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The influence of heptane-1,2,3-triol on the size and shape of LDAO micelles

Implications for the crystallisation of membrane proteins

Peter A. Timmins[1], Jürgen Hauk[2], Thomas Wacker[2] and Wolfram Welte[2]

[1] *Institut Laue Langevin, 156X, 38042 Grenoble Cèdex, France* and [2] *Institut für Biophysik und Strahlenbiologie, Albertstrasse 23, 78 Freiburg, Germany*

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The presence of small amphiphiles has been found to be necessary in the crystallization of several membrane-protein/surfactant complexes. It has been suggested that the role of the small amphiphile may be to reduce the size of the surfactant belt around the protein, making the formation of crystals easier. Thus far it was not known if this would involve changes in micellar size in general or whether the small amphiphile would merely replace LDAO during crystal growth. In the present study we have used small angle neutron scattering to study mixed micelles of lauryldimethyl amine oxide (LDAO: hydrogenated and deuterated) and heptane-1,2,3-triol (HP). Our results show that with increasing overall HP concentrations mixed LDAO/HP micelles of decreasing mass and radius are formed. The composition of these micelles has been determined. HP thus may decrease the size of the surfactant belt around a protein before crystallisation by insertion into a host micelle. As HP is a 'small amphiphile' compared to the surfactants used for solubilization of membrane proteins, the curvature of the host micelle will be increased by its insertion.

Membrane protein crystallization; Surfactant micelles; Mixed micelles; Small amphiphiles; Small angle neutron scattering

1. INTRODUCTION

The crystallisation of several integral membrane protein compexes over the last few years offers the prospect of understanding the structures of this class of protein. To date, all such crystals have been grown from mixed protein-surfactant micellar solutions using similar precipitants as for water-soluble proteins. In a number of cases [1] the addition of small amphiphilic molecules was reported to be necessary for such crystallisation. Often in the absence of small amphiphiles, phase separation phenomena are observed [2–4] and in their presence crystal growth is observed. The first use of a small amphiphile was for the crystallisation of the reaction centre of *Rhodopseudomonas viridis* [5]. Here, 1, 2, 3-heptanetriol was used as an additive to the reaction centers solubilized with lauryl dimethylamine oxide (LDAO). The success of this method led Michel to propose a 'small amphiphile concept' [6] in which he suggested that small amphiphilic compounds might replace the surfactant during the process of insertion of protein-surfactant micelles into the growing crystal. Due to its small size it could form a smaller surfactant belt than LDAO around the hydrophobic zone of the reaction centre. In this manner it would allow for sterically unimpeded growth of the crystal which explains its propitious influence on the crystallisation. Alternatively he proposed that these molecules might act by forming, with the surfactant mixed micelles which are smaller than the pure surfactant micelles [6,7].

As a first step towards exploring these different hypotheses we have used small angle neutron scattering (SANS) to investigate the effect of the addition of heptane-1,2,3-triol (HP) on the size and shape of LDAO micelles. We have already characterised pure LDAO micelles as part of a systematic study of surfactants used in membrane protein crystallisation [8]. SANS is the ideal technique for such structural investigations as the different components can be independently contrast matched (for review see [9]).

In order to fully exploit this technique we used both hydrogenated LDAO and LDAO in which the lauryl moiety was perdeuterated. In this way the contributions to the scattering of the LDAO and HP could be effectively separated.

2. MATERIALS AND METHODS

2.1. *Surfactants and small amphiphiles*

Critical micellar concentrations (cmc) were determined with a Kruss-Digital-Tensiometer K-10.

Hydrogenated LDAO (H-LDAO) was purchased from Fluka (Neu Ulm, Germany) and used without further purification. The water content was guaranteed as $< 7\%$.

Deuterated dodecanoic acid (CD_3-$(CD_2)_{10}$-COOH) was purchased from the Centre d'Etudes Nucleaires de Saclay, France. D-LDAO was synthesized using undeuterated dimethylamine. The amineoxide ($C_{14}D_{23}H_8NO$) was obtained by oxidation with hydrogen peroxide. The cmc was 0.0148% w/v at room temperature.

Correspondence address: P.A. Timmins, Institut Laue-Langevin, 156X, 38042 Grenoble Cedex, France

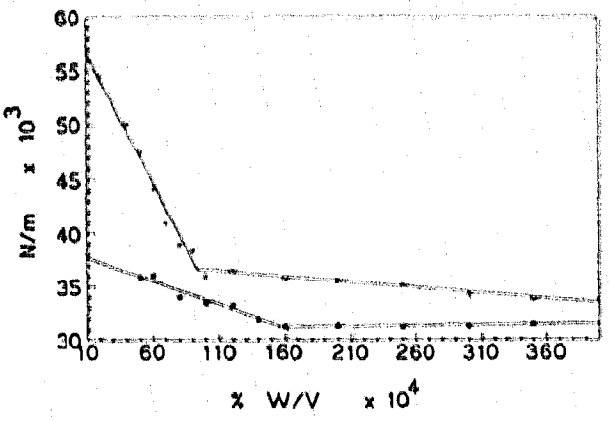

Fig. 1. Surface tension measurements with pure H-LDAO solutions (○) and H-LDAO solutions containing 3% (w/v) HP (●).

Heptane-1, 2, 3-triol (HP) high melting-point isomer was purchased from Fluka BioChemika, Neu-Ulm, Germany.

2.2. *Small angle neutron scattering (SANS)*

SANS experiments were carried out on the instrument D11 [10] at the High Flux Reactor of the Institut Laue-Langevin (Grenoble, France). Samples were contained in standard 1 or 2 mm pathlength quartz cuvettes (Hellma, France). Surfactant concentration was 1% w/v (10 mg/ml) and HP concentration 0%, 2%, 5% or 10% (w/v). Data were measured both in the Guinier range [11], using a sample-detector distance of 2.5 metres and incident wavelength (λ) of 10 Å, and at higher angles with sample detector distance of 1.2 metres and incident wavelength 5 Å. The wavelength spread ($\Delta\lambda/\lambda$) was 8% (FWHM). Data from the 64 × 64 cm^2 multidetector were radially averaged about the incident beam direction and corrected for differential detector response by the scattering of a 1 mm thick sample of H_2O using standard programs [12]. Final scattering curves of neutron intensity ($I(Q)$) versus scattering vector, Q ($Q = 4\pi \sin\theta/\lambda$, 2θ = scattering angle) were obtained by subtracting from the scattering of each sample its corresponding solvent background.

3. RESULTS

Fig. 1 shows surface tension measurements which were made to determine the concentration of LDAO monomers in equilibrium with micelles. In the absence and in the presence of 3% HP values of 0.009% (w/v) and 0.016% (w/v) respectively were found.

In order to interpret unambiguously the effects of heptane triol on LDAO micelles it was necessary to carry out two different sets of experiments. In a first series data were measured from samples of H-LDAO in D_2O to which were added various amounts of HP. In these experiments the LDAO has a very high negative contrast with respect to the water. If, however, HP is included in the micelle then it too will have a very high negative contrast which would contribute strongly to the scattering. Therefore in a second set of experiments data were measured from D-LDAO in H_2O to which was added HP. In this situation the LDAO has high positive contract (6.099×10^{-14} cm·Å^{-3}) but the HP has a very low contrast (0.745×10^{-14} cm·Å^{-3}). All samples were measured at low angles (Guinier range) from which were obtained the radius of gyration (R_g) and zero angle intensity ($I(0)$), and at higher angles to obtain more detailed information on the shape. The scattering length densities calculated for both surfactant molecules and the various solvents, calculated from their chemical composition, are listed in Table I.

The contrast variation behaviour of H-LDAO in H_2O has already been described [8]. Briefly, the micelles were found to be close to spherical with a radius of gyration at infinite contrast of 16.2 ± 0.2 Å. In order to interpret the data on D-LDAO in the presence of HP it was also necessary to characterise the D-LDAO in a similar manner. From measurements carried out at 0%, 40%, 60%, 80% and 100% D_2O the D-LDAO was found to have a neutron match point corresponding to 0.897 mole fraction D_2O. From this we can estimate the partial specific volume to be 1.053 cm^3·g^{-1} consistent with the value measured for H-LDAO (1.134 cm^3· g^{-1} [8]) taking into account the replacement of 25 hydrogen atoms by deuterium which makes the molecular weight of the deuterated molecule

Table I

ϱ (10^{-14} cm·Å^{-3})		$\varrho-\varrho_s$ (10^{-14} cm·Å^{-3})	
D-LDAO ($\nu = 1.053$)	5.690	D-LDAO in H_2O	6.252
		H_2O/3%HP	6.223
H-LDAO ($\nu = 1.134$)	−0.169	H_2O/5%HP	6.203
		H_2O/10%HP	6.161
H_2O	−0.562		
H_2O/3%HP	−0.533	H-LDAO in D_2O	−6.577
H_2O/5%HP	−0.513	D_2O/3%HP	−6.397
H_2O/10%HP	−0.471	D_2O/10%HP	−5.977
		HP in D_2O	−6.225
D_2O	6.408		
D_2O/3%HP	6.228	HP in H_2O	0.745
D_2O/5%HP	6.108		
D_2O/10%HP	5.808		
HP	0.183		

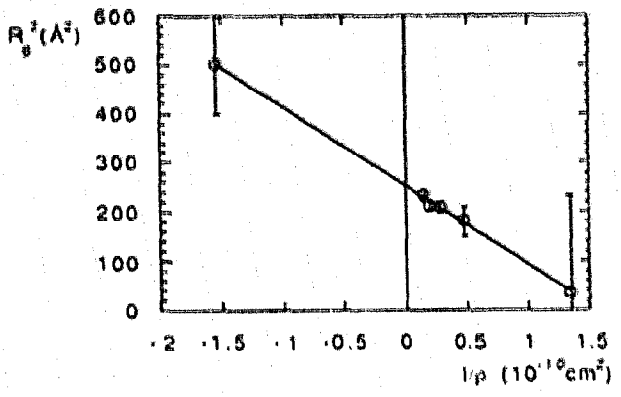

Fig. 2. Plot of R_g^2 versus $1/\varrho$ (reciprocal contrast) for D-LDAO micelles. The points can be fit with a straight line as shown, indicating the higher scattering density (polar heads) at the periphery of the micelle. The radius of gyration at infinite contrast is given by the intercept on the R_g^2 axis at $1/\varrho = 0$.

(252.3) 11% greater than that of the non-deuterated (229.8). The match point for H-LDAO is at 0.057 mole fraction D_2O (incorrectly printed as 0.57 in [8]). The variation of radius of gyration with inverse contrast is shown in Fig. 2 and leads to a radius of gyration at infinite contrast of 16.2 ± 0.1 Å in perfect agreement with that found for H-LDAO. The high angle scattering curves of both H- and D-LDAO at high contrast are also essentially identical indicating that the micelle structure is independent of the deuteration of the surfactant. The $I(0)$ value obtained for the deuterated surfactant led to an unreasonably low micellar mass (corresponding to 49 monomers). In view of the identity of size and shape found we ascribe this difference to the difficulty of accurately weighing small amounts of hygroscopic surfactant (D-LDAO) and assume the aggregation number to be the same as that for H-LDAO.

The low angle scattering curves for H-LDAO in D_2O and D-LDAO in H_2O (1% w/v) with varying amounts

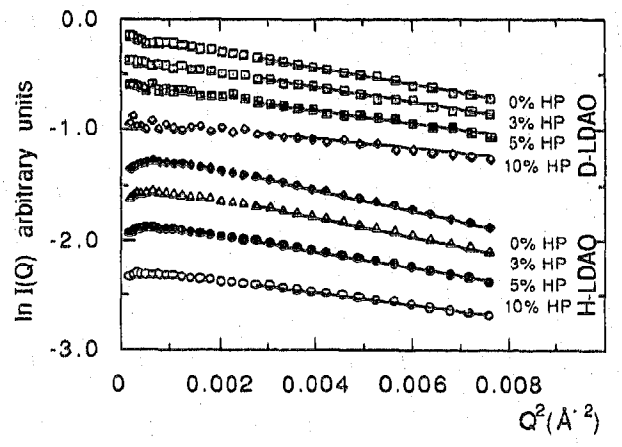

Fig. 3. Guinier plots for H-LDAO in D_2O/HP and D-LDAO in H_2O/HP. The linear regression straight lines give the radius of gyration and the intensity at $Q=0$. Due to inter-particle interference effects fits used only points at $Q>0.05$ Å^{-1} (see text).

Table II

	%HP	R_g (Å)	$I(0)$
D-LDAO/H_2O	0	15.4 ± 0.1	1.00
	3	14.4 ± 0.2	0.80
	5	13.6 ± 0.2	0.63
	10	12.1 ± 0.3	0.46
H-LDAO/D_2O	0	16.1 ± 0.1	1.00
	3	15.7 ± 0.1	0.96
	5	14.7 ± 0.1	0.93
	10	12.9 ± 0.1	0.77

of HP are shown in Fig. 3. A slight inter-particle effect is evident at the lowest angles due to the rather low ionic strength of the solvent. Measurements were repeated for a number of samples after addition of NaCl to 100 mM. The resulting Guinier plots (not shown) demonstrated that the interference effects are only significant for $Q<0.05$ Å^{-1} and therefore this region was not used in the Guinier plots.The results of the Guinier analyses are summarized in Table II. The $I(0)$ values given are normalized to unit transmission, thickness and sample pathlength and given relative to the value of the pure micelle.

The most striking observation is the decrease in the radius of gyration on addition of HP, both for the hydrogenated and the deuterated micelle. It was shown previously for H-LDAO [8] and confirmed here for D-LDAO that the LDAO is not very elongated and therefore such a decrease in radius of gyration must reflect a decrease in volume (or in mass) rather than a simple compaction of the micelle. This is confirmed in the combined low and high angle curves shown in Fig. 4. For the pure micelle there is a clear minimum in the scattering curve close to $Q=0.25$ Å^{-1}. For particles of the same shape but decreasing dimensions we would expect this minimum to shift to larger angles. Although the statistical accuracy of the data at the highest Q-values is rather poor (such that sharp changes in intensity between adjacent points are not significant) it can be seen that on addition of HP the minimum does indeed

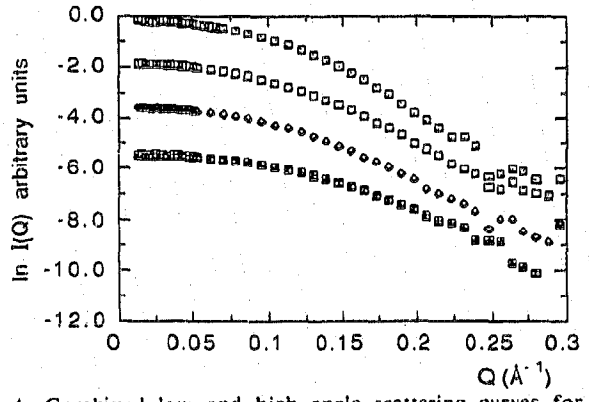

Fig. 4. Combined low and high angle scattering curves for H-LDAO/HP in D_2O. (⊡) 0%HP (□) 3%HP (◇) 5% HP (⊞) 10%HP.

move to higher Q and for 5% and 10% HP even beyond the measured Q-range.

Having noted this decrease in micelle size in the presence of HP it remains to elucidate the structure and composition of these micelles. This may be done by a quantitative analysis of the observed $I(0)$ values which are an indication of the micellar mass.

In a small angle scattering experiment we can write:

$$I(0) \propto N(\Sigma b - \varrho_s V)^2$$

where N = number density of scattering micelles; Σb = sum of the atomic scattering lengths of the scattering micelle; ϱ_s = scattering length density of solvent; V = micellar volume.

N can be calculated from the concentration C (weight %) of the surfactant with the following formula:

$$N = 10\,(C - \mathrm{cmc}) \cdot N_A/(A \cdot M) \qquad \text{in particles/l}$$

where cmc is the critical micelle concentration in weight percent, A the number of surfacant molecules per micelle, M the molecular weight of one surfactant molecule and N_A Avogadro's number.

As mentioned above, there was some difficulty in measuring correctly the absolute concentration, particularly of the D-LDAO. We therefore use relative $I(0)$ values, i.e. $I(0)$ normalized to the $I(0)$ of pure micelle without HP. We then place the derived quantities on an absolute scale by assuming that both H- and D-LDAO pure micelles consist of about 70 surfactant molecules. For the relative $I(0)$ we get the following general expression;

$$\sqrt{I(0)_{\mathrm{rel}}} = \frac{\sqrt{N_1}[m_1(\Sigma b - \varrho V)_{\mathrm{LDAO}} + n(\Sigma b - \varrho V)_{\mathrm{HP}}]}{\sqrt{N_0}[m_0(\Sigma b - \varrho V)_{\mathrm{LDAO}}]}$$

N_0 and N_1 are the number densities of the pure LDAO and LDAO/HP micelles, respectively; m_0 and m_1 are the number of LDAO molecules per pure and mixed micelle, repectively; n is the number of HP molecules in the mixed micelle; $(\Sigma b - \varrho V)$ are the excess scattering lengths per LDAO or HP molecule and can be calculated from the excess scattering length densities $\varrho - \varrho_s$ using the volume per LDAO molecule (432.7 Å^3) and per HP molecule (218 Å^3[13]).

For the case of D-LDAO micelles in H_2O/HP the presence of HP within the micelles would have little effect on the scattered intensity as the contrast is approximately 8 times lower. Indeed we calculate that for a mixed D-LDAO/HP micelle of molar ratio 1:1 the intensity at zero angle would only be ~3% greater than in a pure D-LDAO micelle.

Using $N_0 m_0 = N_1 m_1$, the formula then reduces to:

$$\sqrt{I(0)_{\mathrm{rel(D\text{-}LDAO)}}} = \frac{m_1\sqrt{N_1}}{m_0\sqrt{N_0}} = \frac{\sqrt{m_1}}{\sqrt{m_0}}$$

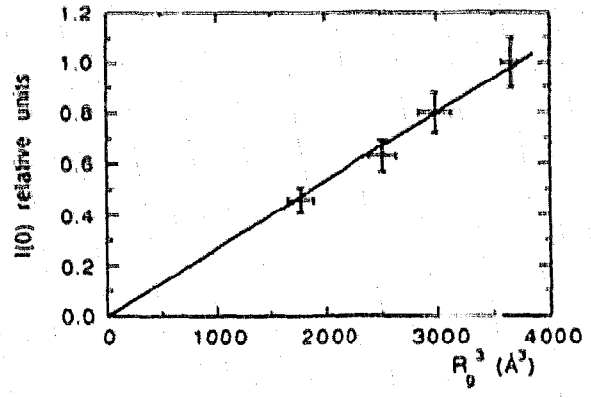

Fig. 5. Variation of $I(0)$ with R_g^3 for D-LDAO micelles in H_2O/HP.

We can, therefore, use the $I(0)$ for D-LDAO in H_2O/HP as an indication of the mass of LDAO in the micelles. If we plot $I(0)$ versus R_g^3 (Fig. 5), we obtain a straight line passing through the origin indicating that the mass of LDAO in the micelle is directly proportional to its volume (within the HP concentration range investigated).

In the case of H-LDAO in D_2O/HP we would expect a decrease in $I(0)$ with respect to the value in pure D_2O arising from two different effects: (i) reduction of the effective contrast on addition of HP to D_2O (due to the proton content of HP); (ii) decrease in micelle volume and hence LDAO mass on addition of HP. The first effect may be calculated from the $\varrho - \varrho_s$ values of H-LDAO with and without 10% HP. Considering that the $I(0)$ values are proportional to the square of the contrast, $I(0)$ will be decreased by a factor of 0.826.

The second effect can be estimated from the data of D-LDAO in H_2O with and without 10% HP. $I(0)$ will be decreased by a factor of 0.485.

Taking both effects together one would expect a relative $I(0)$ for H-LDAO in D_2O with and without 10% HP of 0.401. The observed value, however, is 0.77, i.e. the decrease in $I(0)$ is much smaller than predicted from the contrast and LDAO mass changes. This difference must arise from an insertion of HP into the LDAO micelle increasing the effective scattering mass.

The above-mentioned theoretical relations can be used to calculate the number of LDAO and HP molecules in the mixed micelles from the experimental data. The square root of the ratio of the relative $I(0)$ values in H-LDAO and D-LDAO gives:

$$\frac{\sqrt{I(0)_{\mathrm{rel\ (D\text{-}LDAO)}}}}{\sqrt{I(0)_{\mathrm{rel\ (H\text{-}LDAO)}}}} = 1 + \frac{n(\Sigma b - \varrho V)_{\mathrm{HP}}}{m_1(\Sigma b - \varrho V)_{\mathrm{H\text{-}LDAO}}}$$

As $m_0 \approx 70$ [8] and as n/m_1 and m_1/m_0 can be determined from the experimental data, the composition and size of the mixed micelles can be calculated as given in Table III. We find, for example, that in D_2O/10% HP the overall composition is 34 molecules of LDAO and

Table III

	0% HP	1% LDAO + 3% HP	5% HP	10% HP
Total surfactant (mol)	0.0435	0.249	0.381	0.718
Mole fraction LDAO of total surfactant	1	0.177	0.144	0.061
Mole fraction LDAO in micelles	1	0.826	0.716	0.596
No. of LDAO molecules per micelle	70	57	48	34
No. of HP molecules per micelle	0	12	19	23
Volume LDAO + HP (Å^3) per micelle	31073	27899	25468	20308
Micelle volume from R_g^2	38318	36229	29814	20261

23 molecules of HP. This would lead to a total micelle volume of 20185 Å^3 which corresponds to a radius of gyration of 13.1 Å. The radius of gyration of H-LDAO in D_2O/10% HP is 12.9 ± 0.2 Å and the radius of gyration at infinite contrast would be somewhat greater than this. The data are, therefore, consistent with a rather spherical micelle of LDAO/HP in which the micelle envelope is occupied almost entirely with LDAO and HP and which therefore contains very little water between the headgroups. Small departures from sphericity would increase the volume for the same R_g and thus allow the inclusion of more water molecules. These data as well as those for other HP concentrations are summarized in Table III. In pure LDAO micelles it was calculated [8] that there were a little over 2 molecules of water per LDAO molecule within the micelle envelope. The results of this study suggest that in HP solutions these inter-headgroup water molecules are progressively replaced by HP molecules. As contrast variation experiments were not carried out for each water/HP mixture we assume that the best approximation to the radius of gyration of the micelle envelope is given by the value for the hydrogenated LDAO and that the radius of gyration at infinite contrast is about 0.2 Å greater than this (as is the case for the pure micelle [8].

4. DISCUSSION

From the data in Table III and the concentration of LDAO monomers in equilibrium with micelles in the presence of 3% HP (see Fig. 1), a picture of the mixed surfactant system can be obtained. The LDAO concentration is approximately 10^2-fold higher than the concentration of monomeric LDAO which is in equilibrium with the micelles. Thus, practically all the LDAO (0.0435 M) is in micelles. On the other hand, it can be seen from the mole fractions that although the overall mole fraction of HP is much higher than that of LDAO, only a very small amount of the HP is in the micelles, less than the LDAO. The transfer of HP from solution to the mixed micelles is therefore thermodynamically unfavorable. We think that this is mainly due to the higher overall polarity of the HP as compared to LDAO.

In order to find a theoretical description of the phenomenon, the mixed micelles and the surfactant monomers in solution can be considered as a two-phase system in equilibrium. This analogy was used [14] to predict the concentrations of surfactant monomers which are in equilibrium with a mixed surfactant micelle when the overall concentration of surfactants, the cmc values of the pure surfactant micelles and their interaction energies in mixed micelles are known. The theory is valid both for ideal mixing of surfactants in micelles as well as for non-ideal mixing due to interactions. For the 1% LDAO/3% HP solution, the interaction parameter of LDAO and HP in micelles $\beta = (W_{11} + W_{22} - 2W_{12})/kT$ can be calculated from our data to be +25. This result restates quantitatively the conclusion which was deduced already, that there is an unfavorable interaction between the two components of the mixed micelle, so that only at high overall concentration ratios of [HP]/[LDAO] will HP partition into the LDAO micelle.

The LDAO/HP/water system bears some resemblance to the lauryldimethylamineoxide/ethanol/water system studied by Hermann and Benjamin [15]. In both systems the addition of HP or ethanol, respectively, decreases the micellar mass and represses the phase separation [1,15]. Hermann and Benjamin argued that by changing water structure and lowering the dielectric constant of the aqueous phase, ethanol could increase electric interactions and consequently the temperature of phase separation. The qualitatively similar behaviour of the phase separation boundary of the LDAO/HP/water system [1] may possibly be explained in the same manner by a change in water structure caused upon addition of HP. Such a change in water structure by one component is not within the framework of the aforementioned theory.

The events occurring upon approaching the phase separation boundary and the shift of this boundary upon addition of precipitants which are used for protein crystallisation have been studied by other authors. Phase separation phenomena seem to be due to non-directed mixed micelle interactions [16,17]. They are promoted by precipitants which are used for lowering the solubility of the protein-surfactant mixed micelles, such as e.g. polyoxyethylenes [4,18] but nevertheless can be protein induced [19]. A reduction of the dielectric constant by HP thus could increase the temperature

of the phase separation boundary. Alternatively a change of water structure by HP could have the same effect.

We have shown that the addition of heptane-1,2, 3-triol to solutions of LDAO leads to a decrease in the size of the micelle whilst maintaining the overall (rather spherical) shape. Evidently, HP, by intercalating into the LDAO micelles, increases the curvature of the mixed micelle and decreases the diameter. Our data show that the intercalation of HP into LDAO micelles is thermodynamically unfavorable and driven only by a large overall excess of HP over LDAO.

We cannot yet establish whether exactly the same process takes place in a solution of crystallizing membrane protein but this would seem likely. The results here show that by the addition of appropriate amounts of HP the curvature and hence dimensions of the LDAO micelle can be finely tuned. In this way it may be possible to adjust the size of the micelle adhering to the membrane protein in such a way as to optimize protein/protein contacts whilst keeping the hydrophobic surface of the protein covered with surfactant.

The only direct structural information concerning the role of surfactant micelles in protein crystals comes from a neutron diffraction study of crystals of the photo-reaction center from *Rhodopseudomonas viridis* [20]. These crystals were grown from reaction centers solubilized in LDAO with the addition of 3% HP. 2.5 M ammonium sulphate was added as a precipitant. The neutron diffraction study shows the LDAO to be disposed in a belt around the hydrophobic membrane traversing helices. The surfactant ring thickness perpendicular to the supposed membrane plane is 25–30 Å. This is of interest as it is somewhat less than twice the length of a fully extended LDAO molecule (~40 Å) and also the probable diameter of a pure LDAO micelle [8]. It is, however, closer to the minimum diameter we would estimate from our measurements of LDAO/3% HP micelles (~34 Å).

This is consistent with Michel's hypothesis [6] that the role of HP in this case, or small amphiphiles in general, is to reduce the surfactant micelle size to the dimensions of the hydrophobic trans-membrane surface in a manner such that the formation of a crystal lattice by interactions of polar surfaces of approaching proteins is not impeded. It should be remembered (as noted in [20]) that the surface present in the isolated reaction center may not be exactly the same as that in the membrane where the reaction center is also associated with light-harvesting proteins.

Another feature of the reaction center structure is the existence of surfactant bridges connecting the protein surfactant complexes. It is clearly of importance that the formation of these bridges (which are presumably not structured at the atomic level) should not inhibit the protein/protein interactions necessary for crystal formation. The HP could therefore play a role in defining the structures of these bridges. It is also of interest to speculate as to whether the HP is homogeneously distributed throughout the surfactant phase or whether it is concentrated in the lipid simulating belt or the intermicellar bridges. A neutron diffraction experiment using deuterated LDAO in reaction center crystals could perhaps resolve this question.

Further experiments to investigate the effect of added HP on protein/surfactant complexes should help to clarify this.

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