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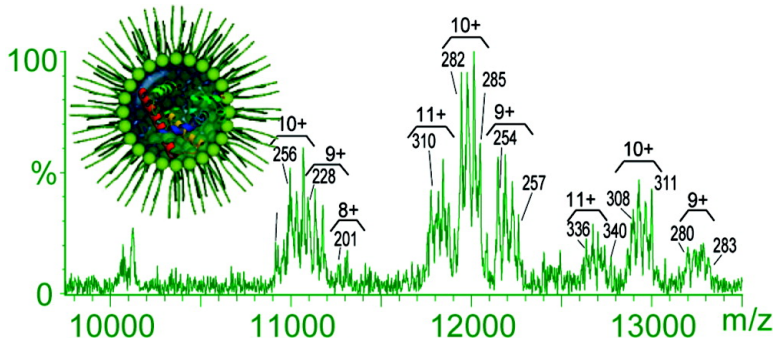

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Evidence for Micellar Structure in the Gas Phase

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Abstract: We have compared micelles, reverse micelles, and reverse micelles encapsulating myoglobin using electrospray mass spectrometry. To enable a direct comparison, the same surfactant (cetyltrimethylammonium bromide (CTAB)) was used in each case and micelle formation was controlled by manipulating the aqueous and organic phases. Tandem mass spectra of the resulting micelle preparations reveal differences in the ions that dissociate: those that dissociate from regular micelles have undergone >90% exchange of bromide ions from the headgroup with acetate ions from bulk solvent. By contrast, for reverse micelles, ions are detected without exchange of bromide ions from the headgroup, consistent with their protection in the core of the micellar structure. Tandem mass spectra of micelles and reverse micelles reveal polydispersed assemblies containing several hundred CTAB molecules, indicating the coalescence of the micellar systems to form large assemblies. For reverse micelles incorporating myoglobin, spectra are consistent with one holo myogolobin molecule in association with ~270 CTAB molecules. Overall, therefore, our results show that the solution-phase orientation of surfactants is preserved during electrospray and are consistent with interactions being maintained between surfactants and an encapsulated protein.

Introduction

It is widely recognized that soluble protein complexes ionized by electrospray (ES) and analyzed by mass spectrometry (MS) retain many noncovalent interactions[1−3] and even aspects of their solution-phase structure.[4] For hydrophobic membrane proteins their tendency to aggregate requires high concentrations of organic solvent and acids to maintain solubility. As a consequence of these conditions, the protein is denatured and lipid interactions are destroyed. Micelles formed in solution, however, entrap hydrophobic environments, mimicking closely the membrane environment. Such micelles are widely employed in the structure elucidation of membrane proteins.[5]

Recently we applied tandem MS to a channel-forming membrane protein (EMRE) involved in cationic drug transport.[6] The channel is formed from two EMRE molecules, and cations bind within the channel. Tandem MS of the ternary submicelle complex formed between EMRE, the detergent *n*-dodecyl β-D-maltoside (DDM), and the cation tetraphenyl phosphonium (TPP) showed that the TPP cation was released prior to EMRE during collisional activation. The results implied that protein−cation interactions as well as clusters of up to 20 DDM molecules were maintained in the gas phase. This, together with other observations of clusters of surfactant[7−10] and protection from hydrogen exchange of a channel-forming peptide,[11] suggests that aspects of micellar structure can be preserved in the mass spectrometer but the extent to which these gas-phase micelles resemble those formed in solution remains unclear.

We set out to investigate the electrospray MS of micelles using the surfactant cetyltrimethylammonium bromide (CTAB) (Figure 1). This surfactant has the ability to assemble into micelles and reverse micelles, depending on the proportion of water and organic solvent in the solution, and even to entrap proteins within reverse micelles. In aqueous solutions CTAB molecules self-assemble with alkyl chains forming the inner core and polar headgroups facing the outer surface to form micelles. In apolar solvents, with low water content, an inverse structure is formed with the polar headgroups forming a central core and the hydrophobic tails directed outward. Such reverse micelles mimic the cellular environment, by forming an effective barrier between the bulk solvent and ordered water molecules, and thus can be used for delivery of water-soluble active enzymes.[12] Here we show not only that the differences between the two types of micelle are preserved in the gas phase, but also that tandem mass spectra are consistent with the entrapment of myoglobin within reverse micelles.

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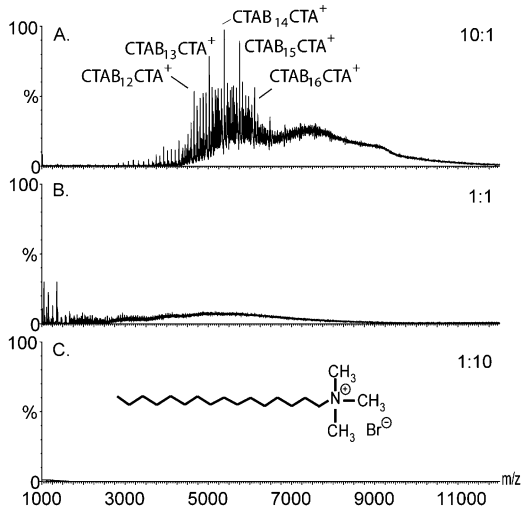

Figure 1. ES mass spectra of regular CTAB micelles at different ratios of CTAB:water. Very broad peaks are observed for ratios of CTAB to water of (10:1) or (1:1) (A and B, respectively), both of which are above the critical micelle concentration (1:1). Superimposed upon this broad signal is a spectrum of singly charged clusters (labeled) with intervening peaks assigned to charged clusters up to 5^+ (A). Inset, lower panel: CTAB molecule. No spectrum can be recorded for solutions below the cmc (1:10) (C), only low molecular weight clusters.

Experimental Methods

Micelles. A range of cetyltrimethylammonium bromide (CTAB) solutions was prepared by dissolution of CTAB in 0.2 M ammonium acetate at pH 7.0 or pH 8.0. Different ratios of CTAB in water above and below the critical micelle concentration (cmc) of 0.85 mM[13] were investigated. A very broad distribution of species was observed in mass spectra consistent with that previously attributed to a distribution of surfactant molecules in micelles[6] (Figure 1). This characteristic was observed only above the cmc. Consequently, the concentration of CTAB in solution used in our investigation was at least 10 times higher than the cmc value to ensure micelle formation in solution.

Reverse Micelles. The phase diagram described by Ahmad et al.[14] was used to obtain the appropriate concentration ratios. CTAB (0.2 g) was dissolved directly into hexanol (0.86 mL) under sonication. Aliquots of water, 10 mM ammonium acetate, or 200 mM ammonium acetate were added to enhance the solubility of the surfactant (these quantities were included in the hydration degree calculations, $[H_2O]/[\text{surfactant}]$ (W_o)). After equilibration for 24 h, aliquots of the aqueous solutions, or 7 μL of horse heart myoglobin (Sigma) at a concentration of 500 μM in H_2O, pH 8.0, were added to achieve a W_o value of 10. Solutions were sonicated to create a transparent micellar solution. We also explored reverse micelles formed under a range of different solution conditions by manipulating the organic and aqueous phases, changing pH, ionic strength, and proportion of organic phase. Full details of all the different solution combinations explored can be found in the Supporting Information.

Mass Spectrometry ES-MS and Tandem MS. Analyses were preformed on nanoflow ES mass spectrometers, Q-ToF2 (Micromass) or QSTAR XL (Sciex), both modified for transmission and isolation of high mass ions.[15,16] The following experimental parameters were used to record mass spectra of micelles and reverse micelles on the Q-ToF2 instrument: needle voltage of 1.4 kV, cone voltage of 100 V, and pressure of 6.0×10^{-3} mbar in the ion transfer region. Conditions on the QSTAR were capillary voltage 1.0 kV, declustering potential 40 V, focusing potential 200 V, second declustering potential 15 V, and MCP 2350 V. For tandem MS maximum transmission was used throughout. This corresponds to an isolation window of ~90 *m*/*z* units. Argon was used as a collision gas, and voltages between 40 and 100 V were applied to the collision cell. All spectra were calibrated externally by use of a solution of cesium iodide (100 mg/mL). The spectral assignment procedure and estimation of the average error in mass measurement are detailed in the Supporting Information.

Results

To optimize the preparation and analysis of micelles, we investigated the effect of changing the CTAB-to-water ratio and defined the optimal MS conditions. Only above the critical micelle concentration (cmc), when micelles have been shown to form in solution, and under high-pressure conditions, commonly employed to observe noncovalent protein complexes in MS,[15] could we observe very broad mass spectral peaks consistent with heterogeneous assemblies (Figure 1). We noted that the *m*/*z* values of the broad peaks were highly dependent on the MS conditions. This observation is in line with our previous experience with micellar systems[6] and is attributed to variation in the desolvation/aggregation processes that occur during electrospray, which in turn affects the *m*/*z* values of the micelle distribution.

We examined the broad peaks observed using tandem MS on a Q-ToF modified for high mass operation.[15] We isolated packets of ions at regular intervals from *m*/*z* 4000 to 16 000. For reverse micelles the predominant dissociation product is assigned to $[(\text{cetyltrimethylammonium})_2Br]^+$ $(\text{CTAB-CTA})^+$ (*m*/*z* 647/649) (Figure 2A,B). By contrast $[(\text{cetyltrimethylammonium})_2Ac]^+$ $(\text{CTA}\cdot\text{Ac-CTA})^+$ (*m*/*z* 627), in which bromide ions have exchanged with acetate ions from the bulk solvent, is the predominant dissociation product from regular micelles (Figure 2C). We attribute this difference in dissociation product to exposure of the headgroup in solutions of regular micelles, allowing exchange of Br^- with Ac^- in the ammonium acetate buffer. Conversely, no exchange of Br^- can be detected in the ordered aqueous environment of reverse micelles.

To investigate this phenomenon of exchange of counterions from the CTAB headgroup in detail, we prepared a series of reverse micelle containing solutions, exploring systematically changes in the organic phase (from 27% to 95% hexanol) as well as replacement of hexanol by octanol/octane. In the aqueous phase we examined the effect of increasing pH (from pH 7 to 8) and of increasing ionic strength (from 10 mM to 1 M ammonium acetate). It is important to note that reverse micelles are known to form under all these solution conditions.[14,17,18] Tandem MS of these different reverse micelle containing solutions led to dissociation of $(\text{CTAB-CTA})^+$ ions with the retention of the Br^-, even in the presence of 50% H_2O (see Supporting Information). In the reverse micelle the Br^- counterion is oriented toward the core of the ordered aqueous environment such that exchange with acetate ions in solution and in the electrospray droplet is prevented. This demonstrates that, under all solution conditions investigated in which reverse micelles are known to form, the orientation of the headgroup is maintained.

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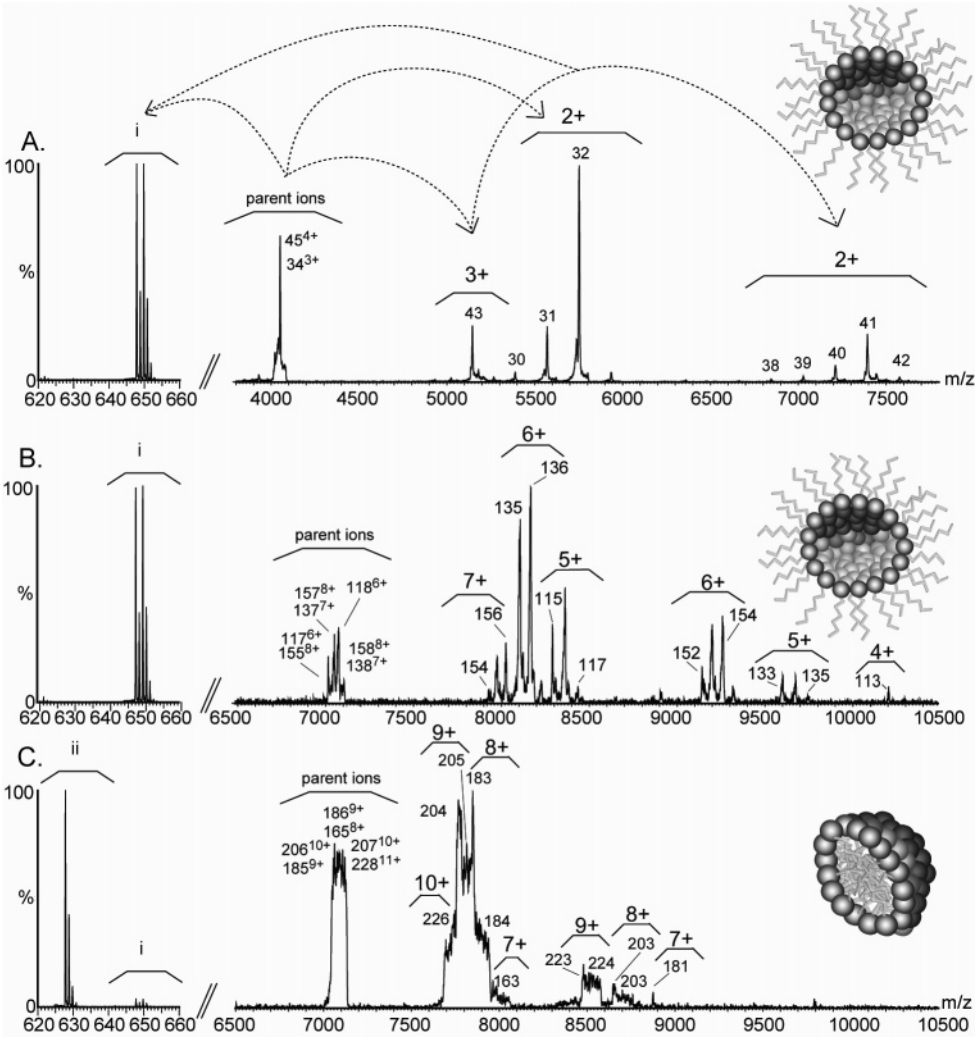

Figure 2. Tandem MS of ions at *m*/*z* 4000 and 7000 from solutions of reverse micelles (A and B, respectively) and at *m*/*z* 7000 for regular micelles (C). At low *m*/*z* values, predominant peaks correspond to $(CTAB\text{-}CTA)^+$ (i) and $(CTA{\cdot}Ac\text{-}CTA)^+$ (ii) for reverse and regular micelles, respectively. The product ions corresponding to stripped complexes (above the *m*/*z* values of the isolated ions) are formed by expulsion of the low mass CTAX-CTA ions and are labeled with the number of CTAB molecules (one peak labeled within each charge state cluster). For reverse micelles, neighboring peaks within each cluster differ by one CTAB molecule. Reverse micelles were prepared in water, and regular micelles were prepared in 0.2 M ammonium acetate solution. Arrows are used to illustrate the dissociation pathway. The diagrams on the right are not intended to describe the respective gas-phase species but are designed to illustrate the solution-phase orientation of the headgroup and demonstrate how exchange of Br^- for Ac^- can occur from regular micelles but is prevented from reverse micelles by burial of the headgroup.

For the stripped complexes that form as a result of dissociation of $(CTAX\text{-}CTA)^+$, at higher *m*/*z* values than the isolated ions, the spectra are very complex with multiple overlapping series. Assigning these spectra is therefore a formidable task. We began the assignment process for the smallest clusters that we could generate for reverse micelles at the lowest charge states, where minimal overlap of peaks was apparent (Figure 2A). For simplicity we consider first the most intense peaks assigned to the 2^+ stripped complexes. Neighboring peaks within each cluster have the same charge but differ by the number of CTAB molecules. In this case a range from 30 to 33 is evident, with 32 being the predominant species. Therefore, the predominant precursor ion that was selected, from which $(CTAB\text{-}CTA)^+$ ions have been expelled, must have had 34 CTAB molecules with three positive charges prior to dissociation. Similarly, the 4^+ species in the original isolation gives rise to $(43\ CTAB)^{3+}$ and $(41\ CTAB)^{2+}$ species by sequential expulsion of $(CTAB\text{-}CTA)^+$ ions, demonstrating that the original assembly contained 45 CTAB molecules in the 4^+ charge state. Interpretation of this spectrum of low molecular mass species enables us to apply this assignment protocol to higher *m*/*z* species. Tandem MS at *m*/*z* 7000 gives rise to spectra that are notably more complex: the number of overlapping species has increased considerably. Using the same procedure, however, we are able to assign this spectrum to assemblies ranging from 117 to 158 CTAB molecules; see Figure 2B.

If we consider now the dissociation of regular micelles, we can see that the spectra have a number of notable differences from those of reverse micelles (Figure 2C). Despite the fact that the same *m*/*z* value was isolated for tandem MS, the resulting spectra are difficult to assign as the peaks are broader than those of reverse micelles. This is most likely the result of the heterogeneity caused by exchange of counterions from the exposed headgroups with the bulk solvent. The nature of the diffuse peaks means the optimum region of the spectrum for assignment is at the highest *m*/*z* values, where separation between adjacent peaks is greatest. The spacing between the different charged clusters again corresponds to $(CTAB\text{-}CTA)^+$,

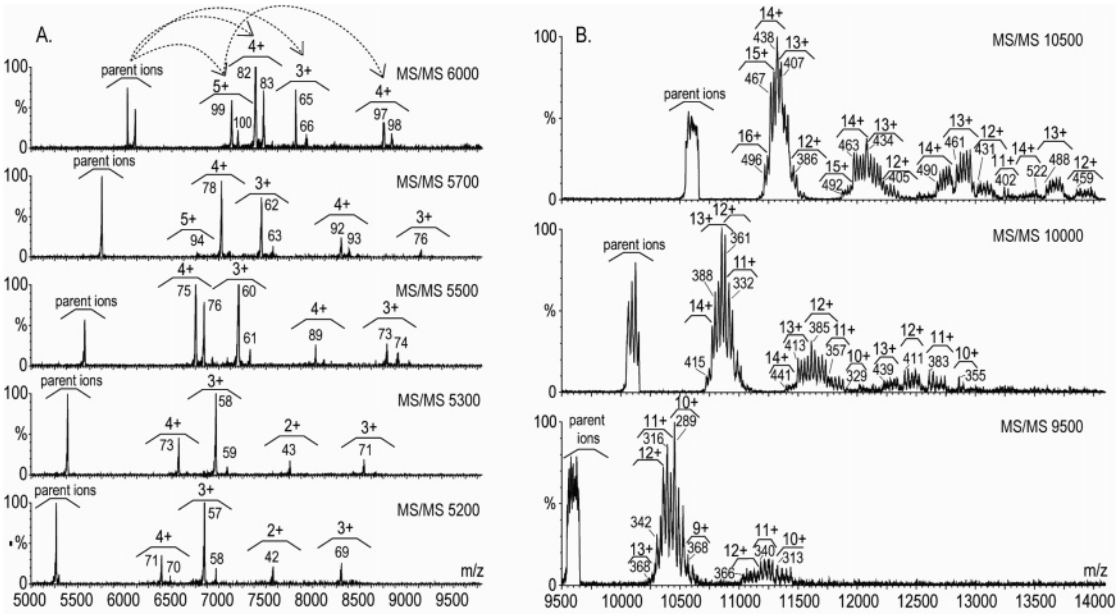

Figure 3. Comparison of tandem mass spectra for reverse micelles recorded after incrementing the isolation value in 100−300 *m*/*z* intervals for the low *m*/*z* region (A) and intervals of *m*/*z* 500 for the high *m*/*z* region (B). These spectra confirm the reproducibility of the dissociation pattern and demonstrate a stepwise increase in polydispersity, size, and charge.

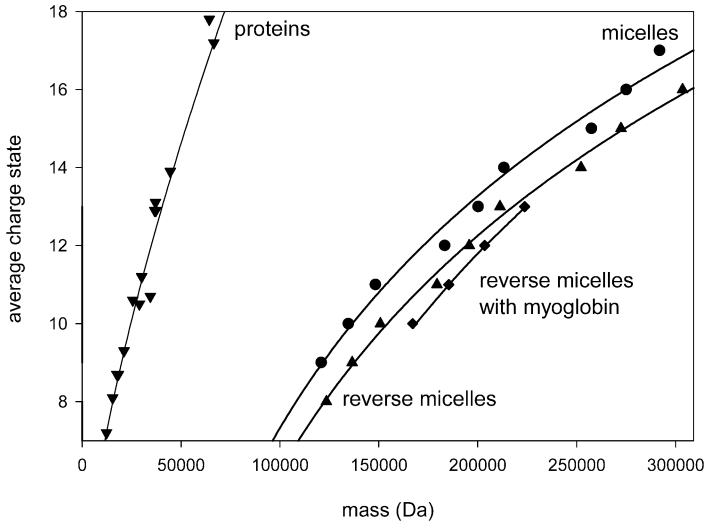

Figure 4. Plot of charge state against mass of various CATB micelles. For comparison a similar plot is included for the average charge state of proteins and their noncovalent complexes. For regular and reverse micelles data were obtained from tandem mass spectra at three different isolation values. The same micelle preparations and identical MS settings were used in each case. For reverse micelles with myoglobin only one isolation could be used since only at this *m*/*z* value sufficient CTAB molecules are available to encapsulate myoglobin (see below).

but in this case with partial exchange of the Br^- ions represented by $(CTAX\text{-}CTA)^+$. However, since peaks in the spectrum are closer to one another than in the spectrum recorded for reverse micelles, the difference in *m*/*z* must be smaller. Therefore, both the charge and number of CTAB molecules must be greater for regular micelles than for reverse micelles, when identical *m*/*z* values are isolated. Consistent with this we find that polydispersity has increased, compared with reverse micelles, with assemblies containing from 165 to 228 CTAB molecules for isolations at this *m*/*z* value.

To investigate these dissociation processes in more detail and to assess the reproducibility of this dissociation pattern, we varied systematically the *m*/*z* value of the packets of ions we isolated (Figure 3). The results show that, as the *m*/*z* value of the isolation window increases, the spectra are highly reproducible. The only differences are the significant increases in the polydispersity, charge state of the assemblies, and number of CTAB molecules associated. A graph of the increase in average charge as a function of the mass of the various micelle species is shown in Figure 4. For comparison a similar plot is included for globular proteins and their noncovalent complexes.[19] Comparison of the plots shows that the rate of increase in charge is much greater for proteins than for the micellar systems studied here. Moreover, we find that regular micelles are able to accommodate more charge, for comparable mass, than reverse micelles or those incorporating myoglobin.

Charging in electrospray takes place during the droplet phase and for proteins is dictated not only by their mass but more precisely by their surface area.[20] However, despite the exposure of many ionizable groups in micelles, the extent of charging is

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less than that observed for globular proteins of similar mass. This difference is likely to result from the inherent instability of highly charged gas-phase micelles leading to disruption of micellar structures during electrospray. By contrast, amino acids in globular proteins are linked by covalent amide bonds and higher order interactions are maintained by salt bridges and electrostatic, hydrophobic, and van der Waals interactions. Globular proteins or protein complexes are therefore likely to be more resistant than micellar structures to the surface charging that takes place during electrospray. The observation that the micellar structures are less resilient to charging therefore implies that their overall gas-phase stability is less than that of a globular protein complex of comparable mass.

In theory, exposed hydrophobic chains of reverse micelles would constitute a greater surface area than regular micelles of similar mass. In practice, however, despite their higher surface area, reverse micelles carry less charge than the same number of CTABs in a regular micelle. This observation is consistent with higher ionization efficiency of the exposed headgroups in regular micelles compared with those buried within the core of reverse micelles. For reverse micelles incorporating myoglobin, moreover, charging is less than for their unoccupied counterparts, consistent with the encapsulation of neutral myoglobin molecules within the protected interior of reverse micelles.

For tandem mass spectra with isolations around *m*/*z* 10 000, the extent of polydispersity and multiple charging of the micellar species render the spectra extremely complex with numerous overlapping charge states. To illustrate the assignment, we have carried out a simulation of the spectra. In this simulation we have calculated the expected *m*/*z* values of the individual clusters and adjusted their intensities to fit the experimental data. Summation of the various charge state series calculated for the different assemblies leads to the final spectrum, as shown in Figure 5. From this simulation we can deduce that reverse micelles isolated at this *m*/*z* value contain at least six overlapping charge state series.

As a further investigation of the effect of the electrospray process on micellar structures, we prepared solutions of reverse micelles in which myoglobin had been incorporated and compared these with reverse micelles and micelles in the absence of myoglobin. Interestingly for a number of tandem mass spectra in which isolations were between *m*/*z* 4000 and 9000, the spectra of reverse micelles with myoglobin incorporated were indistinguishable from those of reverse micelles in the absence of myoglobin (data not shown). This implies that at this *m*/*z* value reverse micelles are unoccupied, similar to the situation in solution where under the conditions commonly used for preparation most reverse micelles are unfilled (i.e., without protein).[21] Isolation at *m*/*z* 10 000 and tandem MS, however, reveals significant differences between spectra recorded for the occupied and unoccupied reverse micelles as well as the regular micelle (Figure 6). For micelles, tandem MS at this *m*/*z* value shows assemblies containing >500 CTAB molecules while for reverse micelles lower aggregation numbers are deduced, from 336 to 419 CTAB molecules. For the reverse micelles incorporating myoglobin we used the same assignment and simulation protocols described above and simulated spectra

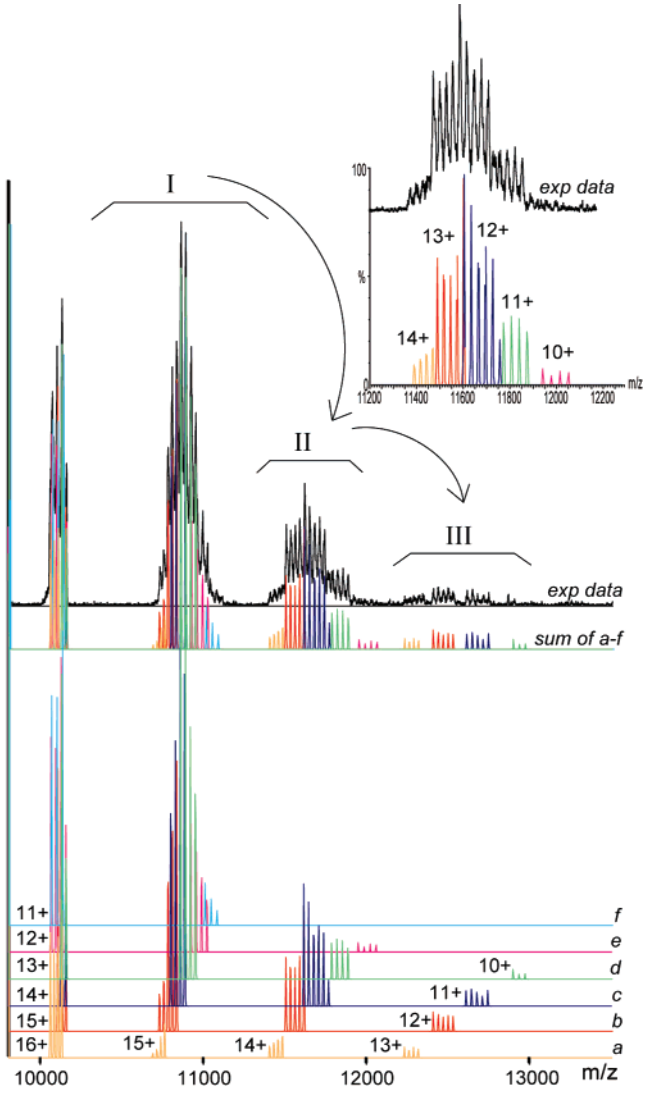

Figure 5. Comparison of simulated and experimental tandem mass spectra recorded from a solution containing reverse micelles. Overlapping charge states from 11^+ to 16^+ are isolated at *m*/*z* 10 000. The intensities are adjusted to match those of the tandem mass spectrum, and simulated spectra (a−f) are summed. Successive dissociation of $(CTAB\text{-}CTA)^+$ (processes I, II, and III) leads to a reduction in charge state. Inset: Expansion of the simulated and experimental data for the region corresponding to loss of two $CTAB\text{-}CTA^+$.

with one, two, or three myoglobin molecules, in apo and holo forms. Results showed that only one simulation was an adequate fit to the data: one molecule of holo myoglobin in association with ~270 CTAB molecules.

Only at *m*/*z* ~10 000 was it possible to isolate assemblies sufficiently different from those prepared in the absence of myoglobin, implying that at this *m*/*z* value sufficient CTAB molecules are available to encapsulate the protein. In solution aggregation numbers determined for reverse micelles incorporating proteins depend critically on the water:surfactant ratio as well as the surface area of the encapsulated protein.[12] Using a probe corresponding to the cross-sectional area of the $N(CH_3)_3$ headgroup of the CTAB molecule, we estimated that a minimum of ~200 CTAB molecules are required to fully encapsulate the accessible surface area of myoglobin in the absence of water. This value is of the same order as the 270 CTAB molecules determined from our tandem MS experiments. The increase in the number determined experimentally could be attributed to

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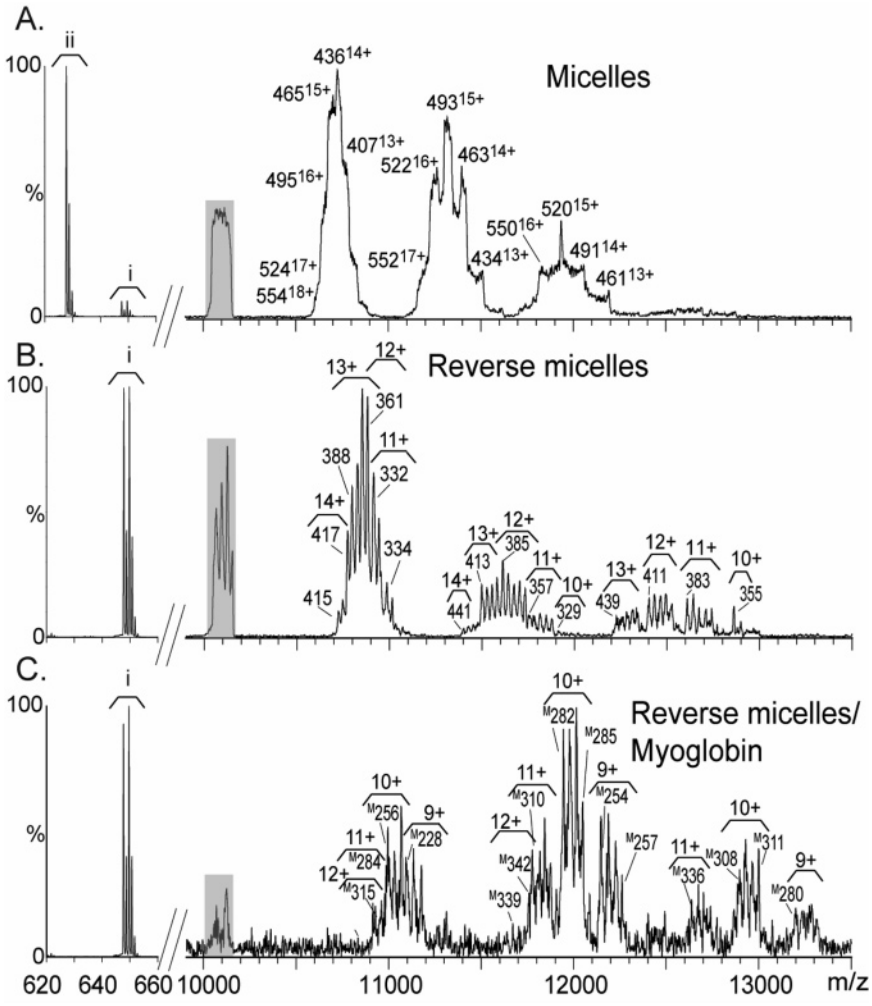

Figure 6. Comparison of tandem mass spectra at *m*/*z* 10 000 for regular micelles in 200 mM ammonium acetate (A), reverse micelles in H_2O (B), and reverse micelles incorporating myoglobin in 10 mM ammonium acetate (C). At low *m*/*z* bromide ions are exchanged from $(CTAB\text{-}CTA)^+$ in the regular micelle (ii) while they are retained for the reverse micelle (i), in accord with Figure 2. The stripped complexes at *m*/*z* values >10 000 reveal characteristic differences. Diffuse peaks are observed for regular micelles, while a shift in the charge states is evident for the unoccupied reverse micelles relative to those encapsulating myoglobin.

the contribution of ordered water molecules to the surface area of the encapsulated protein. These ordered water molecules are known to be present within reverse micelles in solution[13] but would be readily dissociated upon activation in the gas phase during tandem MS.[22] The majority of the CTAB molecules, however, remain associated with each other as well as with the protein. This scenario would give rise to the observed distribution of 230−317 CTAB molecules remaining associated with one molecule of holo myoglobin.

Discussion

We have shown that micelles formed from the same surfactant but under different solution conditions exhibit markedly different mass spectra. Specifically, we have shown that while exchange of the bromide counterion takes place readily from micelles, exchange is prevented by the orientation of the headgroup toward the ordered core of the reverse micelle. At high *m*/*z* values differences in the charging of the micellar structures is evident, with regular micelles carrying more charge than reverse micelles of the same mass. The stripped complexes formed during dissociation are consistent with the encapsulation of myoglobin within reverse micelles.

In solution regular CTAB micelles have a typical aggregation number of 90,[23] a lower value than the aggregation numbers of several hundred deduced here. For reverse micelles incorporating myoglobin our data are consistent with one molecule of holo myoglobin associated with 230−310 CTAB molecules. In the absence of myoglobin, however, the number of molecules within each assembly ranges from 40 through to >500 CTAB molecules. Why then are these gas-phase species larger than those formed in solution? We anticipate that maintaining CTAB micelles with low aggregation numbers (∼90) is likely to be experimentally challenging due to their tendency to aggregate. Assemblies with such aggregation numbers are therefore likely to be present in very low numbers in the electrospray droplet. Support for this hypothesis comes from solution-phase studies where changes in concentration of the micellar solutions have been shown to cause conversions from spherical micelles to megadalton rodlike structures.[23,24] These transitions are conceiv-

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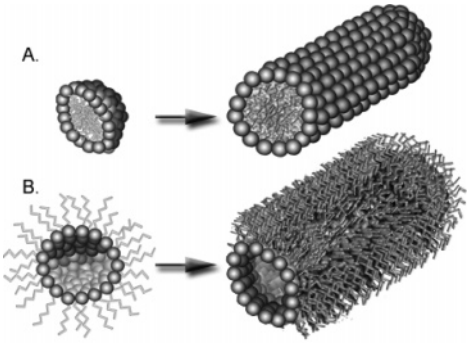

Figure 7. Schematic diagram to show the structural transitions that occur from the well-defined regular and reverse micelles (A and B, respectively) through to the rodlike structures which are believed to form in response to changes in concentration. We propose that a similar process could occur during evaporation of the electrospray droplets.

able during the evaporative steps of ES[25] which would lead to changes in the solution concentration and hence aggregation and coalescence of micelles into large rodlike structures, accounting for the high aggregation numbers determined here (Figure 7).

To our knowledge, these data represent some of the largest surfactant clusters reported to date and contrast with previous experiments below the cmc, where extensive interactions of surfactant molecules were not observed.[9,10] Taken together, our results demonstrate that micellar structure can survive the phase transition, without changing the orientation of the surfactant molecule, and imply that protein molecules remain encapsulated within reverse micellar structures.

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Supporting Information Available: Tandem mass spectra of reverse micelles formed from different solution conditions and concentrations of CTAB surfactant; further details of the assignment protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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