the collision energy past the threshold for release of membrane proteins from detergent micelles, highly charged monomer subunits are generated which upon further activation fragment, yielding sequence information.

Results and Discussion

Three integral membrane proteins were natively reconstituted into detergent micelles and studied by native top-down MS. The gating mechanism of the pentameric mechanosensitive ion channel of large conductance (MscL)[15] and the oligomeric state of the tetrameric Kirbac3.1 ion channel have been studied previously,[36] whereas the hepatitis C Virus (HCV) p7 viroporin had not been investigated before. As

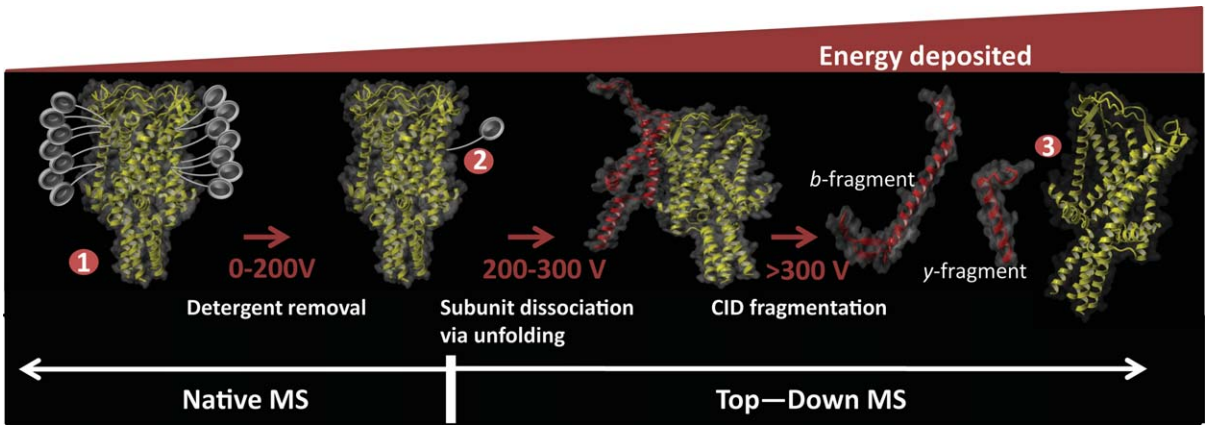

Figure 1. Effect of different energy regimes used in mass spectrometry of membrane proteins under native conditions when using sugar based detergents, such as the commonly used detergent DDM. The apparent protective ability of detergent micelles in the gas-phase allow noncovalent interactions to be maintained for membrane proteins at levels (up to 200 V) at which soluble protein complexes would already undergo significant dissociation. Increasing the collision energy above the threshold for membrane protein release leads to collision induced unfolding, followed by dissociation and even fragmentation, yielding sequence information. Collision energies reported are typical values for DDM, but will vary based on the protein and detergents used.

detergent micelles are quite heterogeneous in size, native MS analysis of embedded membrane proteins is not possible without releasing them, by using collision-induced dissociation (CID) to remove the attached detergent. For the commonly used nonionic detergent dodecyl-maltoside (DDM), collision energies of 100–200 V are required to remove the detergent molecules (Fig. 1), but values can vary depending on protein size, surface area of the membrane-embedded regions and the strength of the protein-detergent interaction (see below).

The ability of detergent micelles to protect natively reconstituted membrane protein complexes from gas-phase dissociation is believed to be due to absorbing the collision energy, leading to ejection of detergent molecules instead. Indeed, it has been shown that the stability of oligomeric membrane proteins towards dissociation in the gas-phase can be correlated to the extent of their membrane spanning regions.[36] Furthermore, especially when stripping the last remaining detergent molecules, the strength of interaction between detergent and IMP becomes another factor to take into account. When the interactions between the subunits of an IMP are weaker than those of the subunits with the detergent, dissociation of the complex will precede detergent stripping. Such effects can be observed for example when subunit interactions are hydrophobically driven. Although a strong stabilizing factor in solution, such interactions are absent in the gas-phase due to the loss of the hydrophobic effect in the vacuum of the mass spectrometer.

Another factor defining membrane protein stability, the net charge carried by the ion, is believed to be correlated to the soluble area of the membrane protein. Membrane proteins reconstituted in detergents appear to carry as much charge after release from the detergent micelles as would be predicted for a soluble protein the size of the soluble region of the IMP.[37] As a consequence IMPs typically yield charge states that are lower than observed for soluble proteins of a similar size. The increase in internal energy in CID is dependent on both the acceleration offset (collision energy) as well as the net charge the ion carries. Thus, although the settings typically used for IMP release from detergent micelles are seemingly harsh, the lower charge states in combination with the protective capabilities of the detergent often allow for the release of intact membrane protein complexes. Increasing the collision energy beyond the threshold for the release of IMPs yields dissociation similar to that observed for soluble proteins.[36] Dissociation of a complex with CID proceeds in an asymmetric fashion, via unfolding of a single subunit. Unfolding coincides with redistribution of charges, yielding a highly charged monomer and a compact lowly charged oligomer, missing a single subunit.[38] Generation of an unfolded, highly charged monomer is crucial to the ability to sequence the membrane protein, as it facilitates efficient fragmentation.

Figure 2(A) shows a mass spectrum for the ion channel Kirbac3.1 under conditions optimized for maximal sequence coverage obtained by top-down fragmentation (see methods and materials). There is still tetrameric Kirbac3.1 remaining, but the spectrum is dominated by the charge state distribution of the ejected monomer (indicated by red circles). Detergent release, subunit dissociation and subsequent top-down fragmentation follow the scheme shown in Figure 1, but the actual collision energies required for these steps depend on detergent, charge and mass

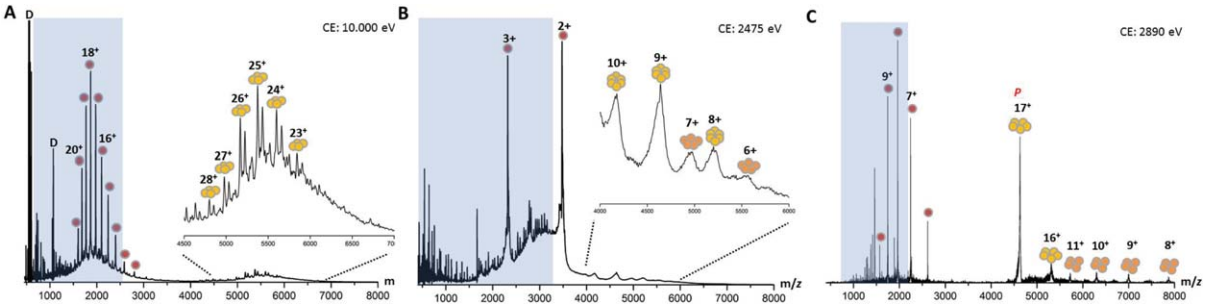

Figure 2. Mass spectra for the oligomeric membrane proteins (A) Kirbac3.1 solubilized in tridecyl-maltoside (3DM) at the maximal operating voltages of the instrument and (B) P7 in triton X-100 at an elevated collision energy level (275 V) yielded monomer and oligomer information as well as sequence-specific fragments. The oligomer size is indicated by colored circles, and areas highlighted in blue indicate the regions where CID fragments are observed. (C) The 17+ charge state of MscL G22C was isolated prior to fragmentation to abolish interference from remaining detergents with the fragment identification. Remaining detergent peaks in any spectra are denoted as D.

of the complex. The energy threshold, where detergent removal and protein release occur, strongly depends on the strength of interaction between protein subunits, protein and detergent and the nature of the detergent itself. At higher energies, unfolded monomers are ejected (complex dissociation), which yields well-resolved peaks in the low m/*z* region that can be used to obtain accurate masses for the protein. For Kirbac subunits a mass of 33,586 ± 30 Da was found, which is in reasonable agreement with the theoretical mass of 33,577 Da (Table I). An additional advantage of such high acceleration voltages is the disassembly of detergent clusters to monomers and dimers, thus leading to minimal interference with the fragment identification.

Native MS of p7 solubilized in triton X-100 showed predominantly monomer, with mainly hexameric and small amounts of pentameric p7 present [Fig. 2(b)]. Triton X-100 is a prominent example of a detergent which is unable to form an extensive network of hydrogen bonds, and it has been shown that MscL pentamers are released at much lower collisional activation.[15,17] Nevertheless, with p7 in triton x-100 we still require collision energies of 175 V to observe oligomers, although monomers are already seen at lower collisional energies (Supporting Information Fig. S1). These observations can be explained in two ways: either p7 is present both as oligomeric as well as monomeric species within the triton X-100 detergent micelles in solution, where monomeric p7 is more easily removed from its detergent cover in the gas-phase. This would match our experience with p7 as a polydisperse protein which forms assemblies of varying size. On the other hand, the rather weak subunit interaction in p7 might suggest that the observed p7 monomers are already dissociating from the oligomeric complex upon increasing collision energies. The collision energy required to rid a membrane protein from its detergent cover in the gas-phase may well already disrupt protein-protein interactions, if they are weak; with electrostatic interactions and hydrogen bonds acting as stabilizing factors *in vacuo* whereas hydrophobic interactions are lost. If the interaction between detergent and protein is stronger than between protein subunits, this might lead to preferential dissociation of the complex before stripping of detergents is complete. Although the latter could explain why such broad peaks are observed for p7 oligomers (due to remaining detergents attached), our current experiments cannot distinguish between these possible explanations. In comparison, MscL (G22C mutant) reconstituted in triton X-100 detergent was previously shown to release as a pentameric complex already at very low collision energy (10V)[15]. For fragmentation of MscL the 17+ charge state was isolated in the quadrupole, prior to dissociation of subunits and subsequent fragmentation. As CID fragmentation of MscL was obtained at rather low collision energies, significant signal from detergent clusters survives, which due to the heterogeneous composition of triton X-100 led to spectral overlap with MscL top-down CID fragments. By using mass selection prior to CID, the interference

Table I. *Masses Observed for the IMPs p7, MscL, and Kirbac3.1*

	Theoretical monomer mass	Observed monomer mass	Theoretical oligomer mass	Observed oligomer mass
P7 (6-mer)	6952.39	6954.39 ± 3	41714.34	41717 ± 157
MscL (5-mer)	15694.51	15695.02 ± 6	78485.55	78882 ± 42
Kirbac (4-mer)	33577.57	33586.48 ± 30	134310.28	134371 ± 142

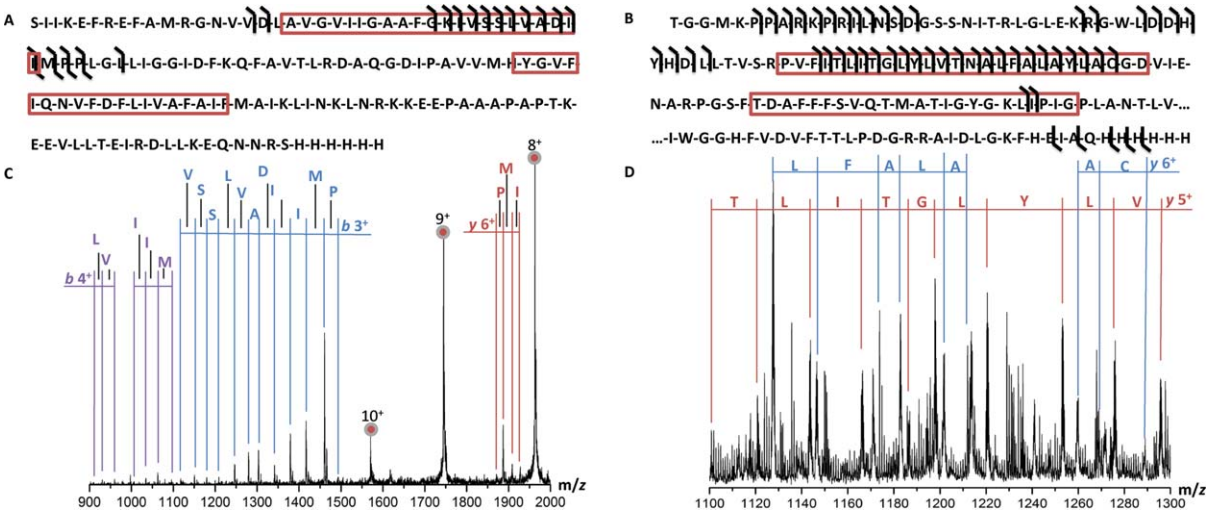

Figure 3. Primary structure of membrane proteins investigated using top-down CID. (A) Sequence of MscL G22C with *b*- and *y*-fragments indicated and (B) Excerpt (1–112 and 265–300) from the sequence of Kirbac3.1. Predicted membrane spanning regions are indicated with a red box. (C) Part of the spectra observed for MscL and (D) Kirbac3.1 displaying CID fragments.

of detergent was circumvented, allowing facile assignment of fragments in the lower m/z regions [Fig. 2(c)].

Increasing the acceleration voltages well past the thresholds for detergent removal and subunit dissociation yielded CID fragments for all three ion channels (Fig. 2 blue boxes). As already mentioned, the energy required to induce top-down CID fragmentation depends on the detergent, but also on the mass and charge of the protein. To facilitate comparison of acceleration voltages between different systems, the laboratory frame energy (in eV) is often used which can be obtained by multiplying the ion charge with the acceleration (collision) voltage. The energies required for CID fragmentation differ drastically between proteins, with laboratory frame energies between 2.20 and 2.75 keV for p7, whereas for Kirbac the values range from 9.2 to 11.2 keV [for the charge states in Fig. 2(a,b)]. One factor for these different fragmentation thresholds in CID, which is an ergodic process, is the mass of the protein, as larger proteins have more degrees of freedom over which the increase of internal energy can be distributed. For membrane proteins however such a relationship is more difficult to assess, as the stripping of detergent in the gas-phase is the crucial step prior to fragmentation. Although both p7 and MscL were reconstituted in triton x-100, they require similar energy levels for fragmentation (2.20-2.75 keV for p7 and 2.89 keV for MscL 17+), even though pentameric MscL is almost double the mass of the p7 hexamer. Another complicating factor for sequencing of membrane proteins by CID is that membrane proteins tend to have lower average charge states than soluble proteins, most likely as a result of being partially embedded in micelles at the beginning of the electrospray process[37]. Fortunately, this effect is compensated by the asymmetric dissociation induced by CID which produces highly charged monomers and top-down fragments (Fig. 3). In hetero-oligomeric complexes though, typically only the smallest exposed subunit dissociates while taking a disproportionate amount of charge with it – thereby limiting the use of top-down CID sequencing to this subunit. In such cases it might be beneficial to first produce subcomplexes in solution by destabilization with chaotropic agents, prior to asymmetric dissociation and sequencing[39,40]. The 17+ MscL precursor displayed b-fragments with a charge of predominantly 3+ and 4+, although some 2+ fragments were observed as well, and a small series of 6+ y-fragments [Fig. 3(a−c)]. Kirbac shows some singly charged C-terminal y-fragments, but otherwise mainly b- fragments with charge states ranging from 1+ up to 7+ [Fig. 3(b)]. The high prevalence of b-ions for these spectra is remarkable, as they are considered to be much more labile than y-ions due to the possibility to undergo subsequent rearrangements, resulting in a-fragments.[41] It should be noted though that conditions here were somewhat less energetic than in standard Q-TOF CID, as the voltages used are near the maximum possible on the instrument and contribute to a large part to detergent removal.

The top-down CID fragments of different native charge states were found to be clustered around limited stretches of the protein, with a sequence coverage for MscL and Kirbac of 11% and 18%, respectively [Fig. 3(a,b)]. Fragmentation for both these proteins appears mostly in the N-terminal part of the protein sequence, with little to no fragments from the middle or end of the sequence. Such limited fragmentation efficiency is quite common in top-down studies and has also been reported for

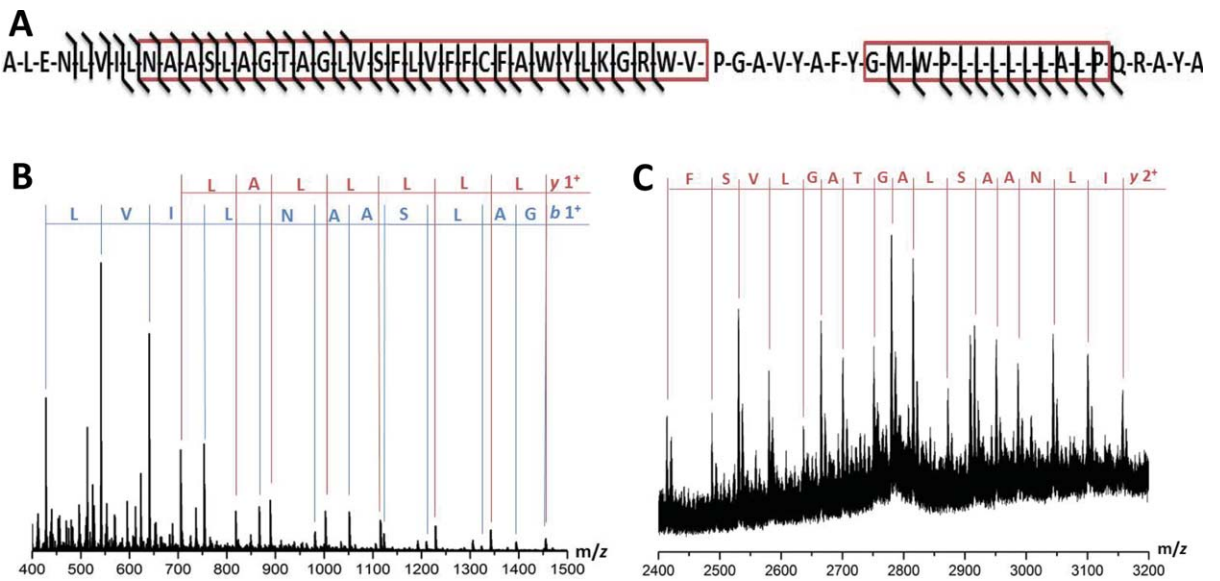

Figure 4. Top-down CID fragments observed for the viral porin p7 at 275 V collision energy. (A) Fragmentation observed for the sequence of p7 with membrane spanning regions indicated by a red box. Fragments observed in the (B) low *m/z* region and (C) high m/z region of p7.

larger IMPs before. It has been postulated that even under denaturing conditions these proteins maintain some residual structure,[42] which results in the limited fragmentation efficiency for larger IMPs. The p7 viroporin on the other hand, which is much smaller than the other two proteins, showed more intense CID fragments (Fig. 4). The sequence coverage was 73%, which would indicate the absence of residual structure due to its smaller size. Fragmentation was neither observed in the N- or C-terminus nor in amino acids 36−44 in this case.

Recent top-down proteomics work by Kelleher *et al*. on 126 denatured membrane proteins has shown that CID has a four-fold preference for transmembrane domains.[43] As all membrane proteins studied here were known to have membrane-spanning alpha-helices, we used the sequences of the proteins to predict which regions where embedded in the lipid bilayer.[44,45] Such approaches are often used to identify membrane regions when no X-ray or NMR structure or homology model is available. Although high-resolution structures of Kirbac3.1 and a homologues structure of MscL are available, the exact structure of the oligomeric p7 viroporin is still under debate.[46–48] Comparing the observed fragmentation sites with the predicted transmembrane domains, we note that all three proteins displayed fragmentation within their transmembrane-spanning domains [Fig. 5(a)]. For MscL and p7, cleavage seems to occur predominantly in membrane regions, whereas Kirbac displays sites in soluble regions as well. As transmembrane prediction approaches should be used with caution, we validated our observations by plotting the observed CID fragments onto the available high-resolution structure of Kirbac3.1. To provide a similar graphical representation for p7 and MscL, we used homologous structures, as there are currently no high-resolution structures available for these IMPs [Fig. 5(b)].

Top-down fragmentation of these membrane proteins delivers extensive sequence tags, which is quite remarkable as CID of highly charged proteins tends to favor cleavage sites which are specific to certain combinations of amino acids. Preferential C-terminal cleavage after an aspartic acid for lowly charged, and N-terminal cleavage before a proline residue for highly charged proteins have been reported.[49] Such preferred cleavages often limit the sequence information obtained and make the spectra difficult to interpret, due to the considerable differences in fragment intensity. This does not seem to be the case however for the three IMPs studied here, where a more equal fragmentation propensity was observed. As CID strongly depends on charge localization and side-chain charging is expected to be absent in the transmembrane helices, this would suggest that protons can move towards these regions when becoming mobile during the CID process. It has been postulated that rather than interacting with basic side chains as expected for soluble proteins, the mobile protons are primarily attached to the amide backbone in such hydrophobic stretches of the amino acid sequence.[43,50] As such interactions are less specific than interactions with a basic side chain, top-down CID of membrane proteins yields not only a greater number of backbone cleavages, but also over a larger number of backbone positions.[43]

Here we have shown how a single set of experiments yields the oligomeric state, accurate subunit

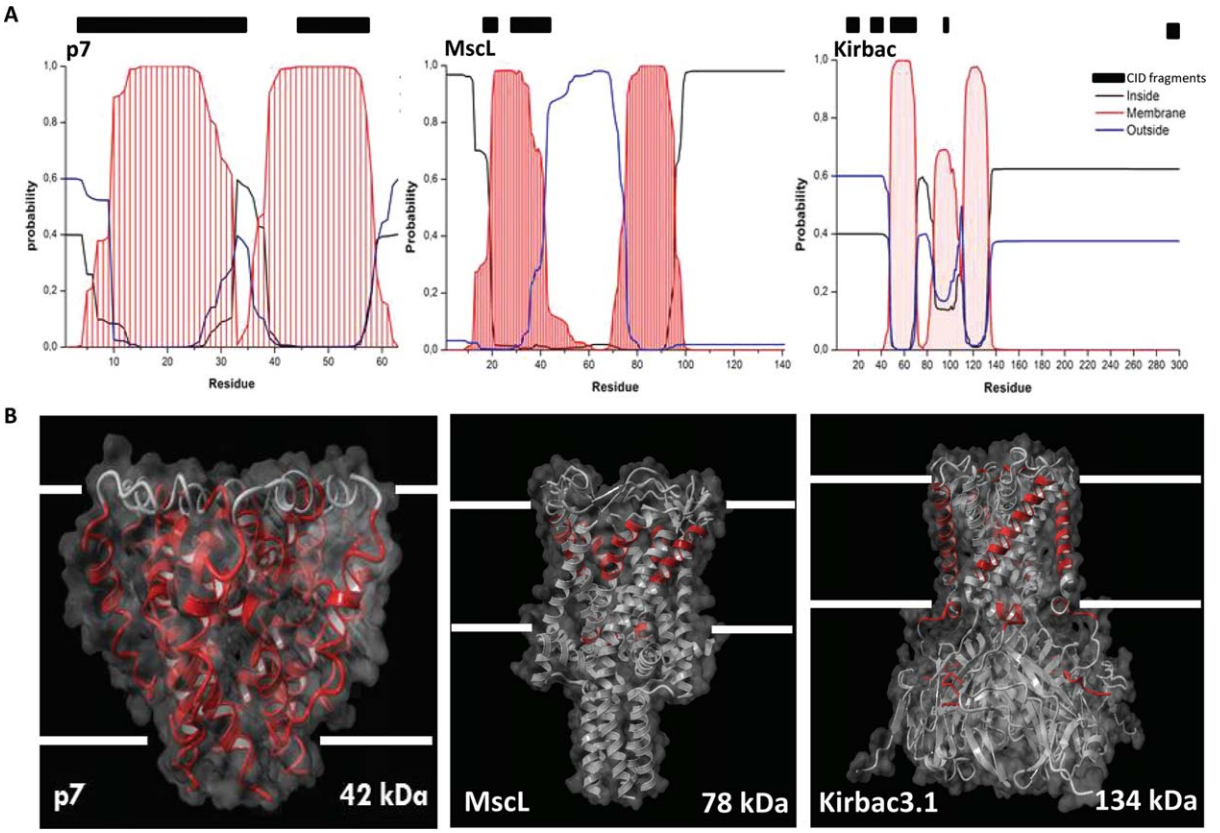

Figure 5. Top-down CID of membrane proteins (detergent micelles; native ESI) leads to fragmentation predominantly in membrane-embedded regions. A: Transmembrane prediction for each of the three proteins reveals potential membrane-embedded areas (red curves); comparison with observed CID fragmentation sites (black bars) shows a preference for membrane regions. B: Mapping the observed fragmentation sites onto the 3D-structures of the three membrane proteins, confirms the results obtained by TMH prediction. Peptide fragments observed are mapped (in red) onto the structures of the ion (in gray). PDB IDs 2M6X, 2OAR and 3ZRS, were respectively used for p7, MscL, and Kirbac3.1 visualization.

mass and (partial) sequence information on membrane proteins, possibly even indicating membrane-embedded parts of the sequence. The main advantage of such an experimental approach is that the interdependency between amino-acid sequence (proteoform) and higher-order structure can be investigated simultaneously, even in heterogeneous samples. In comparison to the reported sequence coverage of 9–22% for top-down CID of denatured membrane proteins with multiple transmembrane domains on a Q-TOF instrument,[42] we obtain similar values in top-down CID of native, oligomeric IMPs embedded in detergent micelles. Although currently the experimental approach is not yet suitable for high-throughput studies, recent developments in coupling size exclusion chromatography with electrospray ionization[51] or automated sampling[52] might significantly increase the viability of such attempts in the context of structural proteomics. As the experimental strategy applied here is easily integrated with the current methodology used when studying IMPs by native MS, it opens up new opportunities to study the effect of PTMs and sequence variations on the higher-order structure of membrane proteins and their complexes.

Materials and Methods

Sample preparation

Kirbac3.1 was expressed overnight at 19°C in E. coli BL21 CodonPlus (DE3) RP (Agilent) and purified according to the protocol described previously,[53] with the exception of the gel-filtration step that was performed in 200 m*M* ammonium acetate and 0.5 m*M* 3DM detergent. The protein was subsequently concentrated to 0.8 mg/mL using Vivaspin concentrators with molecular weight cutoff of 50 kDa. In these experiments, we used the W46R mutant as it shows an increased stability compared to the wild-type Kirbac. P7 samples were prepared by dissolving 0.1 mg lyophilized p7 (H77 strain with the H17A mutation) in 100 μL triton X-100 (10x CMC), vortexed, and subsequently spun down to remove any large remaining particles prior to analysis. Pentameric MscL ($G22C_5$ mutant) reconstituted in triton X-100 was prepared as reported earlier.[15]

Mass spectrometry

All samples were introduced into the vacuum of the mass spectrometer without further desalting using nanoESI with gold-coated borosilicate capillaries at

capillary voltages of 1.2−1.6 kV. Spectra were recorded in positive ion mode on a commercially available travelling wave ion mobility mass spectrometer (Synapt G2 HDMS, Waters) in TOF-mode. The instrument was fitted with a 32 k quadrupole and settings optimized for transmission of large complexes. Gas pressures were 6.0−9.0 mbar, 1.5 e^{-2} mbar and 4.6 e^{-2} mbar for backing, source and trap cell, respectively. Collision energies were optimized for each membrane protein specifically in order to obtain maximum CID fragmentation, whilst maintaining some of the original oligomeric protein present. Voltages used were 150/150/10/125 for p7, 125/10/10/160 for MscL and 200/200/10/200 for sampling cone, trap CE, trap DC bias and transfer CE, respectively. The m/z scale of all mass spectra was externally calibrated using a 10 mg/mL CsI solution. The spectra were processed using Masslynx 4.1 (Waters). Transmembrane predictions were performed using the TMHMM server, as described elsewhere.[39,40]

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