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THE INTERACTION OF SURFACTANTS WITH THE CHLOROPLAST THYLAKOID MEMBRANE AT SUB-SOLUBILIZING CONCENTRATIONS

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The interactions of nonionic, anionic and zwitterionic surfactants with the chloroplast thylakoid membrane of wheat (*Triticum aestivum*) were examined. Measurements of the surface tension of the solution and behavior of the membrane material during centrifugation were used to identify solubilizing concentrations of the surfactants. Studies at sub-solubilizing concentrations indicated that the primary interaction between the surfactant and the thylakoid membrane may involve adsorption at the membrane/solution interface rather than insertion of surfactant molecules into the membrane. This hypothesis was supported by a lack of increase in the fluorescence yield of chlorophyll at sub-solubilizing surfactant concentrations, indicating that interactions between the photosynthetic pigment-protein complexes are not extensively disrupted. Difference spectroscopy revealed that long-wavelength (greater than 690 nm) chlorophyll molecules are perturbed by the surfactant. We conclude that at sub-solubilizing concentrations, surfactants specifically interact with exposed Photosystem I components on the surface of the thylakoid membrane. It appears that use of sub-solubilizing concentrations of surfactants has the potential to provide much useful information about the structure and organization of the thylakoid membrane.

Introduction

The utilization of surfactants to study the organization and constituents of biological membranes is a routine technique [1]. Much of our knowledge about the molecular architecture of the higher plant photosynthetic membrane is derived from studies using surfactants. Differential solubilization of the chloroplast thylakoid membrane has been used to probe the lateral distribution of photosynthetic components (e.g., see Refs. 2–5) and has contributed to the laterally segregated model for the photosynthetic membrane [6]. Also useful has been the more general solubilization of the thylakoid membrane with anionic or mixed anionic and nonionic surfactants followed by electrophoretic fractionation of the resulting pigment-protein complexes (e.g., see Refs. 7 and 8). However, in all of these studies, the use of surfactants is empirical. The optimal type or species of surfactant for the production of a given effect is generally not predictable in advance. The membrane concentrations used are usually high and under these conditions the ratio of surfactant to membrane may be more important than the surfactant concentration [1]. Membranes bind a significant amount of added surfactant, thereby altering the monomeric concentration in the bulk phase.

Because of the nature of the various chemical groups which comprise the surfactant molecules, there is the potential for specific interaction with

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Abbreviations: Chl, chlorophyll; PS, photosystem; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

particular membrane components or domains. As an example, the interaction of surfactants with the negatively charged [9] thylakoid membrane may depend on the ionic nature of the surfactant molecules [10]. We have previously suggested [11] that better understanding of the interaction of surfactants with the thylakoid membrane would greatly increase their usefulness as specific tools for the study of its supramolecular organization.

This study examines the interaction of several surfactants with low concentrations of thylakoid membrane. Use of low membrane concentrations is intended to: (i) minimize the alteration in the bulk phase concentration of monomeric surfactant molecules; (ii) facilitate spectrophotometric and spectrofluorometric analyses of the membrane pigments; and (iii) permit the surface tension of the solution to be used as a criterion for surfactant binding and for predicting the concentration by which solubilization should have occurred. To our knowledge, the only other systematic study conducted on low concentrations of thylakoid membranes was that of Hoarau and Remy [12]. These authors observed differential effects of high and low concentrations of Triton X-100 on changes in the chlorophyll difference spectra. We corroborate these findings and provide evidence that the effect observed at sub-solubilizing concentrations of surfactants is due to the interaction with surface-exposed Photosystem I components.

Materials and Methods

Wheat plants (*Triticum aestivum* var. Brule) were grown for 7–12 days in a growth chamber. Leaves were harvested, washed with an ice/water mixture, and ground in a Waring blender using an ice-cold solution of 0.4 M sorbitol, 10 mM NaCl and 25 mM Na^+-Tricine (pH 7.6). All further steps until the addition of surfactant, were carried out at 0–4°C. The brei was filtered through four layers of Miracloth (Calbiochem) and centrifuged at $3000 \times g$ for 1 min. The pellet was suspended in 50 mM Tris-HCl (pH 8.0), disrupted with a Tenbroeck homogenizer, and centrifuged at 30000 $\times g$ for 10 min. The membranes in the pellet were washed and pelleted again as above. The membranes were then resuspended and homogenized in 50 mM Tris-HCl (pH 8.0) and an aliquot was removed for chlorophyll determination. The membrane suspension was diluted to twice the chlorophyll concentration desired in the final incubation with surfactant. At this stage the membranes had approx. 7 mg protein/mg Chl.

To study the interaction of surfactants with the thylakoid membrane, 6 ml of surfactant solution in 50 mM Tris-HCl (pH 8.0) were added to 6 ml of membranes, both being twice the desired final concentration. The mixture was incubated at 25°C for 10 min prior to analysis. All surfactant solutions were prepared on a $g \cdot ml^{-1}$ basis. Absorption spectra normally were determined using a Varian DMS 90 spectrophotometer. Difference spectra were determined with a Cary 219 spectrophotometer interfaced to an Apple II computer using a 1 nm band pass and scanning at a rate of 1 $nm \cdot s^{-1}$. Surface tension was measured with a Du Nouy tensiometer. Fluorescence emission was determined with a Perkin Elmer MPF 44A spectrofluorometer; the excitation was at 445 nm with a 4 nm band pass and emission was monitored with a 2 nm band pass. Solubilization was determined by centrifuging the surfactant/thylakoid mixtures ($300000 \times g$, 20 min) at 15°C and measuring chlorophyll in the supernatant fraction.

Concentrations of chlorophyll were determined by the method of Arnon [13]. Protein was measured by the procedure of Lowry et al. [14] as modified by Dulley and Grieve [15], except that color development was monitored at 750 nm to avoid interference from chlorophyll. Buffers, SDS, Nonidet P-40 and the Triton series of surfactants were purchased from Sigma Chemical Co., St. Louis, MO. The Zwittergent series of surfactants were purchased from Calbiochem-Behring Corp., La Jolla, CA. Magnesium dodecyl sulfate was the generous gift of Alcolac, Inc., Baltimore, MD.

Results and Discussion

Addition of increasing amounts of surfactants to an aqueous solution results in a decrease in the surface tension at the air/liquid interface until the critical micelle concentration is reached. In the presence of membranes, the surface tension versus surfactant concentration plot will be displaced toward higher surfactant concentrations. Such an experiment is shown in Fig. 1A using the surfac-

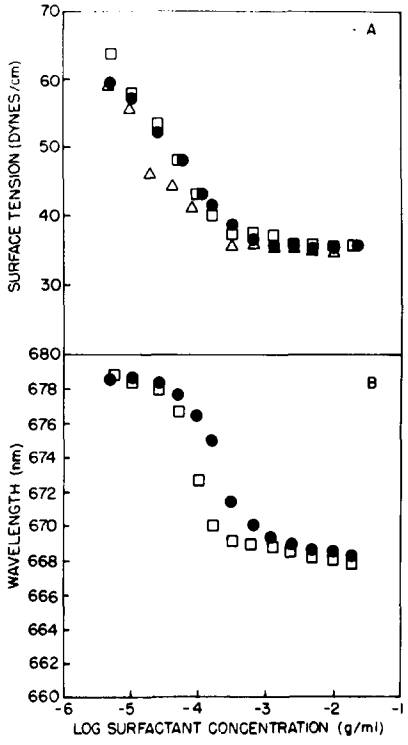

Fig. 1. Effect of added Triton X-100 in the absence and in the presence of two concentrations of thylakoid membranes. (A) Effect of surfactant concentration on the solution/air interface surface tension. (B) Effect of surfactant concentration on the wavelength of maximal chlorophyll absorption. Membranes were added to a final concentration of 0 (△), 6 (□) or 30 (●) μg Chl·ml^{-1}.

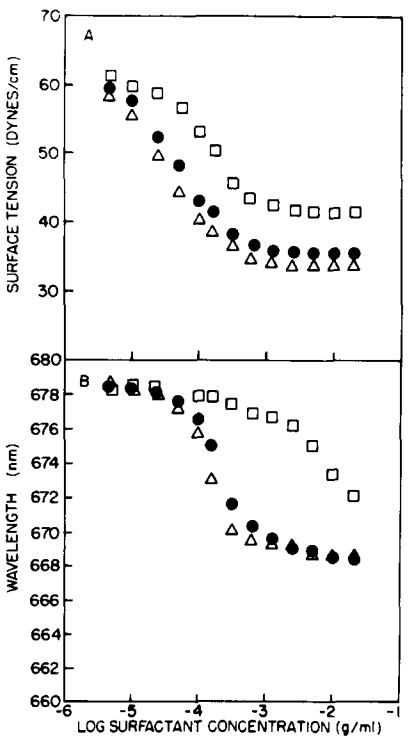

Fig. 2. Effect of increasing concentrations of Triton series surfactants on surface tension (A) and wavelength of chlorophyll absorption maximum (B). Membrane concentration was 30 μg Chl·ml^{-1}. (△) Triton X-114, (●) Triton X-100, (□) Triton X-165.

tant Triton X-100. Because membranes remove surfactant molecules from the bulk phase of the solution, membrane concentration can have an effect on the studies. This is shown in Fig. 1B where the decreases in the wavelength of the maximal absorption by chlorophyll in the red region of the visible spectrum depends on membrane concentration. All of the chlorophyll in the thylakoid membrane exists bound to proteins in the form of specific pigment-protein complexes [7,8]. The wavelength of chlorophyll absorption in the red region of the visible spectrum depends to a large extent on the polarity of its immediate environment [16]. Furthermore, the in vivo chlorophyll absorption spectrum is a composite of a number of different spectral forms, each representing chlorophyll molecules in a different, unique environment [17]. As the thylakoid membrane and its constituent pigment-protein complexes are perturbed, the resulting blue shifting of the chlorophyll absorption spectrum in the red spectral region is due to the perturbation of individual chlorophyll spectral forms [17]. The differential susceptibility of particular pigment-protein complexes to membrane-disrupting agents can result in the alteration of specific spectral forms. In the present study, we attempted to control carefully the concentration of membranes and utilize them at sufficiently low concentration that we can observe effects due to surfactant concentration, as well as the stoichiometry of surfactant to membrane.

Using three members of the Triton series of surfactants, we examined the effect of differing hydrophile-lipophile balance [1,18] on the interaction of surfactants with the thylakoid membrane (Fig. 2). These Triton surfactants are a series of

nonionic compounds with similar structures that have increasing lengths of polymerized ethylene oxide (a hydrophilic group) in the order Triton X-114 < Triton X-100 < Triton X-165. As can be seen, the nature of the surfactant strongly influenced the decrease in the surface tension of the solution and the perturbation of the chlorophyll absorption spectrum at any given surfactant concentration, as well as in the maximal extent to which the surface tension could be decreased.

An ideal plot of log surfactant concentration versus surface tension at concentrations below the critical micelle concentration should approximate a straight line. However, in the presence of membranes (Fig. 2) this is not the case; at the lowest concentrations the points depart from linearity. This departure represents concentrations where binding to the membrane's high-affinity sites results in nonproportionality of monomeric surfactant molecules with increasing surfactant concentration [19]. As these sites are saturated, the relationship becomes linear [19]. The region of the plots in which increasing surfactant concentrations no longer decrease the surface tension can be extrapolated to intersection with the proportional region to approximate a value for the critical micelle concentration. This value will not be a true critical micelle concentration because of the formation of mixed micelles between the surfactant and the membrane components, but is useful in assessing the concentration of any particular surfactant by which the membrane structure will be disrupted and the constituent components will be solubilized. The approximate critical micelle concentration values obtained with our specific membrane and buffer concentrations are shown in Table I for a number of surfactants. Lowering the membrane concentration did not decrease the approximate critical micelle concentration for any of the surfactants examined, indicating that the membrane concentration was sufficiently low to prevent differences due to binding large amounts of

TABLE I

APPROXIMATE CRITICAL MICELLE CONCENTRATION OF SURFACTANTS MEASURED IN THE PRESENCE OF THYLAKOID MEMBRANES AT A CHLOROPHYLL CONCENTRATION OF 30 $\mu g \cdot ml^{-1}$

Surfactant	Critical micelle concentration ($mg \cdot ml^{-1}$)
Nonionic	
Triton X-165	0.45
Triton X-100	0.36
Triton X-114	0.28
Nonidet P-40	0.32
Anionic	
Magnesium dodecyl sulfate	0.53
SDS	1.5
Zwitterionic	
Zwittergent 3-10	15
Zwittergent 3-12	0.49
Zwittergent 3-14	0.39
Zwittergent 3-16	0.34

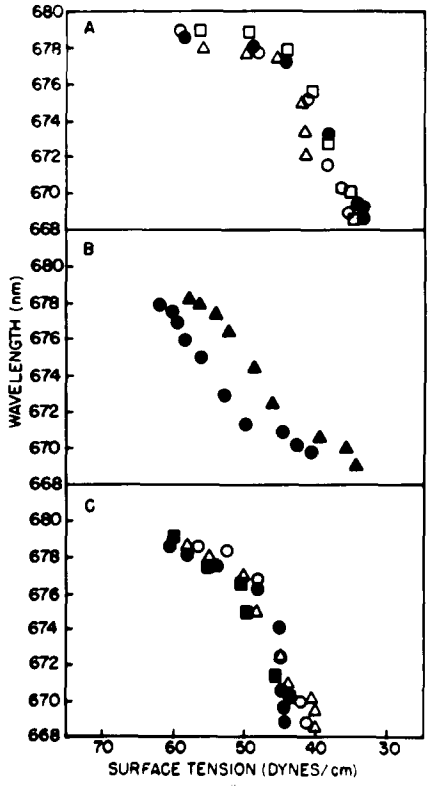

Fig. 3. Correlation between wavelength of maximal chlorophyll absorption and solution surface tension for nonionic (A), anionic (B) and zwitterionic (C) surfactants at a membrane concentration of 30 μg Chl $\cdot ml^{-1}$. (A): (□) Nonidet P-40, (●) Triton X-114, (○) Triton X-100, and (△) Triton X-165. (B): (△) magnesium dodecyl sulfate and (●) SDS. (C): (△) Zwittergent 3-16, (○) Zwittergent 3-14, (●) Zwittergent 3-12 and (■) Zwittergent 3-10.

monomeric surfactant. Two cationic surfactants, dodecyltrimethylammonium bromide and tetradecyltrimethylammonium bromide, were also employed in these studies, but at concentrations between 0.2 and 0.7 mg · ml^{-1} the membranes would aggregate and precipitate from the solution. Therefore, only data using anionic, nonionic and zwitterionic surfactants are reported.

Since the different surfactants in the Triton series differ in hydrophile-lipophile balance, the extent to which they will partition into the membrane from the bulk phase will differ. If the perturbation of the chlorophyll porphyrin rings is due to the insertion of surfactant molecules into the membrane, then the magnitude of change in the wavelength of maximum absorbance as a function of surface tension would be different for the three surfactants. Alternatively, if the shifts are due to perturbations at the membrane surface or changes in the bulk solution, then the changes in the wavelength of chlorophyll absorption maximum would correlate with the change in surface tension regardless of surfactant nature within a particular series. Fig. 3 shows the relationship between change in the wavelength of maximal chlorophyll absorption and surface tension for a number of surfactants. For the nonionic surfactants, Triton X-100, Triton X-114, Triton X-165 and Nonidet P-40, the change in chlorophyll maximal absorption is apparently a function only of surface tension. The Zwittergent series of zwitterionic sulfobetaines also show a similar relationship between surface tension and the chlorophyll absorption maximum (Fig. 3C). The fact that the Zwittergent series produced a greater relative change in the position of the chlorophyll absorption maximum for a given surface tension may be due to a greater chemical activity at the membrane/solution interface due to its charged nature.

Interaction of surfactants with the membrane surface could explain why cationic surfactants caused thylakoid aggregation. This interpretation would also be consistent with the differences observed between the sodium and magnesium salts of dodecyl sulfate (Fig. 3B). Though the critical micelle concentration of magnesium dodecyl sulfate is lower than that of SDS [19,20], which should increase perturbation of the chlorophyll molecules, the divalent cation appears to protect the pigment-protein complexes from environmental changes. The thylakoid membrane has a net negative charge at neutral pH and charged species in the solution will be attracted to the interface, forming an electrical double layer [19–24]. Mg^{2+} causes a reorganization of components within the membrane into regions of high or low surface charge density at low concentrations [4,23,24]. The magnesium-induced reorganization apparently reduces the sensitivity of the chlorophyll molecules within the pigment-protein complexes to changes in surface tension. Mg^{2+} dramatically reduces the ability of Triton X-100, a nonionic surfactant, to disrupt the thylakoid membrane [25]. In the particular pigment-protein complexes which were being perturbed by sub-solubilizing concentrations of surfactant migrated to regions of greater surface charge density during membrane reorganization, they would be less susceptible to surfactants. The regions of greater surface charge density are thought to be enriched in PS I components [24]. Another possibility is that membrane reorganization changes the exposure of the various pigment-protein complexes to the aqueous environment or to the membrane interior [26].

While the perturbation of chlorophyll molecules within the pigment-protein complexes is a sensitive indicator of change in the local environment, the amount of information that can be obtained from any one such criterion is limited. The ability of membrane components to remain in the supernatant fraction following centrifugation was used as an operational measure of solubilization while shifts in the wavelength of fluorescence emission and increases in the relative yield of chlorophyll fluorescence served as indications of intercomplex and intracomplex disruptions among the pigment-protein complexes. For these studies the membrane concentration was further reduced to 6 $\mu g \cdot ml^{-1}$, sufficiently diluted to obviate fluorescence artifacts caused by self-absorption. The two most commonly used surfactants for these types of studies, SDS and Trition X-100, were employed.

The effect of Triton X-100 and SDS concentration on solubilization is shown in Fig. 4. The amount of membrane remaining in the supernatant fraction after centrifugation at 300 000 × g for 20 min is nearly maximal at the apparent critical micelle concentration. Solubilization begins

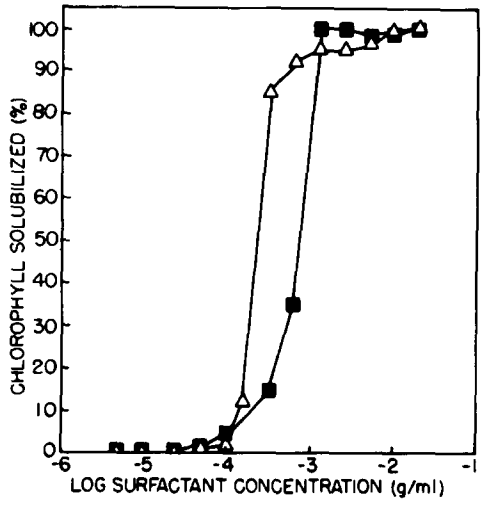

Fig. 4. Effect of surfactant concentration on the solubilization of the thylakoid membrane. Amount of total chlorophyll remaining in the supernatant fraction following centrifugation at 300000 × *g* for 20 min is shown versus surfactant concentration. Thylakoid membrane concentration was 6 μg Chl · ml^{-1}. Surfactants used: (△) Triton X-100, (■) SDS.

with formation of mixed micelles at surfactant concentrations somewhat less than the critical micelle concentration [1]. With both surfactants, there is little apparent solubilization at concentrations of 10^{-4} g · ml^{-1} or less. Between this concentration and the approximate critical micelle concentration values of $3.6 \cdot 10^{-4}$ g · ml^{-1} for Triton X-100 and $1.5 \cdot 10^{-3}$ g · ml^{-1} for SDS, membrane solubilization increased.

It seems reasonable that below 10^{-4} g · ml^{-1} of either surfactant gross membrane structure is little affected. In spite of this apparent retention of membrane structure, the data in Fig. 1B, using an identical membrane concentration, indicate that Triton X-100 at or below 10^{-4} g · ml^{-1} strongly affects the environment near some of the pigment-protein complexes. In some other membrane systems examined [1], the initial high-affinity binding of surfactant results in an insertion of surfactant molecules into the membrane bilayer which significantly increase membrane area. If this were the case in the thylakoid membrane, inter-complex interactions between the constituent pigment-protein complexes should be disrupted, increasing chlorophyll fluorescence yield. As seen from comparing Figs. 4 and 5B, increases in the yield of chlorophyll fluorescence appear with the onset of solubilization, and not at sub-solubilizing surfactant concentrations. The shift in the wavelength of maximal fluorescence emission (Fig. 5A) appears to be minimal at sub-solubilizing concentrations, and then changes markedly as solubilization proceeds in a manner similar to the change in fluorescence yield.

Because the major portion of the fluorescence being monitored in Figure 5 originates from the light-harvesting complex and PS II, it is possible that changes in PS I would not be revealed. Furthermore, since the pigment-protein complexes of PS I absorb light of longer wavelengths than the average light-harvesting complex or PS II complexes [27], a shifting of these components to shorter wavelength due to an altered environment might produce a proportionally greater change in the composite absorption spectrum. Difference

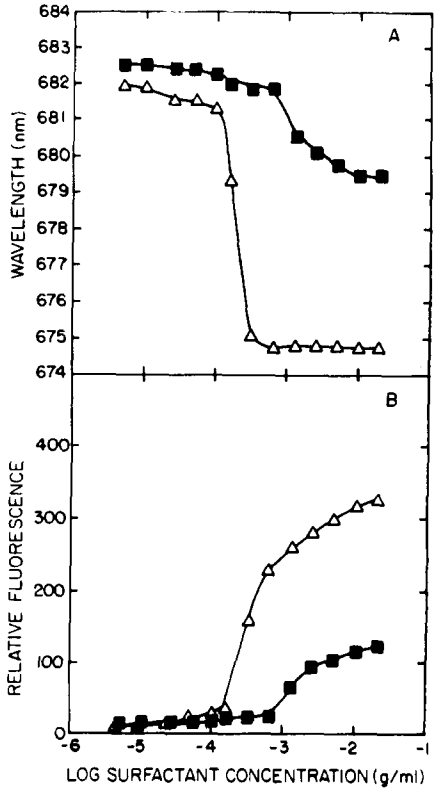

Fig. 5. Effect of surfactant concentration on the wavelength of maximal fluorescence yield (A) and the relative yield of fluorescence (B). Thylakoid membrane concentration was 6 μg Chl · ml^{-1}. Surfactants used: (△) Triton X-100, (■) SDS.

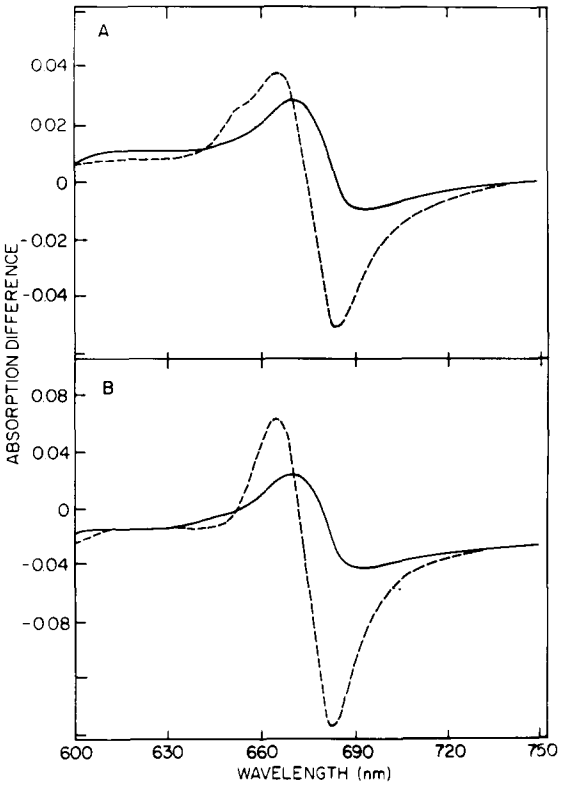

Fig. 6. Effect of sub- and super-solubilizing surfactant concentration on Chl absorption. Thylakoid membranes were used at a concentration of 6 μg Chl·ml^{-1}. Data are shown as difference spectra of surfactant-treated minus untreated samples. Surfactants used: (A) SDS at 0.1 mg·ml^{-1} (solid line) or 1.25 mg·ml^{-1} (dashed line); (B) Triton X-100 at 0.05 mg·ml^{-1} (solid line) or 0.32 mg·ml^{-1} (dashed line).

spectra (surfactant-treated minus untreated) were obtained with the same samples used for the fluorescence studies (Fig. 6). The shape of the difference spectra were similar for both surfactants at all concentrations below the apparent critical micelle concentration but changed to a second characteristic shape at concentrations above the apparent critical micelle concentration. At the lower concentrations, the minimum in the difference spectra, indicating the greatest loss of long-wavelength spectral form, occurred at 694–695 nm with SDS and 691 nm with Triton X-100. The maximum in these spectra, indicating the increase in shorter-wavelength spectral form, occurred at 671–674 nm; the increase in absorption at this wavelength was always greater than the decrease in absorption of the longer-wavelength form, indicating accompanying changes in the oscillator strength of chlorophyll chromophore. At surfactant concentrations above the apparent critical micelle concentration, the difference spectra show maxima and minima of approximately equal magnitude. The maxima and minima occur at approx. 666 and 685 nm, respectively, for both surfactants. This agrees with the report of Hoarau and Remy [12] who investigated the effects of Triton X-100 on the thylakoid membranes of *Nicotiana tabacum*. These authors also reported that if the membranes were first disrupted by French press treatment, the characteristic low concentration spectral changes were not observed, further suggesting that these changes are due to the interaction of surfactant molecules with the membrane surface. Alternatively, the French press treatment may grossly change the arrangement of membrane components [28] which could account for the difference.

We consider that the most likely explanation for the above data is that the addition of low concentrations of surfactants to the thylakoid membrane can selectively perturb PS I components. There is a selective loss of long-wavelength spectral forms of chlorophyll (greater than 690 nm); these forms are usually ascribed to PS I. It has also been reported that surfactants may influence the primary donor of PS I, P-700 [29,30]. The interaction of sub-solubilizing concentrations of surfactant with the thylakoid membrane appears to be largely confined to the membrane/solution interface. This would explain the role of the ionic nature of the surfactant in the interaction [10]. The results also imply that the PS I components which are thus affected, be they photochemical center or antenna, are more exposed to the membrane surface that the other pigment-protein complex. More experiments will be necessary to demonstrate conclusively the selective effect of surfactants of sub-solubilizing concentrations on the PS I pigment-protein complexes.

As the surfactant concentration increases, the type of interaction between the membrane, or its components, and the surfactant changes. At low concentrations of surfactants there is a high-affinity interaction between monomeric surfactant molecules and the membrane, occurring largely at

the membrane/solution interface. Surface-exposed pigment-protein complexes, very likely components of PS I, appear to be susceptible to surfactant-mediated change in environment. As the surfactant concentration increases still further, significant amounts of surfactant are incorporated into the membrane, disrupting intercomplex interactions and changing chlorophyll fluorescence. Still further increases in surfactant produce micelles with a higher proportion of the surfactant and result in solubility. However, the intracomplex interactions between pigment and protein may survive incorporation into surfactant micelles; solubilization of thylakoid membranes with SDS does not necessarily denature pigment-protein complexes and form free chlorophyll molecules [31]. Thus, it appears that sub-solubilizing concentrations of surfactants have the potential to provide much useful information about the structure and organization of the thylakoid membrane.

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