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Effects of non-ionic and zwitterionic detergents on soluble proteins during native mass spectrometry experiments

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ABSTRACT

Detergents are commonly employed for solubilization and stabilization of integral membrane proteins. Their effects on the charge states of detergent-solubilized membrane proteins in native mass spectrometry measurements have previously been described. These effects can be mediated through the transmembrane region directly interacting with the detergent micelle or through the soluble domains of the proteins getting in close contact with detergent micelles during electrospray ionization. Here, we explore the effects of detergent micelles on soluble proteins during native mass spectrometry aiming at distinguishing these two events. Specifically, we employ two non-ionic and one zwitterionic detergents as well as a variety of standard proteins differing in size, oligomeric state and surface hydrophobicity, and evaluate the observed charge states in the absence and presence of detergents. Using ion mobility-mass spectrometry, we assess the proteins' stability as well as the ability to maintain non-covalent interactions with ligands. Finally, we examine lipid transfer from mixed detergent-lipid micelles containing the various detergents to soluble proteins. In summary, we found that C8E4 detergent reduces the proteins' charge states, stabilizes the proteins and facilitates lipid transfer.

1. Introduction

Detergents are amphipathic molecules. Similar to phospholipids, they contain a polar head group and a hydrophobic moiety. Detergents are mainly classified as ionic, non-ionic or zwitterionic [1]. Ionic detergents carry a net charge in their head group. Their hydrophobic group is usually composed of a hydrocarbon chain or a steroidal backbone. Common examples are sodium dodecyl sulfate or bile acid salts. The head groups of non-ionic detergents are hydrophilic and uncharged. They are further classified into polyoxyethylene and glycosidic head groups. Prominent examples are 1-O-(n-Octyl)-tetraethyleneglycol (C8E4) or n-Octyl β-D-glucopyranoside (OG). Zwitterionic detergents such as *N,N*-Dimethyl-*n*-dodecylamine-*N*-oxide (LDAO) carry positive and negative charges in their head group resulting in a net charge of zero [1].

Due to their amphipathic structure, detergents spontaneously form micellar structures in aqueous solution when their critical micelle concentration (cmc) is reached [2]. The cmc is therefore an important characteristic for the individual detergents. A common application of detergents in biology is solubilization and stabilization of proteins and protein complexes [3]. This is of particular importance for membrane proteins which are insoluble in water. During extraction from the phospholipid bilayer of biological membranes, integral membrane proteins are reconstituted into detergent micelles; thereby detergent molecules assemble at the transmembrane region of the protein embedding it in a detergent ring [4].

Importantly, the structure of the detergents affects their solubilization characteristics: Ionic detergents very effectively solubilize membrane proteins, however, they are often denaturing. An exception are bile acid salts which are considered milder detergents with lower denaturing properties. Non-ionic detergents break protein-lipid interactions while protein-protein interactions are mostly maintained; they are therefore comparably mild and non-denaturing detergents. Zwitterionic detergents combine the properties of ionic and non-ionic detergents; they are less inactivating than ionic detergents but more denaturing than non-ionic detergents. Apart from these general guidelines, numerous structural studies on membrane proteins showed that the right detergent has to be chosen for each individual protein [1,4].

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Detergents were also extensively explored for their application in mass spectrometry (MS). This included initial studies exploring their general applicability in MS measurements [5] up to detergent screening for native MS compatibility [6,7]. Specifically designed detergents for native MS of membrane proteins have also been introduced [8,9]. As a consequence, a variety of non-ionic and zwitterionic detergents is nowadays available to study the structural arrangements of membrane proteins by MS [10–13]. During native MS, the intact protein-detergent complex is transferred into the gas phase and the protein is released from the detergent micelle by collisional induced activation [14]. Importantly, the number of charges carried by the released protein was found to be detergent-dependent [7,15]. However, the underlying principles of charge reduction or super-charging of membrane proteins when released from the detergent micelle are largely unknown.

Membrane proteins naturally co-purify with their surrounding or stably-bound lipids; associated lipids as well as the interactions they form with the membrane proteins are then often identified by MS [16]. However, the protein-lipid interactions of peripheral membrane proteins, which dynamically interact with the lipid membrane, are difficult to capture. The transfer of lipids from detergent-lipid micelles to soluble proteins has recently been described, paving the way to study lipid binding to peripheral membrane proteins or lipid-binding soluble carriers [17].

Here we explore the effects of three MS-compatible detergents on soluble proteins during native MS experiments. For this, we chose the non-ionic detergents OG and C8E4 as well as the zwitterionic detergent LDAO (see Fig. S1 for structures). Using standard proteins differing in size, oligomeric state and surface hydrophobicity, we evaluate protein charge states as well as protein stability and ligand binding in the presence and absence of the detergents during electrospray ionization and native MS. We further explore lipid transfer capabilities from the different detergent-lipid micelles to a protein. We anticipate that these experiments will enhance our understanding of charging effects on membrane proteins during native MS when detergents are used for solubilization.

2. Experimental methods

2.1. Materials

Alcohol dehydrogenase (ADH), β-lactoglobulin (β-LG), bovine serum albumin (BSA) and myoglobin (MG) were purchased from Sigma-Aldrich. OG, LDAO and C8E4 were purchased from Glycon Biochem. All proteins were solubilized in 200 mM ammonium acetate solution and mixed with the detergents to a final protein concentration of 10 μM and a detergent concentration of 1-4 × cmc. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) solubilized in chloroform was purchased from Avanti Polar Lipids. Aliquots of POPE were prepared and the solvent evaporated under a stream of nitrogen. Dried lipid aliquots were overlaid with argon and stored at −20 °C.

2.2. Native MS

Native MS analysis was performed using a Waters Synapt G1 HDMS mass spectrometer modified for the transmission of high masses [18] The instrument was operated in TOF mode. The samples were loaded into gold-coated emitter needles prepared in-house [19]. The source pressure was adjusted to 5 mbar using a SpeediValve. Typical instrument parameters were: capillary voltage, 1.5–1.7 kV; sampling cone voltage, 30–80 V; extraction cone voltage, 10–100 V; collision voltage, 8–30 V; nanoflow pressure, 0.00–0.05 bar; trap cell pressure, 3.5–4 mbar. Data analysis was performed using MassLynx v4.1 (Waters). Peak intensities of monomeric and dimeric complexes were obtained using UniDec software [20].

2.3. Native IM-MS

For IM-MS, samples were prepared as described and analyzed on the Waters Synapt G1 HDMS mass spectrometer (see above) in mobility TOF mode. Typical IM-MS settings were: IMS wave velocity, 300 m/s; IMS wave height, 10 V; trap collision voltage, 10–100 V; transfer collision voltage, 10 V. Nitrogen was used as drift gas. Generation of unfolding plots and extraction of drift-time centroids was performed using Pulsar [21]. For acquisition of well-resolved mass spectra in the presence of detergent, we used sampling and extraction cone voltages of 80 V. However, the plot of calibrant drift times corrected for mass-dependent flight time (t'_d) against theoretical CCSs corrected for charge (Ω') did not follow the expected correlation described previously [21]. Therefore, relative drift times were calculated for each protein/detergent combination as follows:

First, peaks with less than 10% of the base peak drift intensity were removed. Then, for each charge state at a defined collisional voltage, relative drift times were calculated by dividing the observed drift time at this collisional voltage by the drift time obtained at 10 V (MG) or at 30 V (ADH). A collisional voltage of 10 V or 30 V was chosen for normalization as these settings delivered well-resolved mass spectra of MG (10 V) or ADH (30 V) for each detergent. Finally, the weighted arithmetic mean of relative drift times across the different charge states was calculated for every protein/detergent combination and collision voltage using the drift intensity as the weight. Accordingly, a weighted standard deviation was calculated. The python code used for data analysis and raw data of extracted spectra and drift times are available at: https://github.com/cschmidtlab/IJMS_Kundlacz2021.

2.4. Lipid binding to β-LG in the presence of detergent

POPE was dissolved in 200 mM ammonium acetate solution containing 2 × cmc detergent to a final concentration of 1 mM POPE. The lipid-detergent mixture was sonicated for 30 min. This stock solution was used for further experiments. To reach the desired lipid concentration, a defined volume of the stock solution was used and mixed with detergent to a final concentration of 2 × cmc (C8E4 and LDAO) or 1 × cmc (OG) in the final reaction volume. The lipid/detergent mixture was added to 10 μM β-LG.

3. Results

3.1. Native MS of soluble proteins in the presence of detergent micelles

To study the effect of detergents on soluble proteins during native MS experiments, we first chose ADH which has been shown to be a suitable standard protein for native MS measurements [19]. ADH assembles from monomeric subunits into a stable homotetramer of approx. 145 kDa. Accordingly, the mass spectrum of ADH shows two Gaussian charge state distributions corresponding to the monomeric protein and the tetrameric protein complex with maxima at the 12+ and 26+ charge states, respectively (Fig. 1). Calculated average charge states (z_{aver}) for these distributions are 11.5+ and 26.0+.

We then mixed ADH with increasing concentrations (i.e., 1, 2 and 4 × cmc) of C8E4, a detergent that was successfully evaluated for native MS studies of membrane proteins [6]. The mass spectrum acquired at low C8E4 concentration (1 × cmc), again, shows a

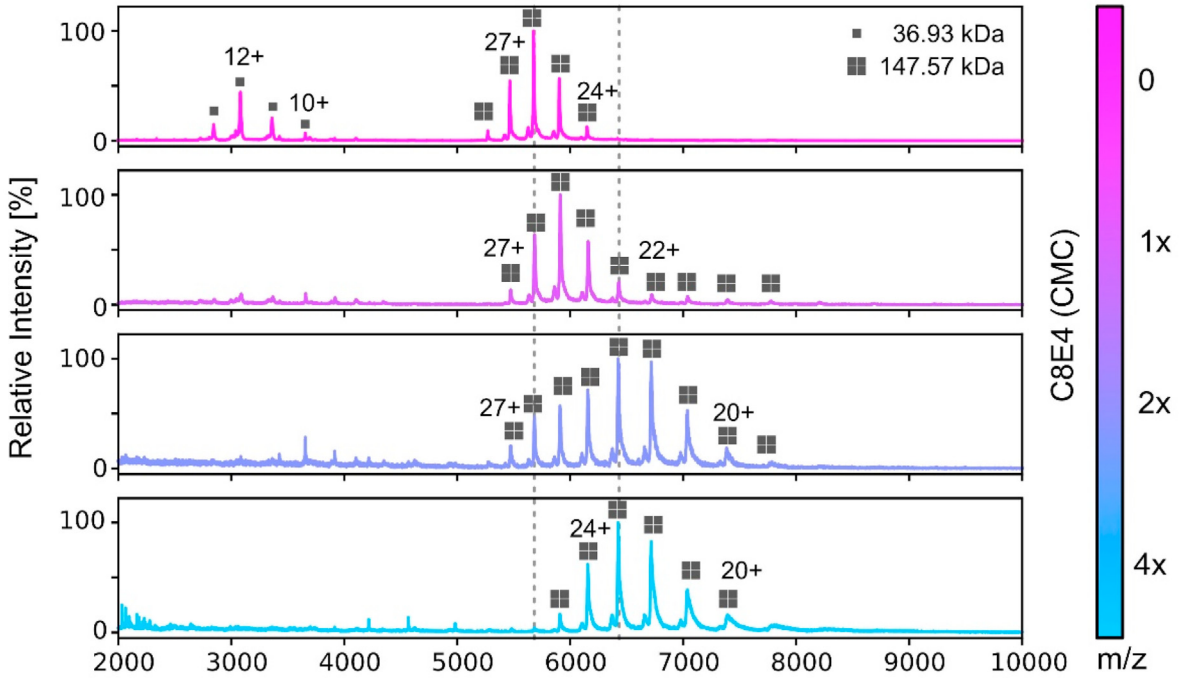

Fig. 1. Native MS of ADH in the presence of C8E4 detergent. 10 μM ADH were mixed with increasing amounts of C8E4 (top to bottom spectra: 0, 1, 2, 4 × cmc). Charge state distributions of monomeric (squares) and tetrameric (four squares) ADH are assigned. The 26+ and 23+ charge states are indicated (dashed lines). Instrument parameters for all spectra: sampling/extraction cone voltage, 100 V/80 V; trap/transfer collisional energy, 10 V/8 V.

charge state distribution corresponding to the tetrameric protein; however, this distribution is extended asymmetrically towards lower charge states; the maximum is now located at the 25+ charge state (z_{aver} 23.0+) (Fig. 1). Interestingly, the intensity of monomeric protein was significantly reduced in this mass spectrum suggesting that the protein complex is stabilized in the presence of C8E4 in solution. At higher C8E4 concentration (2 × cmc), the intensities of lower charge states of the ADH tetramer further increased; the charge state distribution now shows a maximum at the 23+ charge state (z_{aver} 23.0+) in contrast to the maximum at the 25+ charge state observed at low C8E4 concentration. At high C8E4 concentration of 4 × cmc, a charge state distribution at high *m/z* (i.e., lower charge states) with a maximum at the 23+ charge state (z_{aver} 22.5+) was observed (Fig. 1).

We conclude that C8E4 detergent reduces the number of charges acquired during electrospray ionization of a typical soluble and globular protein complex such as ADH. In addition, tetrameric ADH appeared to be stabilized in the presence of C8E4 as indicated by the absence of monomeric protein.

We next investigated the effects of two additional detergents commonly employed in MS experiments, namely LDAO and OG [6,22]. We used the tetrameric ADH complex, the globular lipocalin protein β-LG and the amphiphile carrier protein BSA and compared their charge state distributions in the absence and in the presence of detergents. The mass spectra acquired in the absence of detergent showed charge state distributions with maxima at the 25+ (ADH tetramer, z_{aver} 25.0+), 8+ (β-LG monomer, z_{aver} 8.4+) and 15+ (BSA monomer, z_{aver} 15.0+) charge states (Fig. 2A). For β-LG and BSA, low-intense charge state distributions corresponding to the dimeric proteins were also observed (Fig. S2). As described above, in the presence of 2 × cmc C8E4, all charge state distributions shifted towards lower charge states with maxima at the 22+ (ADH tetramer, z_{aver} 21.0+), 5+ (β-LG monomer, z_{aver} 6.0+) and 14+ (BSA monomer, z_{aver} 12.0+) charge states. In addition, all charge state distributions broadened (i.e., showed additional charge states at higher *m/z*). The effects observed for ADH were therefore confirmed for other soluble proteins differing in size, oligomeric state and surface hydrophobicity.

LDAO was previously characterized as a charge-reducing detergent [7]. However, we did not observe pronounced charge reduction for the standard proteins employed. In the cases of ADH and β-LG, the mass spectra acquired in the presence of LDAO are comparable to those acquired in the absence of detergent; i.e., the acquired mass spectra showed maxima at charge states 25+ (z_{aver} 25.0+) and 7+ (z_{aver} 8.5+), respectively. Note that intensities of the charge states varied for β-LG presumably due to experimental deviation as described previously [23]. Largest effects in the presence of LDAO were observed for BSA; although the maximum of the charge state distribution was only minimally affected (maximum charge state at 16.0+), the distribution of charge states extended asymmetrically towards lower charges (z_{aver} 13.5+). In addition, a series of highly charged BSA ions was observed suggesting unfolding of the protein in the presence of LDAO.

Similar to the observations made for membrane proteins, we found that in the presence of 1 × cmc OG charge state distributions of ADH and BSA shifted towards higher charges with maxima at the 28+ (z_{aver} 27.5+) and 19+ (z_{aver} 18.5+) charge states. For β-LG only minimal effects were observed; the corresponding charge state distribution showed a maximum at the 7+ charge state (z_{aver} 7.5+) (Fig. 2A). Of note, β-LG dimers were not observed in the presence of OG. When compared with charge state distributions of ADH, β-LG and BSA in the presence of C8E4 and BSA in the presence of LDAO, charge state distributions in the presence of OG are comparably narrow including only few charge states. This suggests that these higher charge states are not caused by charge uptake from unfolding of the proteins but rather by supercharging after or during transition into the gas-phase. This is in line with findings that a low number of charge states is observed for folded proteins <100 kDa in ESI-MS [24].

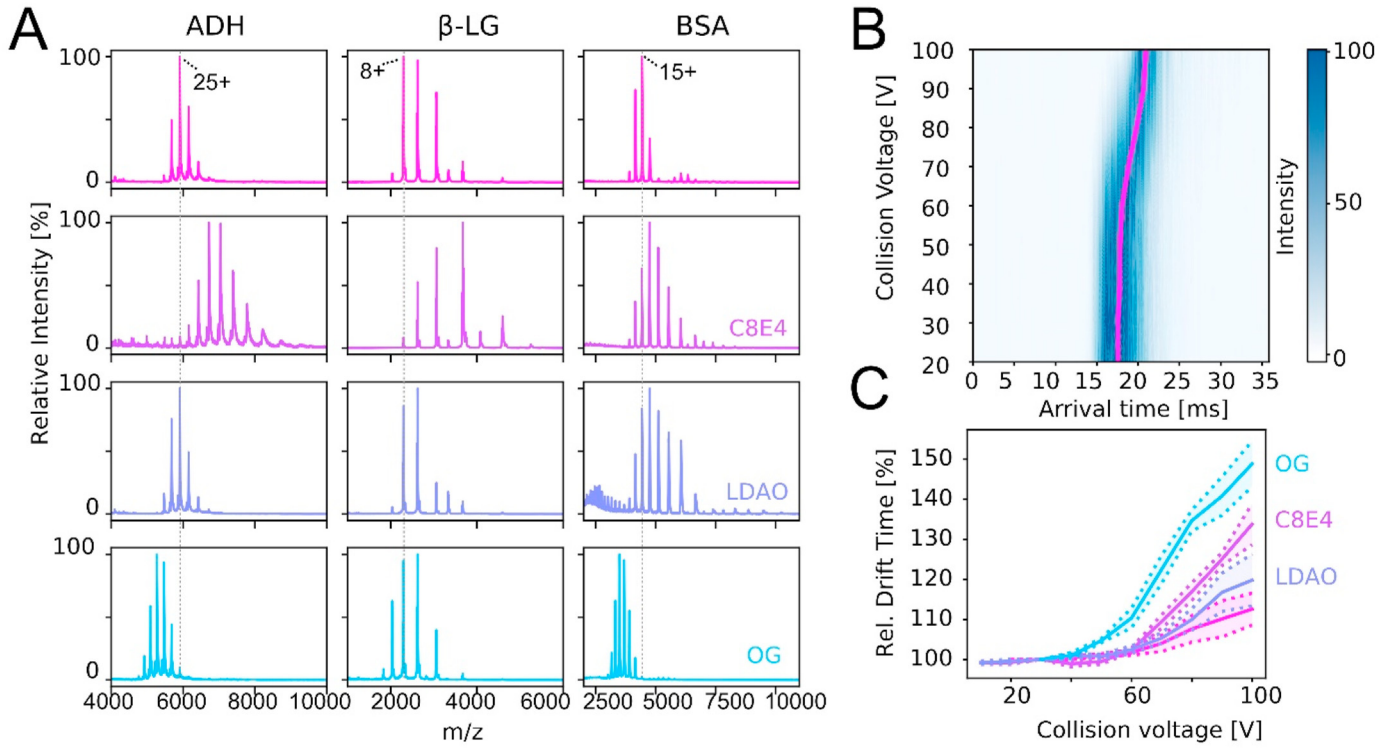

Fig. 2. Native MS and IM-MS of ADH, β-LG and BSA in the presence of various detergents. (A) 10 μM ADH, β-LG and BSA were analyzed by native MS without detergent (pink, top spectra) or in the presence of 2 × cmc C8E4 (magenta) and LDAO (blue) or 1 × cmc OG (cyan; top to bottom mass spectra). The maximum charge state as observed in the absence of detergent is indicated (dashed lines). Instrument parameters for ADH: sampling/extraction cone voltage, 80 V/80 V; trap/transfer collisional energy, 10 V/8 V. Instrument parameters for β-LG: sampling/extraction cone voltage, 100 V/20 V; trap/transfer collisional energy, 10 V/8 V. Instrument parameters for BSA: sampling/extraction cone voltage, 80 V/80 V; trap/transfer collisional energy, 30 V/8 V. (B) Collision-induced unfolding (CIU) of the 25+ charge state of ADH in the absence of detergent. The drift time centroid (pink) is used in further analyses. (C) CIU of ADH in the absence (pink) or presence of 2 × CMC C8E4 (magenta) and LDAO (blue) or 1 × CMC OG (cyan). The intensity-weighted average of drift times divided by the average drift time at 30 V (continuous lines) +/− the standard deviation (dotted lines) is shown (see Methods for details).

To investigate whether charge reduction is induced by collisions with inert gas in the collision cell, we ramped the collisional voltage and monitored the observed charge states of ADH in the absence and presence of C8E4, LDAO and OG (Fig. S3). Again, we found that C8E4 and OG reduced or increased the number of acquired charges, respectively. A reduction of charges at higher collisional voltages was, however, not observed. In fact, we found that the charge states were slightly shifted towards higher charges at higher collisional voltages indicating unfolding of the protein.

In summary, we found that the soluble standard proteins employed here acquire fewer or more charges during electrospray ionization in the presence of C8E4 and OG micelles, respectively. For LDAO, the previously described charge reduction effect of membrane proteins was only observed for BSA while ADH and β-LG remained unaffected. Charging effects were independent of the collisional voltages applied.

3.2. Assessing protein stability in the presence of detergents by collision induced unfolding

To study the effect of the detergents on the structure of the proteins, we performed collision induced unfolding (CIU) of ADH in the absence and presence of detergents. For this, the collisional voltage in the trap cell was ramped from 10 to 100 V, the ions were separated in the IM cell and the arrival time at the detector was recorded. In the absence of detergent, a conformational change from a folded to a more extended conformation was observed at a collisional voltage of approx. 75 V (Fig. 2B). We then compared CIU of ADH in the presence of C8E4, LDAO and OG detergents. The drift time distributions of ADH in the presence of the various detergents were obtained in consecutive measurements employing the same instrument settings. Note that recording of well resolved mass spectra in the presence of detergent required sampling and extraction cone voltages of 80 V. However, these settings interfered with reliable drift time calibration to obtain CCSs. Nonetheless, for studying destabilization of ions in the gas phase, CCSs are not required; changes in the observed drift times are sufficient to monitor unfolding of the protein ions. We therefore, normalized drift times observed in the presence of one detergent to the corresponding drift time obtained at the lowest collisional voltage at which resolved peaks were obtained (see Methods for details).

Plotting these relative drift times of ADH in the presence of the different detergents, we found that ADH in the absence of detergents and in the presence of LDAO and C8E4 detergents follows the same trend (Fig. 2C and Fig. S4); at a collisional voltage of approx. 70 V, a shift towards higher relative drift times was observed. Interestingly, the CIU profile obtained in the presence of OG was shifted to lower collisional voltages indicating a destabilizing effect on the ADH tetramer (Fig. 2C and S4 and S5). This is in agreement with the super-charging effect of OG observed in native MS as a higher charge density on the protein surface is likely to favor unfolding at lower collisional voltage.

To exclude false interpretation due to charge averaging, we compared CIU and drift time profiles of the same charge states (Figs. S6 and S7). Note that the charge states varied in the presence of detergents (see above); we therefore selected charge states that showed reasonable intensities in the absence and presence of detergent. Indeed, drift time profiles of the 23+ and 24+ charge

states of ADH were comparable when acquired in the absence or presence of C8E4. A lower arrival time was observed at a collisional voltage of 20 V, while higher arrival times at a collisional voltage of 90 V indicated destabilization and unfolding of the ions. A slight shift towards lower arrival times in the presence of C8E4 at a collisional voltage of 20 V indicated stabilization in the presence of detergent (Figs. S6 and S7). In the presence of OG detergent, the 26+ charge state showed destabilization at lower collisional voltages (Figs. S6 and S7). In addition, the arrival time distribution obtained in the presence of OG at a collisional voltage of 20 V is lower when compared with that obtained in the absence of detergent suggesting compaction of the ions. These findings were confirmed for all ADH charge states observed in the presence of OG (Fig. S8). Destabilization of the ions through different unfolding states were observed.

As stabilization of ADH in the presence of C8E4 was indicated by native MS (see above, Fig. 1), we further investigated this effect on non-covalent interactions during MS measurements. For this, we selected MG, a monomeric protein of approx. 17 kDa which stably binds a heme. In the absence of detergent, a charge state distribution corresponding to the monomeric apo-protein (16.95 kDa) and a signal for the singly charged heme ligand (*m/z* 616) was observed (Fig. 3). The mass spectrum further revealed low-intense adduct peaks with a mass difference corresponding to binding of heme albeit at very low quantities (Fig. S9).

We next investigated the effects of detergents on heme binding and added 2 × cmc C8E4 and LDAO to the protein solution (Fig. 3A). Interestingly, in the presence of 2 × cmc C8E4, we identified a series of charge states of two populations corresponding in mass to apo-MG (16.95 kDa) and heme-bound MG (17.57 kDa). Both populations showed equal intensities suggesting that binding of heme to MG is stabilized in the presence of C8E4. In the presence of 2 × cmc LDAO, on the contrary, heme-bound MG was only observed at very low intensity comparable with the intensities observed in the mass spectrum acquired in the absence of detergents (Fig. S9). We also investigated the effect of 1 × cmc OG on MG and found that, despite the charge increase of the main peak from 7+ to 8+, the heme-bound protein was present at approx. half the intensity of the apo-form. We therefore assume that some detergents have a stabilizing effect on non-covalent interactions with ligands.

Next, we performed IM-MS analysis of MG in the presence of detergents. Again, as well-resolved mass spectra in the presence of detergent were difficult to record, we normalized the observed drift times as described above. Comparing the CIU profiles of the heme-bound form of MG averaged across the observed charge states, we found that the drift times observed in the absence and presence of LDAO and OG detergents are stable until approx. 70 V (Fig. 3B and Figs. S10 and S11). At higher collisional voltages, the protein collapses as indicated by the decrease in drift times. In contrast, the charge-averaged drift time of heme-bound MG in the presence of C8E4 remains unchanged when increasing the collisional voltage up to 100 V. To investigate whether the observed differences are caused by the different charge effects of the detergents, we compared CIU-profiles of the 8+ charge state which was observed under all conditions (Fig. 3C and Figs. S11 and S12). Again, we found that, in the absence of detergent as well as in the presence of LDAO and OG, drift times are constant until approx. 70 V whereas in the presence of C8E4 the change in drift time shifts towards higher collision voltages. Note that, even though our findings suggest stabilization of the holo-protein in the presence of C8E4, drift times of the 8+ charge state decrease at collisional voltages above 80 V, indicating collapse of the protein (Fig. S12). We assume that the overall stabilizing effect of C8E4 observed when averaging across charge states (Fig. 3B and Fig. S10) results from both charge reduction as well as interactions with the protein in the electrospray droplet and during the desolvation process (Fig. 3C).

The absence of charge states corresponding to the holo-protein in the absence of detergent suggests that the relatively harsh ionization conditions required for the analysis of soluble proteins in the presence of detergent might induce partial unfolding of the proteins during analysis under the same instrument settings in the absence of detergent. Indeed, IM-MS spectra of MG acquired at capillary, sampling and extraction cone voltages of 1.3 kV, 30 V and 0.3 V, respectively, revealed holo-MG even in the absence of

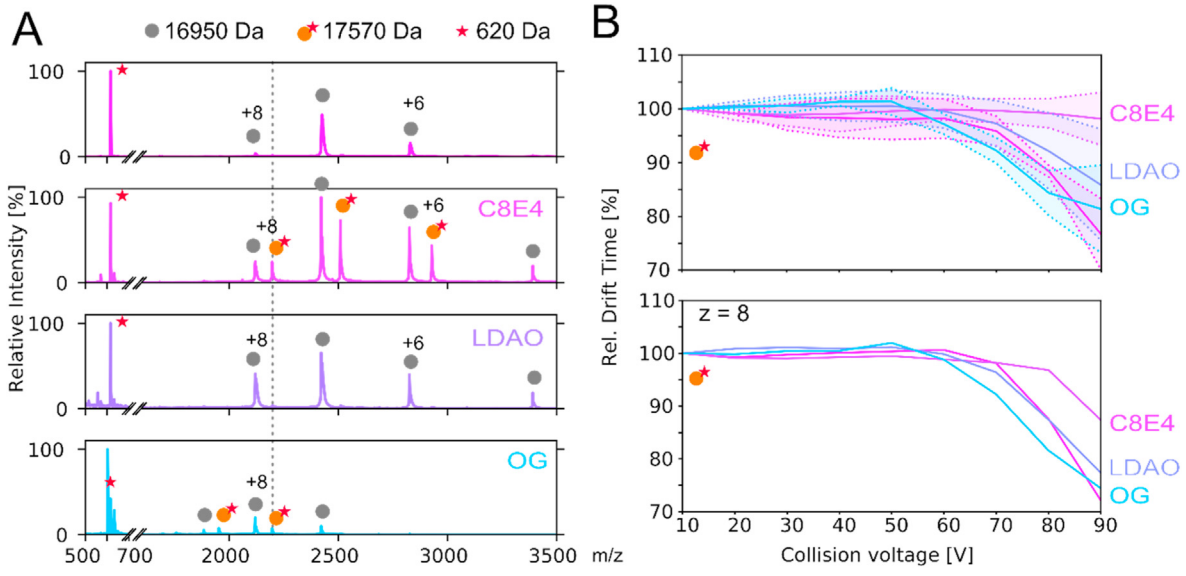

Fig. 3. Native MS and IM-MS of MG in the presence and absence of various detergents. (A) 10 μM MG were analyzed by native MS in the absence (pink) and in the presence of 2 × cmc C8E4 (magenta) and LDAO (blue) as well as 1 × cmc OG (cyan). Instrument parameters: sampling/extraction cone voltage, 30 V/10 V; trap/transfer collisional energy, 10 V/8 V. (B) 10 μM MG were analyzed by native IM-MS in the absence (pink) and in the presence of 2 × cmc C8E4 (magenta) and LDAO (blue) as well as 1 × cmc OG (cyan). The intensity-weighted average drift time divided by the average drift time at 10 V (continuous lines) +/− the standard deviation (dotted lines) of holo-MG is shown (top panel, see Methods for details). The bottom panel shows drift time profiles of the 8+ charge state. Instrument parameters: sampling/extraction cone voltage, 30 V/10 V; trap/transfer collisional energy, 10–90 V/8 V.

detergent (Fig. S13) suggesting that non-covalent interactions can also be maintained when soft ionization conditions are applied.

3.3. Transfer of lipids from the detergent micelle to soluble proteins

Interactions between detergent micelles and soluble proteins in ESI droplets were recently described to facilitate lipid transfer from the detergent micelle to the protein [17]. We therefore evaluated lipid binding to β-LG in the presence of C8E4, LDAO and OG. Phosphatidylethanolamine binding to β-LG was previously described and we, therefore, chose POPE as a model lipid [25]. As phosphatidylethanolamines are poorly soluble, we slightly modified the previous protocol and first dissolved the lipid in the detergent and then incubated detergent-lipid micelles with the protein (see Methods for details). Fig. 4 shows that POPE binding was only observed when C8E4 micelles were employed. Mass spectra acquired in the presence of LDAO resembled the mass spectrum acquired in the absence of detergent. Using OG detergent, we observed a shift of the β-LG charge state distribution to higher charges. However, adduct peaks indicating lipid binding were also not observed when using this detergent. Importantly, the number of lipid adducts depends on the concentration of POPE present (compare Fig. 4A and B). An increase in the POPE concentration in the presence of LDAO and OG, however, did not remarkably affect lipid binding (Fig. 4C). Possible explanations are: (i) C8E4 functions as a charge carrier and the resulting lower charge states better accumulate lipid molecules at their surface (charge-dependent effect); (ii) C8E4 more tightly interacts with the protein surface thereby facilitating lipid transfer (surface interaction effect) or (iii) C8E4 captures excess energy after collisional activation or during gas-phase transfer in the source region (energy-reducing effect). The latter two assumptions are supported by our findings that non-covalent interactions are stabilized in the presence of C8E4.

4. Discussion

In this study, we investigated the effects of non-ionic and zwitterionic detergents on the charge states of soluble proteins during native MS experiments. We found that the non-ionic detergents employed here reduced (C8E4) or increased (OG) the number of charges acquired during electrospray ionization. Similar observations were made previously for membrane proteins [26]. We therefore assume that charge-reducing and super-charging effects are at least partially induced by the soluble parts of membrane proteins rather than their transmembrane regions alone. Whether charging effects are also mediated through hydrophobic transmembrane regions remains to be elucidated in future studies employing protein variants that lack their soluble regions. Zwitterionic LDAO, on the contrary, appears to affect the number of charges only when a transmembrane domain is present. Additional high charge states of BSA in the presence of LDAO, as observed here, further indicate destabilization of the protein. In agreement with previous studies, showing that LDAO reduces the number of acquired charges of membrane proteins [7,22], we assume that LDAO preferentially binds hydrophobic regions of BSA and thereby induces unfolding of the protein.

Importantly, we realized that charging effects do not depend on protein size and oligomeric state of the proteins. Note that only minor effects were observed for smaller proteins such as β-LG; these proteins acquire only few charges during the ionization process and charge reduction or increase are accordingly less pronounced. Various groups of charge-reducing agents for native MS have been described in previous studies, including additives with high gas-phase basicity that compete with the protein ion for protons just before ionization [27] and ammonium compounds and amine oxides that were suggested to remove charges upon gas-phase collisions with the protein ion [28,29]. For the detergents investigated here, we could not observe collisional voltage-dependent charge reduction, indicating that gas-phase activation in the collision cell of the mass spectrometer is most likely not involved in the underlying mechanism. However, collision induced charge reduction of the ions during transfer into the gas-phase cannot be excluded and transient interactions of the detergents with the proteins during transfer into the gas-phase might be involved in charge manipulation as previously suggested for charge-reducing polyamines [30]. Another possibility is that the

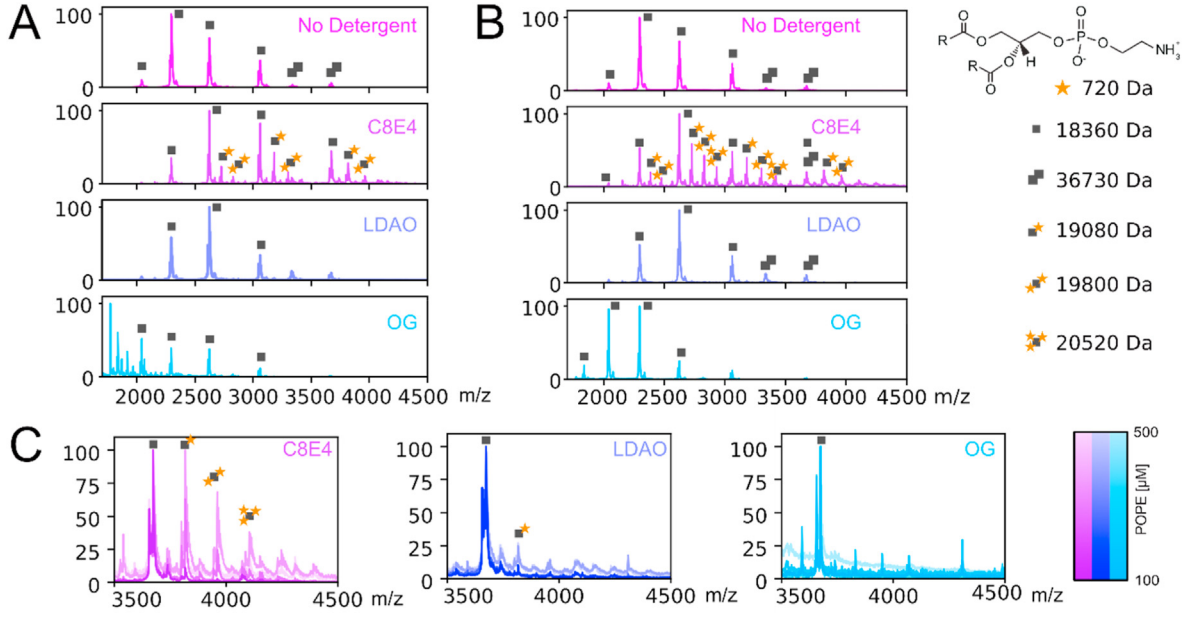

Fig. 4. Detergents modulate POPE binding to β-LG. 10 μM β-LG were analyzed by native MS in the absence or presence of detergents. Instrument parameters: sampling/extraction cone voltage, 30 V/10 V; trap/transfer collisional energy, 10 V/8 V. Color legend: No detergent/lipid, pink; 2 × cmc C8E4, magenta; 2 × cmc LDAO, blue; 1 × cmc OG, cyan. (A). 100 μM POPE were added in the presence of detergents. (B) 200 μM POPE were added in the presence of detergents. (C) Zoom on the 5+ charge state of β-LG in the presence of C8E4 (left panel), LDAO (middle panel) and OG (right panel). 100–500 μM POPE were added (see color legend for POPE concentrations).

detergent micelle changes the properties of the electrospray droplet thereby manipulating the charges of the protein acquired during ionization. However, an early study showed that micellar structures are preserved during transfer into the gas-phase [31] making charging effects due to a different micellar arrangement in the electrospray droplet of soluble proteins unlikely. Nonetheless, the same study suggests that highly-charged gas-phase micelles are unstable leading to disruption of the micelles and probably to charge reduction of the proteins. We assume that one of these mechanisms or a combination of several effects are the basis for charge reduction of soluble proteins through detergents.

Super-charging observed for OG in this study can, however, not be explained by these assumptions. The process of protein super-charging is, in general, less understood and two basic principles are discussed: First, an increase in surface tension [32,33] and, second, conformational changes of the proteins [34]. Noteworthy, super-charging of protein complexes showed that non-covalent interactions with protein subunits and ligands were preserved [35]. This is in agreement with our findings, that protein ions observed in the presence of OG showed a narrow charge state distribution indicating presence of a folded proteins. In addition, a population of holo-MG was observed confirming binding of a non-covalently bound ligand. On the contrary, the arrival time distribution of ADH ions observed in the presence of OG shifted towards lower drift times indicating structural rearrangements resulting in a more compact ion. We conclude that the mechanism of super-charging appears to be manifold making a simple explanation of one possible charging effect difficult.

Stabilization of ADH and holo-MG in the presence of C8E4 were confirmed by native MS and IM-MS. While only minimal effects were observed for ADH, stabilization of heme-bound MG in the presence of C8E4, and to a lower extent in the presence of OG, was prevalent. However, the underlying principle for stabilization by some but not all detergents remains elusive. We speculate that the observed effects might rely on two principles: First, overall charge reduction of the ions produced during electrospray ionization reduces their internal energy (charge-dependent effect) and, second, lowering the impact of collisional activation; for instance, by shielding the protein (surface interaction effect) or capturing activation energy (energy-reducing effect). In accord with the latter point, dissociation of protein-bound detergent molecules was previously found to prevent conformational collapse of a membrane protein in the gas-phase of a mass spectrometer [36]. Even though we did not identify detergent binding to the soluble standard proteins used here, (transient) interactions with the proteins in the electrospray droplet are most likely and dissociation of these transient and labile complexes might reduce the internal energy of the proteins. This is supported by our findings that holo-MG is at least in part stabilized in the presence of OG (Fig. 3), while highly charged ions produced in the presence of OG are less stable and prone to unfolding (Fig. S8).

Lastly, we investigated lipid transfer from detergent-lipid micelles. Employing the different detergents, we found that only C8E4 micelles were applicable for lipid transfer. This was surprising as similar arrangements in the different detergent micelles are expected. Nonetheless, these findings might provide clues on the mechanisms of detergent-induced charge-reduction and stabilization of ions during native MS experiments. We, consequently, assume that C8E4 micelles more tightly interact with the surface of the proteins thereby facilitating lipid transfer. These interactions might have charge-reducing and stabilizing effects during transfer of the ions into the gas-phase (see above).

Comparing the cmc's of the three detergents employed here, we do not recognize a specific trend for detergent-based lipid transfer. The cmc of LDAO in water is lower (1–2 mM), and the cmc of OG is higher (18–20 mM) when compared with that of C8E4 (8 mM). Note that a comparison of cmc's is most meaningful when the same class of detergents is explored. In this manner, an increase in the number of detergent micelles was found to promote lipid transfer from the detergent micelle to the protein [17]. In our experiments, micelle concentrations of approx. 50 μM (LDAO), 195 μM (C8E4) and 250 μM (OG) were used. However, even though the micelle concentration of OG was the highest, unspecific binding was not observed when OG was employed. Again, this finding confirms that effects of C8E4 are likely to be provoked through interactions of the detergent micelle with the protein in the electrospray droplet.

5. Conclusions

Here, we evaluated the effects of non-ionic and zwitterionic detergents on soluble proteins during native MS experiments. As previously described for integral membrane proteins, embedded in the detergent micelle, we observed charge-reducing or super-charging effects for C8E4 and OG detergents. In addition, we found that the proteins' structural features as well as non-covalent interactions with ligands are stabilized by the non-ionic detergent C8E4 but not OG or zwitterionic LDAO; drift time measurements in the presence of OG and LDAO followed the same trend as the protein in the absence of detergents. In addition, lipid transfer from mixed detergent-lipid micelles to a lipid-binding protein was only facilitated when C8E4 was employed. We propose three (not mutually exclusive) effects that might occur when adding C8E4 to the solubilized protein sample causing stabilization of the ions and favoring lipid transfer: (i) reduction in the number of acquired charges during transfer to the gas-phase might change the properties of the protein ion (charge-dependent effect), (ii) the presence of the detergent micelle in the electrospray droplets might capture activation energy which is directly applied to the naked ions in the absence of detergents (energy-reducing effect) and (iii) interactions of the detergent micelle with the protein's surface might facilitate lipid transfer. We speculate that C8E4 dynamically and transiently interacts with the protein in the electrospray droplet thereby affecting its gas-phase properties. Overall, soluble proteins presented here act as model systems to unravel charging effects observed in the studies of membrane proteins from those specific to the presence of a transmembrane region. Moreover, a thorough understanding of these effects might aid the analysis of peripheral membrane proteins by native MS in future studies.

CRediT authorship contribution statement

Til Kundlacz: Conceptualization, Investigation. **Julian Bender:** Conceptualization, Methodology, Visualization, Writing – original draft, preparation. **Carla Schmidt:** Conceptualization, Supervision, Funding acquisition, Writing – original draft.

Declaration of competing interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

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