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Drug Binding Revealed by Tandem Mass Spectrometry of a Protein−Micelle Complex

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Electrospray mass spectrometry (ES MS) approaches for characterizing membrane proteins in general involve extraction into organic solvents to maintain solubility and enable spectra of unfolded protein molecules to be recorded.[1] Consequently, under such conditions, interactions between proteins and lipids are lost. Strategies that have been developed to overcome these denaturing approaches involve various lipid preparations and have led to MS of peripheral and transmembrane proteins bound to lipid molecules[2,3] as well as observation of an intact membrane protein trimer.[4] It has also been reported that clusters of up to 12 sodium dodecylsulfate molecules, derived from an intact micelle, could be preserved using ES MS.[5] Protein−lipid interactions within a protein−micelle complex have not been demonstrated previously.

Given that it is possible to obtain ES spectra of megadalton particles such as viruses[6,7] and ribosomes,[8] mass and complexity should not preclude study of protein−micelle complexes. It is established that transit from solution phase to low vacuum devoid of solvent molecules is accompanied by significant increase in electrostatic interactions and concomitant reduction in hydrophobic forces.[9] Consequently, this force that drives micelle formation and contributes to stability in solution is expected to be weakened. Previous ES MS experiments have shown that the hydrophobic nature of membrane proteins and absence of formal charge on nonionic lipid molecules lead to low charge states.[2] The combinations of low charge states, as well as the established dynamic nature in solution and potential instability of micelles in the gas phase, represent significant challenges for MS.

Here we show that it is possible to transfer into the gas phase a protein−micelle complex of EmrE from *E. coli* solubilized with the detergent dodecylmaltoside (DDM) while maintaining drug binding within the complex. This 110 amino acid transmembrane protein is involved in multidrug transport. The structure of EmrE in DDM with the cation substrate tetraphenyl phosphonium (TPP^+) has recently been shown by cryoelectron microscopy to involve an asymmetric dimer with four transmembrane helices present in each monomer.[10] One TPP^+ molecule binds simultaneously to two glutamic acid residues, one from each neighboring EmrE monomer.[11] We chose to analyze by ES MS solutions of EmrE in DDM in the presence of TPP^+ at pH8.0 where the off-rate of substrate is established as extremely slow.[11] This complex has also been extensively characterized by a variety of biophysical methods[12] and yielded the highest resolution structures to date.[10]

The MS recorded for EmrE purified in DDM (see Supporting Information) is shown in Figure 1A. Three major distributions are observed labeled peaks 1, 2, and 3. The broadness of these distributions is indicative of a heterogeneous assembly of protein, lipids, water/buffer molecules, and counterions. As such, it is not possible to discern the charge states or consequently the mass of these species.

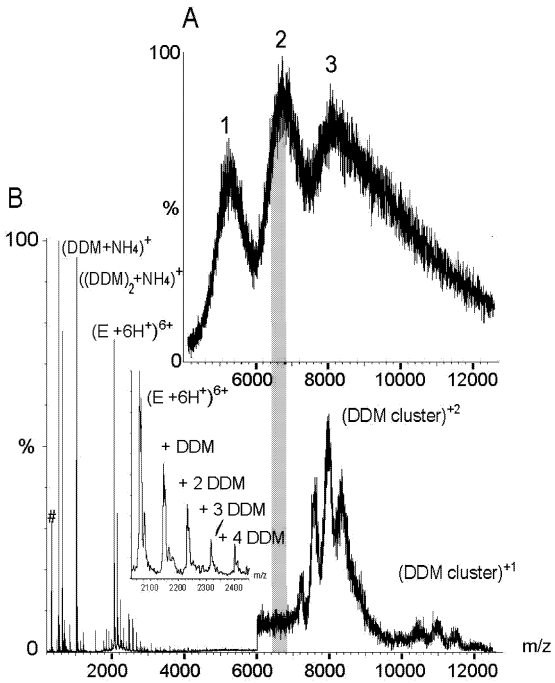

Figure 1. (a) Mass spectrum of EmrE in DDM with TPP^+ recorded with a needle voltage of 1.7 kV, cone voltage of 90 V, and pressures of 4.5−8.0, 9.5×10^{-4}, and 1.7×10^{-6} mbar in the ion transfer, quadrupole, and ToF analyzers, respectively. (b) MS/MS of ion isolated at 6500 ± 100 *m*/*z* units (shaded) and accelerated at 30 V through the collision cell at a pressure of 3.5×10^{-2} mbar. # represents the peak assigned to TPP^+. Inset shows an expansion with ×15 magnification of *m*/*z* 2000−2500 showing EmrE 6+ with 1−4 DDM adducts.

To extract information from this spectrum therefore and determine the composition of the broad peaks we isolated defined *m*/*z* ranges using a modified time-of-flight MS with a low-frequency quadrupole capable of isolating packets of ions at high *m*/*z* values.[13] One such isolation of ions from peak 2 and subsequent acceleration through the collision cell gives rise to the tandem mass spectrum shown in Figure 1B. Two distinct series of peaks at higher *m*/*z* values than the isolated region are observed. Unlike the spectra of proteins or their complexes, neighboring peaks within these series do not differ in charge, rather the difference between each peak is approximately constant, consistent with addition of neutral DDM molecules. These series are assigned therefore to clusters of DDM containing on average 20 ± 2 and 30 ± 1 molecules with one and two charges, respectively.

In the low *m*/*z* region of the tandem MS, DDM monomers and dimers, as well as fragments, can be discerned. A peak at *m*/*z* 2065

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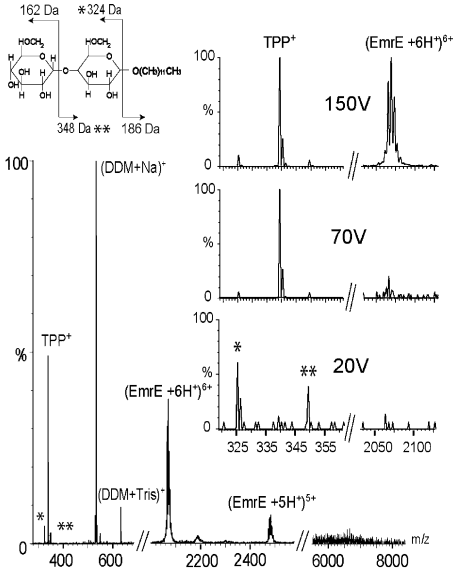

Figure 2. Tandem MS of ions isolated at *m*/*z* 6800 ± 100 and acceleration with a collision cell voltage of 150 V revealing the presence of drug, protein, and lipid. Inset: Expansion of the MS/MS spectra encompassing *m*/*z* range of TPP^+ and the 6+ charge state of EmrE. At 20 V, fragmentation of the DDM (*/**) headgroup is apparent. At 70 V, a peak at *m*/*z* 339 assigned to TPP^+ is apparent, while at 150 V, both EmrE 6+ and TPP^+ are visible.

is prominent and assigned to the 6+ charge state of monomeric EmrE (measured mass 12 388 ± 4, calculated value 12 387 Da).

Also apparent are a series of peaks with 6+ charges assigned to binding of EmrE to 1−4 DDM molecules. Below *m*/*z* 400, the predominant peak at *m*/*z* 339 (labeled #) is assigned to the substrate TPP^+. Examination of the broad peaks (1 and 3) on either side of the isolated peak revealed similar spectra of DDM clusters at high *m*/*z* and DDM molecules at low *m*/*z* values. It was not, however, possible to identify appreciable quantities of EmrE or TPP^+, implying that only the central distribution can be assigned to species containing drug, protein, and lipid that together constitute the functional complex.

To investigate the dissociation of the complex in detail we carried out tandem MS on peak 2 using a stepwise increase in collision cell voltage and monitored the regions of the spectrum assigned to TPP^+ and the 6+ charge state of EmrE (Figure 2). At the lowest collision cell voltage (20 V), peaks corresponding to fragments of DDM arising from cleavage across glycosidic linkages can be discerned. At intermediate voltage (70 V), the peak at *m*/*z* 339 corresponding to TPP^+ is apparent. Further increase in collision cell voltage to 150 V reveals not only TPP^+ but also the 6+ charge state of EmrE. At higher collision cell voltages, it is possible to effect the complete disruption of the protein−micelle complex, yielding all three components: DDM, TPP^+, and EmrE. This stepwise dissociation shows that DDM molecules are dissociated at the lowest collision cell voltage, while release of TPP^+ and subsequently EmrE from the complex requires significantly higher acceleration voltages. It is important to note that only folded dimers of EmrE are competent to bind TPP^+ with high affinity.[14] The fact that TPP^+ is released at lower voltages than EmrE and prior to the eventual disruption of the complex is consistent with the preservation of the associations of EmrE with lipids even after release of TPP^+.

The question arises as to the extent that the protein−micelle complex is maintained in the gas phase. Given the fact that formation of micelles has been shown to be facilitated by evaporation,[5,15] such a process could be considered akin to the early stages of ES. This would therefore enable protein−micelle complexes to be transferred into the gas phase largely intact, stabilized by the hydrogen bonded structure of the DDM headgroups mediated by water molecules. However, the transition from the ES source to the high vacuum of the analyzer might be anticipated to destabilize the protein−micelle complex. Given that the mass of the solution-phase protein−micelle complex has been determined as 137 kDa,[12] ~20 exposed positive charges would be required to satisfy the *m*/*z* value of peak 2 (7000 Da/e). The anticipated low surface charge of DDM molecules, the burial and neutralization of formal charge on the protein with counterions inside the micelle are not consistent with the level of charging required to satisfy this *m*/*z* value. However, the observation that lipid, protein, and drug components are released from species in this *m*/*z* range allows us to propose that these peaks correspond to heterogeneous submicelles, formed from collapse of the intact protein−micelle complex during transmission under high vacuum.

In summary, the protein−micelle complexes can be interrogated using tandem MS in a controlled manner by manipulating the voltage across the collision cell. The three broad distributions observed in the mass spectra are assigned to collapse of intact micelles. The peaks are tentatively assigned to empty submicelles (peak 1), submicelles containing both EmrE and bound substrate (peak 2), and aggregated forms (peak 3). Overall, therefore, our investigation allows us to infer that the functional dimeric species of EmrE must be preserved in gas-phase micelles as in solution. As a result, this study raises the exciting possibility of examining this membrane-associated drug pump in the presence of a wide range of small-molecule drugs. More generally, we anticipate that such investigations will have great value in accessing notoriously elusive membrane proteins and probing the presence of drug molecules within relevant complexes from intact micelles.

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Supporting Information Available: Preparation of EmrE, investigation of composition of DDM clusters, and comparison of protein-bound and free micelles (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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