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Effective detergent/chlorophyll ratio and detergent concentration in the aqueous phase during solubilization of *Phormidium laminosum* membranes

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Abstract

Experiments of turbidity decrease induced by detergents were systematically performed to characterize the solubilization of *Phormidium laminosum* membrane fragments. SDS, Triton X-100 and a mixture of octyl glucoside/decyl maltoside/lithium dodecyl sulfate (OG/DM/LiDS, in a molar ratio of 4.19:2.54:1) were used. The detergent concentration in the aqueous phase (D_W) and the effective detergent/chlorophyll ratio in mixed aggregates (R_e) were determined. Both parameters increased during the solubilization and in an exponential way in the range from 10 to 90% solubilization. At detergent concentrations which caused the complete solubilization, D_W values were close to the described critical micellar concentrations (cmc), but solubilization started at concentrations well below the cmc. At the onset of solubilization five molecules of SDS, one of Triton X-100 and three of the mixture OG/DM/LiDS, per chlorophyll molecule, saturated the membrane fragments. The increase of D_W and R_e values was characterized by two constants. This permits the design of a model to predict the detergent concentration which produces a desired solubilization of thylakoid membrane fragments for a given chlorophyll concentration.

Keywords: Surfactant; Detergent; Thylakoid membrane; Solubilization; Turbidity; Cyanobacterium; (*Phormidium laminosum*)

1. Introduction

The study of biological membrane components requires the use of detergents for the solubilization, ordered fractionation, purification and crystallization of membrane proteins [1,2]. Detergents release membrane proteins from the lipid bilayer and keep them in solution during the isolation and purification processes. When the detergent concentration is raised beyond lytic concentrations, increasing amounts of detergents are bound to membranes. Saturation is reached at some point and gradual disintegration of the membranes starts [3–6].

During solubilization, part of the total detergent concentration (D_T) interacts with membrane components, while the rest is in the aqueous phase (D_W) in form of monomers and micelles. In the solubilized sample, D_W must be maintained throughout the procedure to prevent alterations in the complexes due to changes in detergent binding. A reduction of D_W will induce reassociation of membranes, and an increase will result in the further disruption or even complete separation of lipids and proteins of possible complexes [3].

Criteria for solubilization of biological and model membranes are based on phenomenologically related effects like the decrease in turbidity of membrane preparations, the increase in non-sedimentable material, the disappearance of continuous lamellar membranes as seen in electron microscopy and, in the case of thylakoid membranes, the shift of the chlorophyll (Chl) absorption spectrum [3–8].

Abbreviations: D_T, total detergent concentration; D_W, detergent concentration in the aqueous phase; cmc, critical micellar concentration; R_e, effective ratio, molar ratio of detergent/Chl in the mixed aggregates (vesicles or micelles); Chl, chlorophyll; T_V, maximal change in turbidity; T_{min}, minimal turbidity corresponding to the solubilized material; S, percentage of solubilization, percentage of decrease in T_V; K_{D_W}, water progress constant, average proportion of D_W that increases when solubilization progresses 1%; K_{R_e}, load constant, average proportion of R_e that increases when solubilization progresses 1%; OG, octyl glucoside; DM, decyl maltoside; LiDS, lithium dodecyl sulfate.

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Systematically performed turbidity measurements as a function of the lipid concentration, appear as a simple and useful technique not only for qualitative evaluation of the lamellar to micellar transition but also for quantitative evaluation of the amount of detergent bound either to the bilayer or to the micella [6,7]. These amounts can be calculated from solubilization experiments, noting when D_T produces a certain effect at different lipid concentrations (L) and employing the relationship of Lichtenberg [9]:

$$D_T = D_W + R_e \cdot L \quad (1)$$

where R_e is the effective detergent/lipid ratio which represents the detergent/lipid molar ratio in the mixed aggregates, vesicles or micelles.

The supposedly ideal way to solubilize biological membranes is the addition of enough detergent to free the individual components from the membrane, avoiding the use of excessive detergent, which causes extensive denaturation of membrane complexes [1]. It has been described that a mixture of octyl glucoside (OG), decyl maltoside (DM) and lithium dodecyl sulfate (LiDS) solubilizes thylakoid membranes of the green alga *Chlamydomonas reinhardtii* with very little release of free pigment and a high degree of preservation of subunit-subunit interactions known to exist in the membrane [10]. On the other hand, SDS and Triton X-100 have been used to purify and crystallize the photosystem I of the cyanobacterium *Phormidium laminosum* [11]. Thylakoid membranes of cyanobacteria are functional and structurally analogous to those of higher plant chloroplasts [12].

In this work we have used by the first time the Lichtenberg's relationship to characterize the solubilization of a thylakoid membrane. The results obtained indicate that this relationship should be modified to define the solubilization of thylakoid membrane fragments by SDS, Triton X-100 and a mixture of OG/DM/LiDS. We propose a model that allows the estimation of D_W and R_e throughout solubilization for a given membrane concentration.

2. Materials and methods

2.1. Materials

Triton X-100 specially purified for membrane research was purchased from Boehringer Mannheim (Germany). Other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Organism

P. laminosum (strain OH-1-pCl$_1$) cells were grown autotrophically at 45°C in medium D [13] supplemented with 0.5 g l^{-1} $NaHCO_3$.

2.3. Membrane fragments

Spheroplasts and photosynthetic membrane fragments were obtained by the procedure of Stewart and Bendall [14] as modified by Bowes et al. [15] except that proteinase inhibitors (5 mM ε-amino caproic acid and 1 mM benzamidine-HCl) were added to all buffers employed. Membrane fragments (25–30 mg protein/mg Chl) were resuspended in 50 mM Tris-HCl, pH 8.0, containing proteinase inhibitors, then frozen and stored at −50°C.

2.4. Analytical methods

The concentration of Chl extracted in 80% (v/v) acetone was determined spectrophotometrically using an extinction coefficient of 85.95 ml/mg per cm [16]. Protein was estimated by the method of Peterson [17].

2.5. Solubilization

Solubilization experiments were carried out with SDS, Triton X-100 and the mixture of detergents OG/DM/LiDS (in a molar ratio of 4.19:2.54:1). Before solubilization experiments, membrane fragments were thawed, vigorously stirred and diluted twice as much as the desired final concentration. Afterwards, membrane fragments were mixed with the same volume of the appropriate detergent solutions (in the same buffer) under stirring conditions. The samples were left to equilibrate for 15 min (in the case of solubilization by SDS or Triton X-100) or 30 min (when the mixture OG/DM/LiDS was used) at 25°C. Turbidity was not significantly altered after these times. Membrane solubilization was monitored as a decrease in the light scattered by the suspension. Light-scattering was measured at 750 nm in a Shimadzu UV 260 double-beam spectrophotometer thermostatized at 25°C. All samples were prepared in triplicate and measured in duplicate.

2.6. Fitting

Data were analyzed with the Macintosh programme KaleidaGraph™ 2.1.2 (Abelbeck Software). Plots of turbidity, T, against the logarithm of detergent concentration, $\log D$, were fitted to a descending sigmoidal curve based on the modified equation of the logistic model described by Zwietering et al. [18] for the bacterial growth curve:

$$T = T_V + T_{min} - \frac{T_V}{1 + e^{\left[\frac{4(\lambda - \log D)}{\alpha - \lambda} + 2\right]}} \quad (2)$$

where T_V is the maximal change in turbidity, T_{min} is the asymptote for the minimal turbidity (thus, $T_V + T_{min}$ is the asymptote for the maximal turbidity in the absence of detergent), λ is the $\log D$ producing a T_V decay of 12% and α is the $\log D$ producing a T_V decay of 88%.

The second derivative of the function equalled to zero indicates that the inflexion point of the curve occurred at $\log D = (\lambda + \alpha)/2$, producing a 50% of T_V decay. The tangent line through the inflexion point intercepts the maximal and minimal asymptotes at λ and α, respectively.

Decrease in turbidity is usually described by three parameters, D_{on}, D_{50} and D_{100}, which are defined as the detergent concentration producing the onset, 50% and 100% solubilization, respectively [19]. The intercepts of the tangent in $\log D_{50}$ with the asymptote for the maximal and minimal turbidity are considered as $\log D_{on}$ and $\log D_{100}$, respectively [19]. For the reasons above mentioned, in this paper D_{on} and D_{100} are termed D_{12} and D_{88}.

If the base of Eq. (2) is changed from exponential to decimal, the significance of λ and α is also changed. Here, λ is the $\log D$ producing a T_V decay of 1% and α is the $\log D$ producing a T_V decay of 99%. If the base of Eq. (2) is changed from exponential to binary, λ is the $\log D$ producing a T_V decay of 20% and α is the $\log D$ producing a T_V decay of 80%. The $\log D$ producing a T_V decay of 50% was calculated in any case as $(\lambda + \alpha)/2$.

Base changes affect neither the accuracy of the fit nor the confidence interval obtained with the χ^2 test, which was always higher than 95%. In all linear and non-linear fittings, data were weighted with the standard error of each point.

3. Results

3.1. Solubilization

Addition of increasing amounts of detergent to an aqueous solution of thylakoid membrane fragments resulted in a turbidity decrease. Plots of turbidity against the logarithm of detergent concentration showed a descending sigmoidal profile characterized by the maximal turbidity without detergent, by T_{min} at high detergent concentrations, and by T_V in a range of detergent concentrations. The detergent did not induce size-growth of the membranes at subsolubilizing detergent concentrations, as reported for small unilamellar vesicles and large unilamellar vesicles [5,7].

The maximal turbidity of membrane fragment suspensions depended on the method used to obtain membrane fragments. When they were obtained by hypoosmotic shock of spheroplasts, the extinction coefficient of light-scattering at 750 nm was around 25 000–30 000 M^{-1} (referred to the Chl content). The T_{min} observed at each membrane concentration depended on the detergent employed. Fig. 1

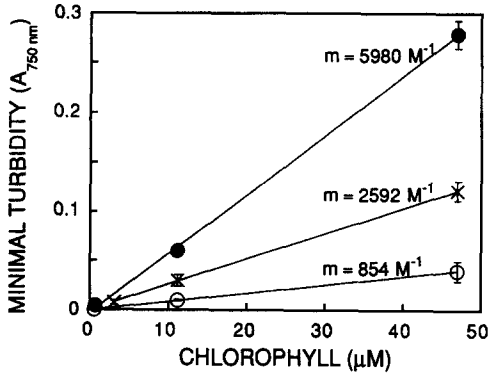

Fig. 1. Minimal turbidity observed after the solubilization of thylakoid membrane fragments by several detergents. Membrane fragments at different chlorophyll concentrations were solubilized by SDS (○), Triton X-100 (●) and a mixture of OG/DM/LiDS (molar ratio, 4.19:2.54:1) (×). Note that minimal turbidity depends on the detergent used and, consequently, the resulting mixed micelles at the end of solubilization are different in size. The slope (m) corresponds to the extinction coefficient at 750 nm.

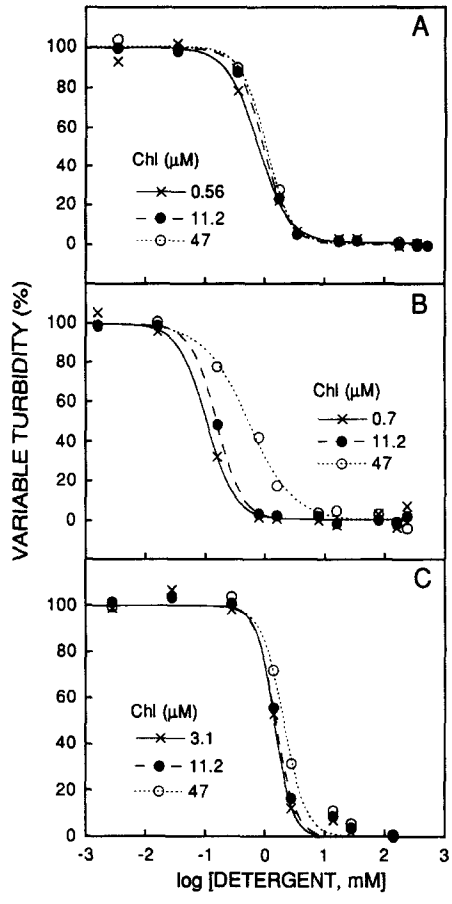

Fig. 2. Change in variable turbidity induced by detergents. Preparations of membrane fragments containing the indicated chlorophyll concentration were incubated for 15 min with SDS (A) or Triton X-100 (B), and for 30 min with a mixture of OG/DM/LiDS (molar ratio, 4.19:2.54:1) (C). Results were fit to a descending sigmoidal curve. Plot of the variable turbidity expressed in a percent base allows the comparison of samples with different chlorophyll concentrations. Increasing chlorophyll concentration, curves shifted to higher detergent concentrations and this shift varied between detergents. Data points are the average of three independent measurements. The standard error of each data and the logarithmic detergent concentrations lower than −3 were omitted for clarity, but they were considered for curve fitting.

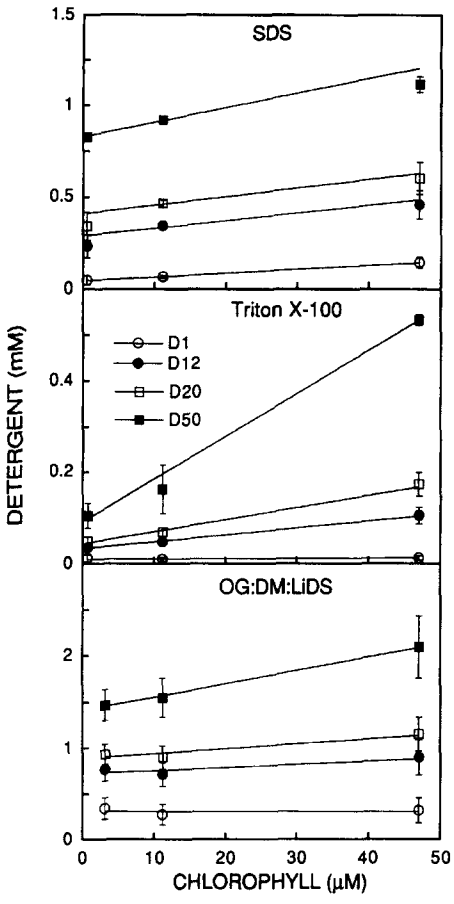

Fig. 3. Determination of R_e and D_W values during solubilization. Total detergent concentrations (D_T) producing a decrease in variable turbidity (as indicator of solubilization) of 1% (D_1, ○), 12% (D_{12}, ●), 20% (D_{20}, □) and 50% (D_{50}, ■) were plotted against the chlorophyll concentration. R_e values were calculated from the slope of the resulting straight lines, and D_W values from the intercept on the vertical-axis. Data and standard errors were obtained from the fittings of Fig. 2. Linear regressions were weighted with standard errors.

shows the extinction coefficient of T_{min} as referred to the Chl content. When membrane fragments were treated with Triton X-100 or the OG/DM/LiDS mixture, the value of T_{min} was 6.3 and 3.9-fold higher, respectively, than that obtained in samples treated with SDS. This indicates that there were differences in size of the mixed micelles resulting at the end of the solubilization.

In the presence of increasing membrane concentrations (in this paper referred to as the Chl concentration), turbidity versus detergent concentration plots shifted toward higher detergent concentrations (Fig. 2). In membranes treated with Triton X-100, the shift was observed in all T_V, while in SDS-treated membranes the shift was more evident during the onset of T_V. In membranes treated with the OG/DM/LiDS mixture, the shift was more important after de onset of T_V. These results indicate that the detergent concentration required to solubilize the membranes increased with membrane concentration and that this increment differed among detergents. The former behaviour has also been observed during the solubilization of higher plant thylakoid membranes by Triton X-100 [20].

3.2. Parameters of solubilization

To estimate the detergent concentration which provoked each decrease in turbidity, a descending sigmoidal curve model was designed. The curve was defined by four parameters and fit the turbidity versus detergent concentration plot. The information obtained from this model was limited because it was not described from biophysical properties. However, it allowed us to estimate the detergent concentration causing a 1, 12, 20, 50, 80, 88 and 99% decrease of T_V, and the standard error arising from this approach. When the concentrations of total detergent obtained (D_1, D_{12}, ..., D_{99}) were plotted against membrane concentration, the straight lines predicted by Eq. (1) were found. Such representations permitted us to calculate the concentration of free detergent in water (D_W) and the effective detergent/Chl ratio (R_e) corresponding to a specific decrease in turbidity. Each straight line intercepted the vertical-axis at a value corresponding to D_W. The slope represented R_e when a certain decrease in turbidity was observed. As an example, values obtained from D_1 to D_{50} with the three detergents tested are plotted in Fig. 3.

Both D_W and R_e increased during membrane solubilization and showed an exponential progression between

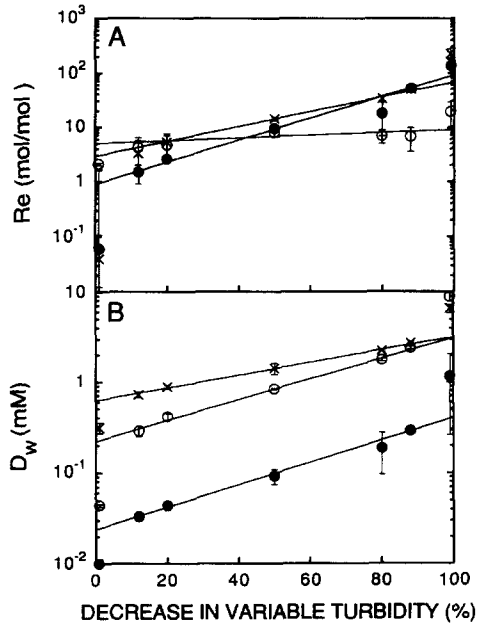

Fig. 4. Variation of R_e (A) and D_W (B) during solubilization. R_e and D_W values obtained from the solubilization of thylakoid membrane fragments by SDS (○), Triton X-100 (●) and a mixture of OG/DM/LiDS (molar ratio, 4.19:2.54:1) (×) were plotted versus the corresponding decrease in turbidity. The straight lines corresponded to the exponential fit of data between 10 and 90% solubilization. The intercepts of this line with 0 and 100% solubilization permit the estimation of R_e and D_W values at the beginning (R_e^{SAT}, D_W^{SAT}) and at the end (R_e^{SOL}, D_W^{SOL}) of solubilization.

Table 1
Parameters used to express solubilization of thylakoid membrane fragments

Parameter	Units	Detergent		
		SDS	Triton X-100	OG/DM/LiDS
R_e^{SAT}	(mol/mol)	5 ± 1	0.9 ± 0.1	2.9 ± 0.3
	(w/w)	1.6 ± 0.3	0.63 ± 0.05	1.1 ± 0.1
R_e^{SOL}	(mol/mol)	9 ± 3	93 ± 15	68 ± 11
	(w/w)	3 ± 1	65 ± 10	27 ± 4
K_R	–	0.005 ± 0.004	0.046 ± 0.002	0.031 ± 0.002
D_W^{SAT}	(mM)	0.22 ± 0.01	0.024 ± 0.001	0.61 ± 0.03
	(%, w/v)	0.0063 ± 0.0004	0.0015 ± 0.0000	0.022 ± 0.001
D_W^{SOL}	(mM)	3.1 ± 0.3	0.4 ± 0.0	3.2 ± 0.2
	(%, w/v)	0.09 ± 0.01	0.025 ± 0.001	0.112 ± 0.007
K_{D_W}	–	0.026 ± 0.001	0.028 ± 0.000	0.016 ± 0.001
$R_e^{1\%}/D_W^{1\%}$	(M^{-1})	48 ± 3	6 ± 5	< 5
R_e^{SAT}/D_W^{SAT}	(M^{-1})	23 ± 6	38 ± 3	4.7 ± 0.6
R_e^{SOL}/D_W^{SOL}	(M^{-1})	3 ± 1	233 ± 40	22 ± 4

Parameters (±S.E.) calculated from Figs. 3 and 4 are summarized and expressed both in molar (used in biomembranes research) and weight (used in photosynthesis research) terms.

10 and 90% of T_V decay, suggesting a first order progression of both D_W and R_e during solubilization (Fig. 4A and B). From the slope of the logarithmic plots, the constants for these phenomena (K_{D_W} and K_{R_e}) can be estimated. The water progress constant, K_{D_W}, was defined as the average proportion of D_W that increases when solubilization progresses 1%. The load constant, K_{R_e}, was defined as the average proportion of R_e that increases when solubilization progresses 1%. (Table 1). K_{R_e} values were very different among detergents, SDS showing the lowest one (0.005 ± 0.004) and Triton X-100 the highest (0.046 ± 0.002). However, K_{D_W} values were similar in all detergents tested.

D_W and R_e values at the onset (D_W^{SAT} and R_e^{SAT}) and at the end (D_W^{SOL} and R_e^{SOL}) of solubilization were determined by extrapolation to 0% and 100% of the exponential progression of both parameters (Fig. 4). These values are given in Table 1.

Consequently, the R_e and D_W values from 10 to 90% solubilization can be defined as follows:

$$R_e = R_e^{SAT} \cdot e^{K_{R_e} \cdot S} \quad (3)$$

$$D_W = D_W^{SAT} \cdot e^{K_{D_W} \cdot S} \quad (4)$$

where S ($12 < S < 88$) is the percentage of decrease in T_V (percentage of solubilization).

4. Discussion

Results indicate that five molecules of SDS per molecule of Chl interacted with the membrane prior to its solubilization. This value is twice or 5-fold those found for the mixture of detergents OG/DM/LiDS or Triton X-100, respectively. The number of detergent molecules which bind to the membrane components during solubilization (K_{R_e}) was negligible in the SDS-treatment but very high in the solubilization by OG/DM/LiDS or Triton X-100. As a consequence, R_e^{SOL} values (mol/mol) of 9, 68 and 93, respectively, were observed (Table 1). This difference could be related to the anionic nature of SDS, whose interactions with the membrane prior to solubilization would be initially favoured by the positive charges of proteins and then avoided after saturation by the incorporated negative charges. In fact, the mixture of anionic and non-ionic detergents OG/DM/LiDS showed R_e^{SAT}, R_e^{SOL} and K_{R_e} values between the anionic SDS and the non-ionic Triton X-100 (Table 1). R_e^{SOL} values (in a mass ratio) were correlated with the difference in T_{min} of solubilized samples (Fig. 1). Thus, this difference in size could be a consequence of differences in the effective detergent/Chl ratio that formed mixed micelles with the cyanobacterial membrane components.

In photosynthesis research, solubilization conditions are routinely expressed as detergent/Chl (w/w). This approaches to the R_e (w/w) value at high Chl concentrations (Eq. (1)). Using an SDS/Chl (w/w) ratio of 10, cyanobacterial thylakoids can be solubilized immediately at 20°C [21] or after 30 min at 0–4°C [3]. However, we found a ratio of 3. This difference can arise from the temperature or time used for sample equilibration, since SDS critical micellar temperature ranges between 10 and 23°C [3]. In the thylakoid of the cyanobacterium *Synechococcus elongatus*, a Triton X-100/Chl (w/w) ratio of 25 solubilized 75–80% of the total Chl when samples were equilibrated for 30 min at 0–4°C [22]. If the parameters described in Table 1 for the solubilization by Triton X-100 were substituted in Eq. (3), a very close R_e value of 20–25 (w/w) for a 75–80% solubilization of the thylakoid membrane of *P. laminosum* could be obtained. Finally, 95–100% of thylakoid membranes from *C. reinhardtii* were solubilized by the OG/DM/LiDS mixture at an R_e (w/w) ratio of 22, when incubated for 30 min in an ice-bath [10]. This value is close to the R_e value of 23–27 (w/w) obtained

when this percentage and the corresponding parameters (Table 1) were substituted in Eq. (3). In the case of Triton X-100 and the mixture OG/DM/LiDS, the effect of temperature would be less important since the critical micellar temperatures of non-ionic surfactants are below 0°C [3].

During solubilization of model membranes and the purple membrane of *Halobacterium halobium*, D_W^{SAT} was similar to D_W^{SOL} and showed a value comparable to the cmc of the detergents in aqueous solutions [4–7]. In contrast to these systems, during solubilization of thylakoid membrane fragments, D_W values increased exponentially from D_W^{SAT} to D_W^{SOL}, and reached values similar to those described for the cmc of the detergents employed. In the solubilization of thylakoid membrane fragments, D_W^{SOL} for SDS and Triton X-100 were, respectively, 3.1 and 0.4 mM (Table 1). These detergent concentrations were in the range described for the cmc value of SDS (from 1 to 10 mM) and Triton X-100 (from 0.2 to 0.9 mM) [3,9]. The cmc for the mixture of detergents OG/DM/LiDS (in a molar ratio of 4.19:2.54:1), determined by the fluorescence dye method in a similar buffer, was 2.8 mM [10]. This value is close to the one obtained in this work (3.18 mM) for D_W^{SOL}.

As in the solubilization of the Semliki Forest virus membrane by SDS [3] and of higher plant thylakoid membranes by Triton X-100 and SDS, solubilization could start at a detergent concentration substantially below the cmc [20]. In this work, as in the solubilization of this viral membrane, the values of D_W increased whereas that of R_e remained almost constant in a range of detergent concentrations (Fig. 4), indicating saturable binding at low SDS concentrations.

The ratio R_e/D_W corresponds to the partition coefficient of the detergent among the membrane, mixed aggregates, vesicles or micelles and the aqueous media. When the smaller decrease in T_V was measured (i.e., 1%) this ratio ($R_e^{1\%}/D_W^{1\%}$) would approach the partition coefficient of the detergent between the thylakoid membrane fragments and the aqueous medium (Table 1). Whereas this partition coefficient was 48 M^{-1} for SDS, the calculated ones for Triton X-100 and the mixture of OG/DM/LiDS were smaller and not completely reliable. However, in the so-defined saturating and solubilizing concentrations [19], SDS partition coefficient decreased whereas those of Triton X-100 and OG/DM/LiDS increased (Table 1). This result indicates that the initial binding of monomeric detergent molecules was responsible for a further, cooperative binding of additional molecules of Triton X-100 and OG/DM/LiDS, but avoided inclusion of SDS molecules in aggregates or mixed micelles.

The partition coefficients R_e^{SAT}/D_W^{SAT} can give us an idea about the feasibility of removing the detergent from membrane components, and R_e^{SOL}/D_W^{SOL} permits us to estimate the feasibility of removing the detergent from mixed micelles. Results indicate that the more appropriate detergent to solubilize thylakoid membrane components was the mixture OG/DM/LiDS, since it showed the lower $R_e^{1\%}/D_W^{1\%}$ ratio, and hence the lower affinity for the membrane components. R_e^{SOL}/D_W^{SOL} was also smaller for this mixture than for Triton X-100, indicating that the former could be removed easier.

Our results support the membrane saturation model described by Lichtenberg [9] as a plausible mechanism for the solubilization of thylakoid membrane fragments since D_W^{SAT} was much lower than cmc. However, this result does not rule out the alternative water saturation mechanism, as cmc is not a single detergent concentration but, rather, a narrow concentration range [3]. The similarity among detergents in the water progression constant could indicate the formation of micelles at increasing detergent concentrations in the aqueous phase.

Taking into account our results, Eq. (1) must be modified to describe the solubilization of thylakoid membrane fragments. From Eqs. (3) and (4), the D_T required to attain a given percentage of solubilization (S) of thylakoid membrane fragments can be written as:

$$D_T = D_W^{SAT} \cdot e^{K_{D_W} \cdot S} + [Chl] \cdot R_e^{SAT} \cdot e^{K_{R_e} \cdot S} \quad (5)$$

This relationship permits us to obtain the same solubilization at different Chl concentrations and it can be extremely valuable when a procedure designed for abundant material must be adapted to small samples, such as in the radioactively labeled membrane components.

Selective solubilization has proved to be especially valuable in the purification of membrane proteins and active membrane components [3]. In the selective extraction of a particular protein or group of proteins by partial or complete solubilization of membranes, followed by biochemical separation, it is advisable to know both D_W and R_e values which permit a given solubilization. D_W must be maintained constant throughout the purification procedure to avoid undesired aggregation or precipitation of membrane components. This concentration is usually slightly above the cmc [2]. Our results indicate that this procedure is correct when the studied component required a complete solubilization of thylakoid membrane fragments. However, it can exceed the required detergent concentration when such a component is extracted by partial solubilization. In this case, D_W for the extraction would be lower than cmc. If this sample is diluted with buffer containing detergent at the corresponding cmc, undesired denaturation of the component can be provoked. This often occurs when samples are diluted during molecular sieve chromatography or when purified concentrated samples are diluted for spectroscopic characterization.

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