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The Role of the Detergent Micelle in Preserving the Structure of Membrane Proteins in the Gas Phase**

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Abstract: *Despite the growing importance of the mass spectrometry of membrane proteins, it is not known how their transfer from solution into vacuum affects their stability and structure. To address this we have carried out a systematic investigation of ten membrane proteins solubilized in different detergents and used mass spectrometry to gain physicochemical insight into the mechanism of their ionization and desolvation. We show that the chemical properties of the detergents mediate the charge state, both during ionization and detergent removal. Using ion mobility mass spectrometry, we monitor the conformations of membrane proteins and show how the surface charge density dictates the stability of folded states. We conclude that the gas-phase stability of membrane proteins is increased when a greater proportion of their surface is lipophilic and is consequently protected by the physical presence of the micelle.*

The study of membrane proteins is challenging primarily due to the solubilization required for isolation from their natural lipidic environments.[1] Mass spectrometry (MS) of intact membrane protein complexes has recently emerged as a complementary biophysical approach, providing new insight into subunit stoichiometry, nucleotide interactions, and peptide or lipid binding.[2] Membrane proteins are transferred into the mass spectrometer encapsulated within detergent micelles by means of nanoelectrospray ionization (nESI), followed by their transmission into vacuum.[3] Here we examine the influence of the detergent micelle, which adds an additional layer of complexity to the process of generating ions when compared to water-soluble proteins.

To explore the relationship between the chemistry of the detergent and charge state of the membrane protein we considered a dataset of 49 different combinations of membrane proteins and detergents, spanning a mass range of 50–430 kDa (Tables S1–S4). In all detergents examined here, membrane proteins displayed charge state distributions (Δz) ≤ 7 consistent with a folded structure in solution.[4] Moreover saccharide detergents consistently gave higher charge states than poly(ethylene glycol) (PEG) and LDAO detergents (Figures 1 and S1, and Tables S5–S7) implying that these differences are due to the nature of the detergent, rather than the protein. However, plotting the observed charge state against properties of the detergent, such as surface tension or micellar mass, shows no correlation (R^2 of 0.01–0.15, Figure S2). Interestingly, in the case of PEG and LDAO detergents, the maximum charge states (z_{max}) were $>20\%$ below the Rayleigh limit (Table S6). Including the micellar mass[5] in our calculations further increases the difference between the Rayleigh limit and z_{max} (Figure 1b, inset), in accord with the water-accessible surface acquiring charge while the micelle protects the remainder of the membrane protein from ionization.[6]

To examine whether proton transfer from protein to detergent causes charge reduction, we monitored the effect of removing individual detergent molecules by collisional activation. MS of the multi-antimicrobial extrusion membrane protein (MATE) gives rise to well-resolved peaks corresponding to binding of individual detergent molecules. The average charge states (z_{ave}) of MATE in DDM, C8E4, and LDAO were 12.7+, 10.0+, and 10.1+, respectively (Figure 2a–c). Detergent molecules were then removed by increasing the accelerating potential into the collision cell until essentially only *apo* protein was observed, with a z_{ave} in DDM, C8E4, and LDAO of 12.4+, 9.8+, and 9.9+, respectively. Therefore the detergent molecules that were attached to the membrane protein in vacuum after droplet evaporation were subsequently lost as neutral species during collisional activation.

To evaluate whether the presence of detergent or the formation of micelles around the protein alters the charging propensity, we examined the soluble protein transthyretin (TTR). As TTR is not encapsulated within detergent micelles it allows the surfactant effect of the detergent to be decoupled from direct interactions with the protein. We found that the z_{ave} of TTR in the presence of DDM (14.5+) did not differ substantially from that in ammonium acetate alone (14.7+), with LDAO causing mild charge reduction (13.8+; Fig-

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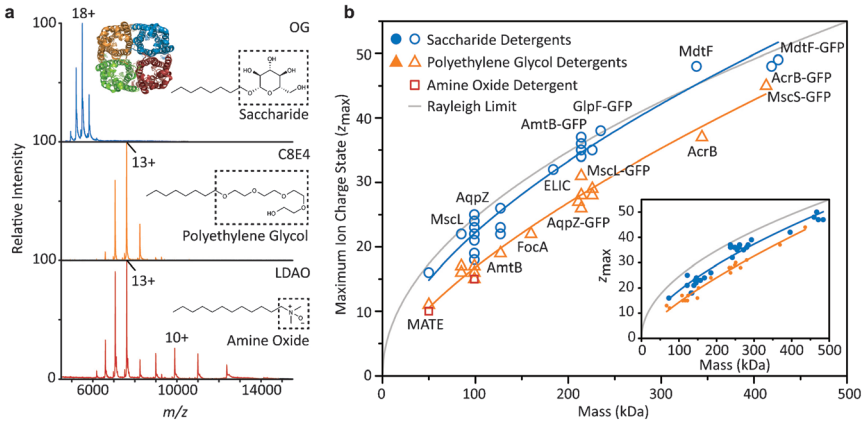

Figure 1. Detergents influence membrane protein charge states in the gas phase. a) Mass spectra of AqpZ in detergents OG, C8E4, and LDAO at maximal activation conditions (200 V) reveal different charge distributions. Detergent head groups are highlighted by dashed-line boxes. Inset is the crystal structure of *E. coli* AqpZ (PDB: 1RC2). The bimodal appearance at maximal activation when in LDAO is discussed in the Supporting Information. b) Maximum ion charge state (z_{max}) post-detergent release as a function of protein mass (M) and protein-micelle mass (inset). The theoretical Rayleigh limit (grey line) is for 200 mM ammonium acetate droplets. Abbreviations used for the membrane proteins and detergents are given in Tables S1 and S2. Data are fitted to the power-law relationship $z = aM^b$. All values and fits are given in Tables S3–S7.

ure 2d). Interestingly, addition of C8E4 led to a significant decrease in the z_{ave} of TTR (to 9.4+).

To dissect the effects of C8E4 on charge reduction, we added the head group of C8E4, tetraethylene glycol (E4, 0.5%), to both TTR and the ammonia channel membrane protein (AmtB) solubilized in DDM. For TTR the z_{ave} decreased from 14.7+ to 13.2+ (Figure 2d), and for AmtB from 21.9+ to 19.1+ (Figure S3). For both proteins, this charge-reduction effect is less pronounced than for C8E4 (9.4+ and 16.3+ for TTR and AmtB, respectively). Thus the properties of the head group are not solely responsible for charge reduction, but rather the amphiphilic properties of the detergent must also play a role. We propose that C8E4 clusters serve as low-molecular-mass charge carriers, resulting in nESI droplets, and subsequently membrane protein ions, with reduced overall charge. This agrees with experimental evidence for the evaporation of PEG ions from highly charged water droplets

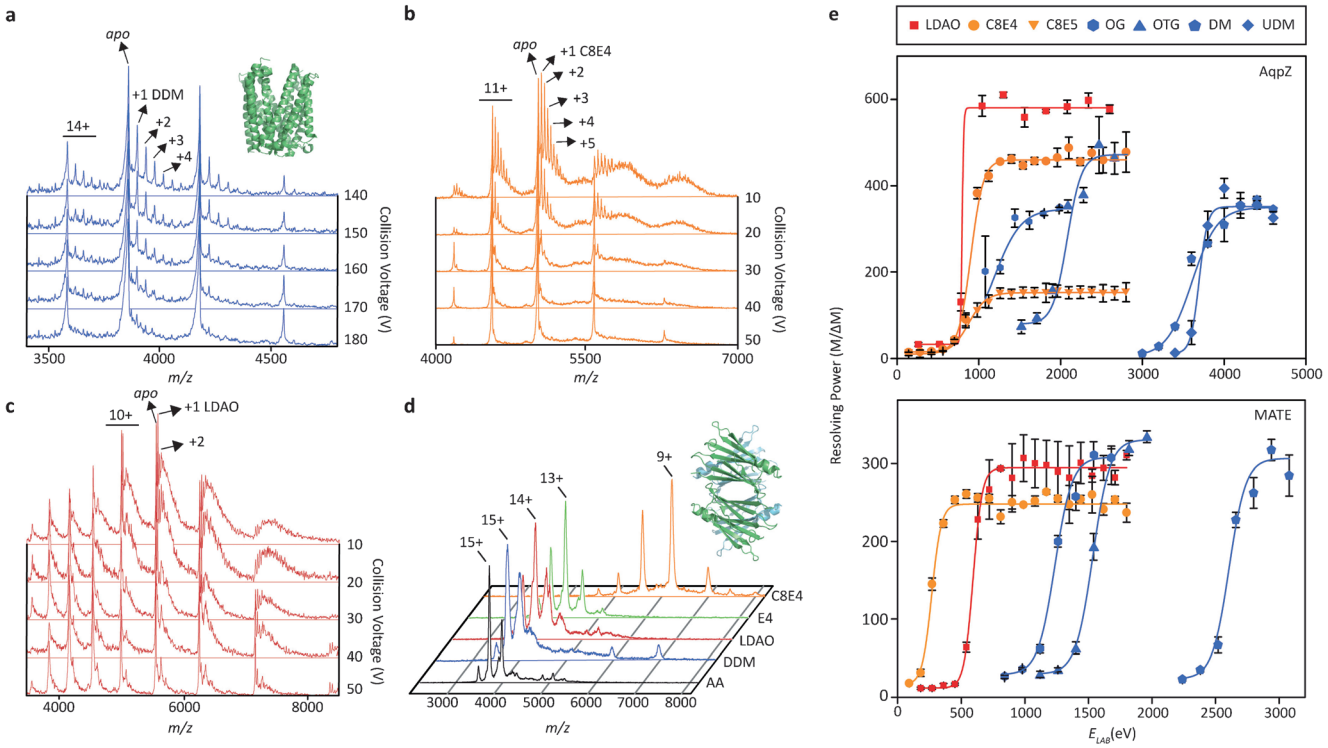

Figure 2. Detergent molecules are lost as neutrals upon collisional activation, with the detergent chemistry influencing both the ionization and release of membrane proteins in the gas phase. Mass spectra of MATE with bound DDM (a), C8E4 (b), and LDAO (c) show detergent removal upon collisional activation. Inset is the crystal structure of *P. furiosus* MATE (PDB: 3VVN). d) TTR in the presence of twice the critical micelle concentration of detergents (DDM and LDAO) and 0.5 % E4 in 200 mM ammonium acetate (AA), and in 200 mM AA only, at 20 V. Inset is the crystal structure of *H. sapiens* TTR (PDB: 4N85). e) The m/z resolving power at E_{LAB} for AqpZ (top) and MATE (bottom). The number of bound detergent molecules is proportional to the m/z peak width (ΔM; Figures S4 and S5). Detergents are colored by class (saccharide, purple; PEG, orange; LDAO, red). Data are fitted with a sigmoidal distribution, and error bars represent the standard deviation from three replicates. Abbreviations used for detergents are given in Table S2.

until a sustainable charge is achieved.[7] Together, therefore, the ionization of membrane protein assemblies is well described by a combined charged-residue ion-evaporation model,[8] with PEG and saccharide detergents primarily emitted as charged clusters or neutrals, respectively.

A major factor that can affect the structure of gas-phase membrane proteins is activation during detergent removal. We therefore investigated the process of detergent release as a function of accelerating potential, V, into the collision cell ($E_{LAB} = zV$),[9] for the membrane proteins aquaporin-Z (AqpZ) and MATE (Figure 2e). For the three detergent classes investigated, an increase in E_{LAB} results in a decrease in both the average number of detergent molecules bound, and a narrowing of the distribution of bound forms (Figures S4 and S5). This reflects a Poissonian process revealing that the detergents behave as nonspecific nESI adducts.[10]

Examining the influence of E_{LAB} on the resolving power and average number of detergents bound reveals clear sigmoidal trends, with sharp inflexions indicating that detergent removal occurs over a narrow energy range (Figures 2e and S4). For both AqpZ and MATE, LDAO and PEG are removed at lower E_{LAB} than saccharide detergents, with the glucoside detergents (e.g. OG) at lower E_{LAB} than the maltoside detergents (e.g. DM).

The observed differences in the ease of detergent removal may relate to protein stability within the detergent micelle: for instance, DM creates a more stable environment for bacterial membrane proteins[1] and requires higher activation energies for removal, whereas LDAO, C8E4, and OG are considered 'harsher' detergent environments, requiring lower activation energies. Additionally we have observed that lipids typically remain bound to protein after the detergent is released.[11] Taken together, this suggests that ease of a detergent's release is inversely correlated with its ability to substitute for natural lipids.

As C8E4 required the lowest E_{LAB} for detergent release, we studied its effect in more detail using four membrane proteins: AmtB, mechanosensitive channel of large conductance (MscL), formate channel (FocA), and acriflavine resistance protein B (AcrB) (Figure S6). To assess their folded state we performed ion mobility MS (IM-MS), which can report on the conformation of an ion through its collision cross section (CCS).[12] IM data obtained as a function of E_{LAB} reveal gas-phase unfolding trajectories that allow extraction of the number of intermediate states populated, and the activation required for 50% depletion of folded states ($E_{LAB}{}^{F}{}_{50\%}$; Figure 3a,b). These parameters describe the stability of each charge state in the gas phase.[11a,13]

We performed such collision-induced unfolding experiments on the four membrane proteins, and considered the increase in CCS of the intermediates relative to that of the folded state ($\Delta CCS_{I\text{-}F}$ (%)) as a function of surface charge density σ ($\sigma = z/SASA$, in which SASA is the solvent-accessible surface area of the protein; Figure 3c). Folded conformations of AmtB, MscL, FocA, and AcrB were observed for the majority of charge states investigated (Tables S8 and S9). In all cases, one or more unfolding intermediates were also observed, with the centroid and width of their CCS distributions varying as a function of charge

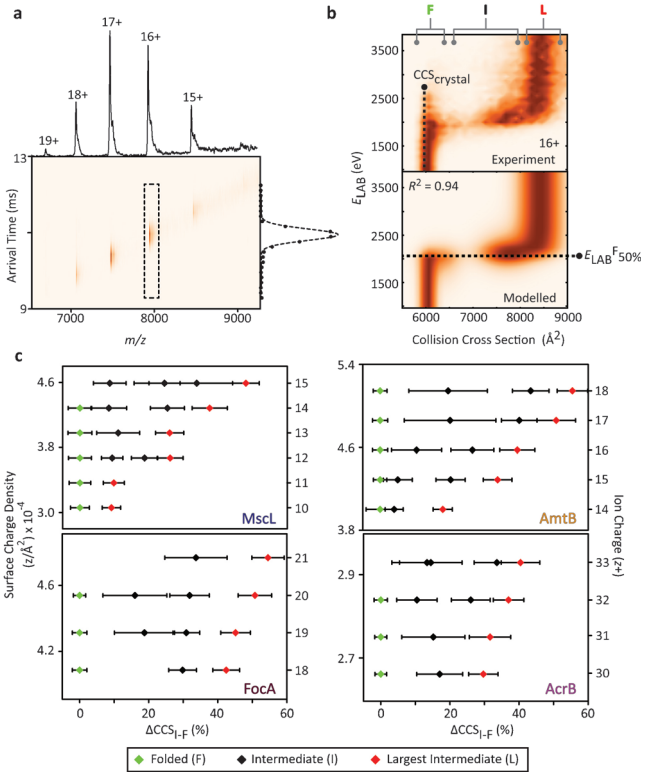

Figure 3. The preservation and unfolding trajectory of folded gas-phase conformations of membrane proteins is dependent on surface charge density and structure. a) IM-MS spectrum of AmtB in the presence of C8E4. Shown are the arrival time distributions of all ions with the raw data of the 16+ ion (dashed-line box) extracted and fitted with a Gaussian distribution (dashed line). Ion mobility is shown in linear intensity scale. b) Gas-phase collision-induced unfolding data and modeled fits of AmtB 16+ ion. The folded native-like state (F, green) is in agreement with the collision cross section (CCS) of the crystal structure ($CCS_{crystal}$). The ions unfold to produce denatured intermediates (I, black) eventually reaching a maximum CCS for an intermediate population before monomer dissociation (L, red). c) The influence of surface charge density on membrane protein unfolding states within a fixed E_{LAB}. The gas-phase populations of MscL, FocA, AmtB, and AcrB with different surface charge densities are expressed as the CCS increase of intermediates (I) from a folded native-like gas-phase state (F). The gas-phase collision induced unfolding for different charge states (z) on the same membrane protein were performed under equivalent conditions: E_{LAB} range for MscL of 900–2400 eV, AqpZ of 675–2700 eV, AmtB of 1080–3360 eV, FocA of 1260–4320 eV, and AcrB of 3900–7200 eV. The intermediates observed are shown with error bars to signify the full width to half maximum of the modeled CCS distributions (Table S9).

state. At similar σ (e.g. $\sigma \approx 4.0$ for AmtB and FocA) the proteins unfold through different intermediates. Gas-phase unfolding pathways are therefore not solely dependent on σ but also reflect the inherent structural characteristics of a membrane protein, as demonstrated recently for monomeric soluble proteins.[14]

In vacuum, proteins unfold due to an increase in internal energy upon collisional activation, during which charge can migrate to the newly exposed area to minimize Coulombic repulsion.[15] An increase in σ indeed lowers the energy

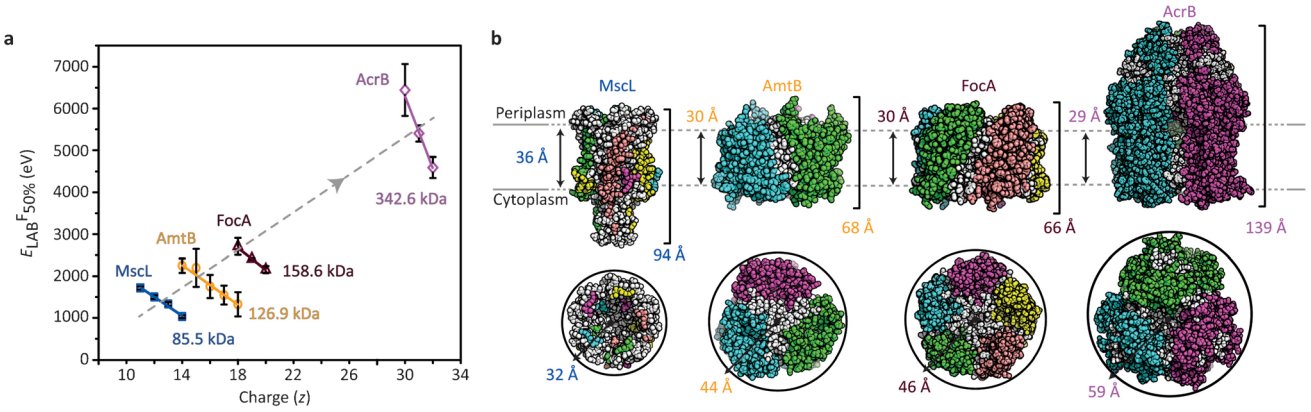

Figure 4. Coulombic repulsion affects the stability of membrane proteins in the gas phase. a) Linear fits of $E_{LAB}{}^{F}{}_{50\%}$ versus charge state (z). The error bars represent the standard deviation from triplicate measurements. As the size of the membrane protein complex increases the energy required to unfold the structure increases due to the ergodic collisional activation process (gray dashed line). The extent of unfolding as a function of charge (m, gradient from linear fits) is $m = -229 \pm 17$, -236 ± 16, -259 ± 15, and -865 ± 78 for MscL, AmtB, FocA, and AcrB, respectively. The errors are the standard error of the linear fits. b) Crystal structures of *M. tuberculosis* MscL (PDB 2OAR), *E. coli* AmtB (PDB 1U7G), *E. coli* FocA (PDB 3 KCU), and *E. coli* AcrB (PDB 2GIF). Reported are approximate protein dimensions quoted in Table S10. The oligomer subunits are uniquely colored, with the residues at the subunit interfaces colored light grey.

barrier for unfolding due to the enhanced Coulombic repulsion (Figure 4a).[16]

To interrogate stability in response to enhanced charging we examined the relationship between SASA and the Coulombic unfolding potential. The latter quantity is determined from the gradients of linear fits (Figure 4a), and describes the extent of change in gas-phase stability upon the addition of charge. We found a negative correlation between SASA and the Coulombic unfolding potential (R^2 of 0.85).

This remarkable observation can be explained by considering the lipophilic surface area of the membrane proteins (Figure 4b and Table S10). By assuming that the trans-membrane regions are cylindrical, one can approximate the lipophilic surface area of MscL, AmtB, FocA, and AcrB to 22%, 23%, 19%, and 9%, respectively. Therefore the smaller the lipophilic surface area the greater (more negative) the Coulombic unfolding potential becomes. This could be due to the protection of the trans-membrane region as membrane protein–detergent complexes are charged primarily on water-exposed regions. Alternatively the presence and release of detergent molecules during collisional activation aids in protecting this lipophilic region to structural unfolding, perhaps by evaporative cooling. This means that the more surface area that is shielded by detergent during ionization, the more stable the ion is in response to charge addition. Collectively, these results suggest that the Coulombic unfolding potential is dependent on the lipophilic surface area.

Here we have presented an understanding of how detergents mediate the ionization and stability of membrane proteins within the mass spectrometer. Specifically we have found that PEG and LDAO produce charge-reduced ions, whereas saccharide detergents lead to charge states closer to the Rayleigh limit. We have also shown that some detergents commonly employed in solution-phase structural studies (e.g., DDM and DM) are not ideal for MS, as they require a higher E_{LAB} for removal compared to other detergents (e.g., C8E4 and LDAO) that mimic the natural lipids less effectively. Observations presented here suggest that membrane proteins with greater detergent-encapsulated area are better able to tolerate collisional activation without unfolding than those in which this area is proportionally smaller. Our investigations of surface charge densities reveal that the higher charge states, and therefore higher surface charge densities, for membrane proteins ejected from saccharide-containing droplets potentially reduce the chances of forming a folded native-like conformation after detergent release.[11a] However, our insights suggest that the use of saccharide detergents may be viable providing a favorable surface charge density is obtained through charge reduction.[17]

Understanding the relationship between the detergent properties and the resultant charging, structural preservation, and unfolding provides a biophysical framework for the interrogation of membrane protein structure in the gas-phase. Moreover we have established that as different intermediates are populated during collisional activation of different membrane proteins, despite similar surface charge densities, gas-phase unfolding must be dependent on the membrane protein structure. This is an important criterion for assessing the effects of ligand binding to this important class of therapeutic targets.

Keywords: ion mobility spectrometry · mass spectrometry · membrane proteins · micelles · structural biology

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