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Effect of Alterations in the Amphipathic Microenvironment on the Conformational Stability of Bovine Opsin. 1. Mechanism of Solubilization of Disk Membranes by the Nonionic Detergent, Octyl Glucoside†

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ABSTRACT: The mechanism of solubilization of the bovine retinal rod outer segment disk membrane by the nonionic detergent, octyl β-D-glucoside, was investigated. Saturation of the membrane by detergent occurred at a level of 1.4 mol of detergent bound per mol of disk membrane phospholipid. Increasing the detergent concentration beyond the level of membrane saturation resulted in the release of rhodopsin–lipid–detergent complexes containing 25–50 mol of phospholipid and 270 mol of octyl glucoside per mol of rhodopsin. The composition of the phospholipids in these initially solubilized complexes was identical (±5%) with that of the native disk membrane, suggesting that rhodopsin does not interact preferentially with any one of the three major classes of phospholipids present in the disk membrane. Direct binding measurements showed that the amount of phospholipid contained in the solubilized rhodopsin–lipid–detergent complexes ranged from 25 mol/mol of rhodopsin in the initial stage of solubilization to 50 mol/mol of rhodopsin at the point of complete disk membrane solubilization. At detergent concentrations above the fully solubilizing level, the amount of rhodopsin-associated phospholipid decreased with increasing detergent concentration. The distribution of phospholipid in the system was as expected for partitioning of phospholipid between the rhodopsin–lipid–detergent complexes and rhodopsin-free lipid–detergent micelles.

The integral membrane proteins, by definition, exist in close association with phospholipid and derive many of their functional and structural properties from this association. This is evidenced by the fact that the activities of membrane-bound enzymes are usually lost when the phospholipid is removed (Helenius and Simons, 1975; Tanford and Reynolds, 1976). In order to better understand the way in which phospholipid acts to stabilize the native conformation of integral membrane proteins, we have measured the changes in the conformational stability of bovine opsin which occur when the native pro-

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tein-lipid interactions of the retinal rod outer segment disk membrane are disrupted by exposure to increasing concentrations of a nonionic detergent.

The disk membrane is well suited to such a study because it contains predominantly the integral membrane protein, rhodopsin (Papermaster and Dreyer, 1974). This protein exists in two forms, unbleached (rhodopsin) and bleached (opsin), which differ markedly in their thermal stability properties (Hubbard, 1958). Rhodopsin can be solubilized and completely delipidated without suffering irreversible changes in its properties (Hong and Hubbell, 1973). Therefore, the composition of the phospholipid-detergent microenvironment of solubilized rhodopsin can be studied while the protein remains in a stable conformation, the major structural features of which likely resemble those of the native state. In contrast, opsin denatures at room temperature in most detergent solutions, as evidenced by its loss of helical secondary structure and regenerability (Stubbs et al., 1976). The existence of these two states of the protein makes it possible to correlate changes in the denaturation rate of solubilized opsin with changes in the composition of the amphipathic microenvironment of the protein measured with unbleached disk membranes under identical conditions of solubilization.

The detergent employed in this study to perturb the native protein-lipid interactions of the disk membrane, octyl β-D-glucoside, was found in a previous study (Stubbs et al., 1976) to be capable of rapidly solubilizing disk membranes and completely delipidating rhodopsin. It was also shown that this detergent did not denature a soluble protein (metmyoglobin) in contrast to several other detergents, suggesting that its effects on membrane protein stability may be entirely attributed to the disruption of the native membrane structure. In addition, octyl glucoside has the technical advantage of being a pure compound in contrast to the more commonly used nonionic detergents, which consist of mixtures of chemical species.

In this paper we describe the changes in the composition of the microenvironment of rhodopsin resulting from the exposure of disk membranes to a wide range of detergent to membrane ratios. The following paper (Stubbs and Litman, 1978) shows how these changes in the amphipathic microenvironment affect the thermal stability of opsin.

Methods

Disk Membrane Preparation. Disk membranes were prepared from frozen bovine retinas (Hormel) by the Ficoll flotation method of Smith et al. (1975). The disk membranes were washed twice and resuspended in buffer consisting of 0.05 M Tris, 0.05 M sodium acetate, and 0.2 M potassium chloride adjusted to pH 7 by addition of HCl. When solubilized in octyl glucoside the resulting disk membranes typically had an A_{280}/A_{500} ratio of 2.3 and contained 70 to 80 mol of phospholipid per mol of rhodopsin. Phospholipid was assayed by the method of Bartlett (1959). The rhodopsin concentration was measured by recording the difference in A_{500} produced by bleaching the sample solubilized in octyl glucoside with a high intensity microscope illuminator for 30 s in the presence of 0.09 M hydroxylamine. The molar extinction coefficient of rhodopsin at 500 nm was taken to be 40 000. All procedures were performed at room temperature (23 °C) under dim red light (Kodak safelight filter no. 1) unless otherwise stated.

Disk Membrane Solubilization. The detergent used, octyl β-D-glucoside, was prepared from acetobromo-α-D-glucose (Sigma) and 1-octanol (Fisher Scientific) by the method of Noller and Rockwell (1938) with modifications described by Baron and Thompson (1975). The amounts of rhodopsin and phospholipid solubilized from disk membranes by octyl glucoside were measured by adding aliquots of disks to solutions of octyl glucoside in Tris-acetate buffer (final volume, 1 mL). The samples were centrifuged 20 min at 100 000*g*, and the supernatants were analyzed for rhodopsin and phospholipid as described above.

Phospholipid Binding.[1] The amount of phospholipid remaining bound to rhodopsin in detergent solution was measured by the use of Sepharose 4B gel beads with covalently linked concanavalin A (Pharmacia). Rhodopsin has a carbohydrate moiety which binds to concanavalin A (Steinemann and Stryer, 1973), allowing the rhodopsin-lipid-detergent complexes to be separated from the phospholipid-detergent micelles[2] in solution. Disk membranes were added to octyl glucoside solutions and centrifuged 20 min at 100 000*g*. The final volume of each sample was 2.25 mL, and the final A_{500} was 0.32. A 1.0-mL aliquot of the supernatant was added to each of two desk top centrifuge tubes containing 0.4 mL of 15 mM octyl glucoside in Tris-acetate buffer and concanavalin A-Sepharose gel beads. The gel bed volume occupied 0.3 mL in each tube. In addition, one of the two tubes also contained 0.25 M α-methyl D-mannoside, and served as a control for nonspecific binding of lipid and protein to the gel. After adding the solubilized disk supernatant to the tubes containing the gel beads, the gel was suspended by vortexing and sedimented in a desk top centrifuge. The supernatant was removed from each tube, diluted 10% with 1.0 M neutralized hydroxylamine, and assayed for rhodopsin and phospholipid. The number of phospholipid molecules associated with each rhodopsin molecule, $\bar{\nu}$, was calculated as follows:

$$\bar{\nu} = \frac{P_m - P}{R_m - R}$$

where P and R are the supernatant phospholipid and rhodopsin concentrations, respectively, in the tubes which did not contain mannoside, and P_m and R_m are the corresponding concentrations for the samples which contained mannoside. Comparison of the concentrations of rhodopsin and phospholipid added with the concentrations measured in the mannoside-containing supernatants showed that no correction for excluded volume of the gel was needed. Significant levels of nonspecific binding of phospholipid and rhodopsin to the gel beads occurred only at low detergent concentrations and always accounted for less than 10% of the total binding. For samples with high detergent concentrations in which the disk membranes were fully solubilized, the 100 000*g* centrifugation step was eliminated, and the membranes, detergent, and gel beads were directly combined at the desired final concentrations.

Phospholipid Analysis. Disk membrane phospholipids were extracted and separated by two-dimensional thin-layer chromatography according to Anderson and Maude (1970). Spots corresponding to phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine were scraped from the plates and analyzed for phosphorus (Litman, 1973). The composition of the phospholipids solubilized with rhodopsin was inferred from the difference in phospholipid composition between native disk membranes and disk membranes from which approximately 50% of the rhodopsin and 25% of the phospholipid had been extracted by octyl glucoside.

[1] The term "bound" refers to the detergent and phospholipid present in the rhodopsin-containing complexes. No implication regarding the structure of these complexes is intended.

[2] The term "micellar" as used in this paper refers to the detergent present in any forms other than monomer in solution.

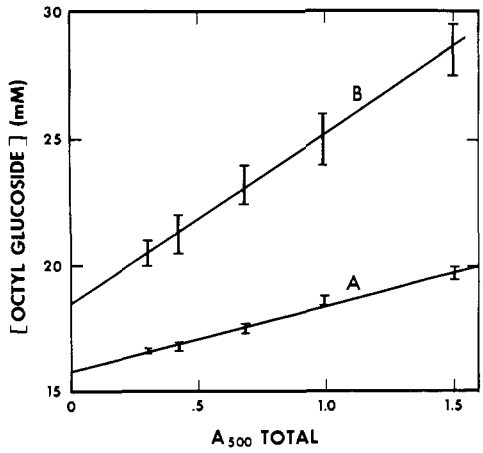

FIGURE 1: (A) The total octyl glucoside concentration corresponding to the onset of rhodopsin solubilization is shown as a function of the total rhodopsin concentration for samples containing five different levels of disk membranes. (B) The total octyl glucoside concentration needed to solubilize all of the rhodopsin in each of the disk membrane samples.

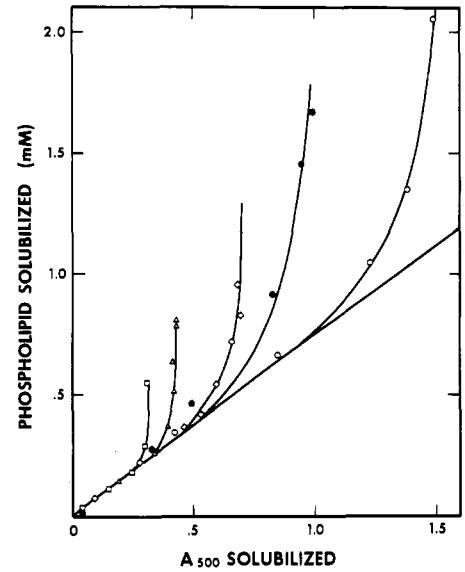

FIGURE 2: The amount of phospholipid solubilized vs. the amount of rhodopsin (A_{500}) solubilized for the five disk membrane samples of Figure 1. The total rhodopsin concentrations were (□) 7.6 μM; (△) 10.6 μM; (◇) 17 μM; (●) 24 μM; and (○) 37 μM.

Results

Rhodopsin Solubilization. The amount of rhodopsin solubilized from disk membranes by octyl glucoside was measured as a function of both the total octyl glucoside and disk membrane concentrations. No rhodopsin solubilization was observed until the total octyl glucoside concentration in each sample exceeded a threshold value. The threshold detergent concentration (which was generally below the critical micelle concentration of the detergent) increased as the total concentration of disk membranes in the sample increased, suggesting that some of the detergent was binding to the membranes prior to the onset of solubilization. These detergent levels mark the points at which the detergent binding capacity of the membranes became saturated; higher detergent levels resulted in solubilization of rhodopsin rather than additional binding of detergent to the disks. At detergent concentrations above the threshold, the amount of rhodopsin solubilized increased linearly with increasing octyl glucoside concentration initially, but gradually plateaued as the fraction of the rhodopsin solubilized approached 100%.

In Figure 1, curve A, the octyl glucoside concentrations corresponding to the onset of rhodopsin solubilization are plotted vs. the total rhodopsin concentration. From the slope of curve A the saturation level of detergent bound to the membranes was determined to be 106 ± 13 mol of octyl glucoside per mol of rhodopsin, or 1.4 ± 0.2 mol of octyl glucoside per mol of disk membrane phospholipid. Extrapolation of curve A (Figure 1) to zero membrane concentration gave a value of 15.7 ± 0.3 mM octyl glucoside, which may be interpreted to be the aqueous monomer detergent concentration in equilibrium with the saturated membranes. The ratio of the detergent concentration in the saturated disk membranes (mol per mol of phospholipid) to that in the aqueous phase (mol per mol of H_2O) yielded a partition coefficient for the distribution of octyl glucoside between the aqueous phase and the membranes of 4900 ± 700 mol of H_2O per mol of disk membrane phospholipid. Direct detergent binding measurements using the method of Roth and Seeman (1972) confirmed that this ratio of bound to aqueous detergent concentrations prevailed throughout the subsolubilizing range of octyl glucoside concentrations.

In Figure 1, curve B, the points represent the total octyl glucoside concentrations at which all of the rhodopsin in the disk membrane samples was solubilized. These points were derived from the beginning of the plateau regions of the solubilization curves. The slope of curve B (270 ± 30 mol of octyl glucoside per mol of rhodopsin) may be interpreted as the amount of detergent bound to the solubilized protein. The equation describing curve B (Figure 1),

$$[\text{octyl glucoside}] = 0.0185 + 270[\text{rhodopsin}] \quad (1)$$

where the brackets refer to total concentrations expressed in mol/L, gives the octyl glucoside concentration needed to solubilize all of the rhodopsin in any given disk membrane sample.

Solubilization of Disk Membrane Phospholipid. In addition to measuring the amount of rhodopsin solubilized in the disk membrane samples, the amount of phospholipid solubilized was also determined. In Figure 2 the amount of phospholipid solubilized is plotted vs. the amount of rhodopsin (A_{500}) solubilized. Phospholipid was initially released from the disk membranes with a stoichiometry of 30 mol per mol of rhodopsin, as evidenced by the initial slopes of the curves. This result suggests that the solubilized rhodopsin was located in protein-lipid-detergent complexes containing a complete boundary layer of phospholipid (Warren et al., 1974). After about 50% of the rhodopsin had been solubilized, additional detergent solubilized proportionally more phospholipid, until the phospholipid to rhodopsin ratio of the soluble supernatant was the same as that of the native disk membranes (~75 mol/mol). Analysis of the phospholipid composition of disk membranes before and after solubilization of a complex containing 50% of the rhodopsin and 25% of the phospholipid revealed no difference (±5%) in the relative proportions of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine present. Thus, the interaction of rhodopsin with these phospholipids in the presence of octyl glucoside appears to be nonspecific.

Amount of Phospholipid Bound to Solubilized Rhodopsin. In order to further define the microenvironment of rhodopsin in solubilized disk membranes, we performed direct measurements of the amount of bound phospholipid over a wide range of detergent to disk membrane ratios by the concanavalin A-Sepharose gel technique described under Methods. This technique allowed us to separate the rhodopsin-lipid-detergent complexes from the free lipid-detergent micelles in the solution

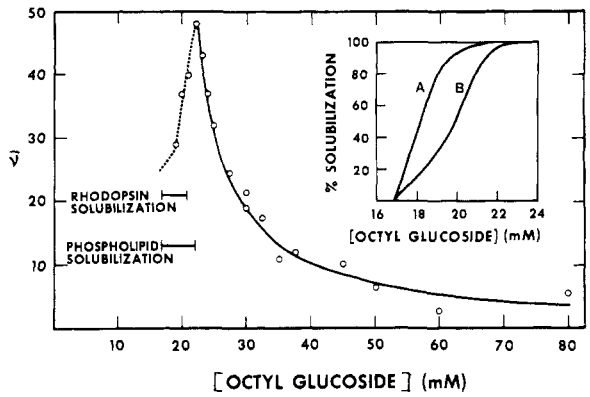

FIGURE 3: The amount of phospholipid bound to solubilized rhodopsin, $\bar{\nu}$ (mol/mol), vs. total octyl glucoside concentration for a disk membrane sample with $A_{500} = 0.32$. The solid line was fitted to the data in accordance with a partitioning model for the distribution of phospholipid in the system. The inset shows the corresponding rhodopsin (A) and phospholipid (B) solubilization curves.

without perturbing the dynamic equilibrium of the system. The resulting data are shown in Figure 3 along with an inset showing the rhodopsin and phospholipid solubilization curves. The initially solubilized rhodopsin was found to be associated with 25 to 30 mol of phospholipid per mol of rhodopsin. As solubilization progressed, the amount increased to a maximum of 50 mol per mol, which occurred at the detergent concentration corresponding to the point of complete disk membrane solubilization. In this range of detergent concentrations the major portion of the solubilized phospholipid was found to be associated with the soluble rhodopsin complexes.

At higher detergent concentrations, in which the membranes were completely solubilized, the amount of bound phospholipid fell gradually toward zero with increasing detergent concentration. It was possible to fit the data in this range of octyl glucoside concentrations with a theoretical curve, which is the solid line shown in Figure 3. The theoretical curve was derived from the following model. We assume that the rhodopsin-lipid-detergent complexes and the lipid-detergent micelles constitute two phases within which the phospholipid may be distributed. In a simple partitioning model, the ratio of the amounts of phospholipid in the two phases should be proportional to the ratio of the amounts of the two phases present. This proportionality is expressed by the equation

$$\frac{[\text{phospholipid}]_r}{[\text{phospholipid}]_f} = K \frac{[\text{rhodopsin}]}{[\text{micellar detergent}]_f} \quad (2)$$

where K is the distribution coefficient, $[\text{phospholipid}]_r$ and $[\text{phospholipid}]_f$ are the concentrations of phospholipid associated with the solubilized rhodopsin-lipid-detergent complexes and the lipid-detergent micelles, respectively, and $[\text{micellar detergent}]_f$ is that part of the total octyl glucoside concentration present in the lipid-detergent micelles. The value of $[\text{micellar detergent}]_f$ was estimated by subtracting the octyl glucoside concentration needed to solubilize all of the rhodopsin in the sample (calculated by eq 1) from the total octyl glucoside concentration. This calculation assumes that the monomer detergent concentration and the amount of detergent bound to the solubilized rhodopsin remained unchanged as the total detergent concentration was increased.

Equation 2 may be rearranged to give

$$\frac{1}{\bar{\nu}} = \frac{1}{K} \frac{[\text{micellar detergent}]_f}{[\text{phospholipid}]_{\text{total}}} + \frac{[\text{rhodopsin}]}{[\text{phospholipid}]_{\text{total}}} \quad (3)$$

where $\bar{\nu}$ is the average number of bound phospholipid molecules per solubilized rhodopsin molecule. According to eq 3, a plot of $1/\bar{\nu}$ vs. the ratio of $[\text{micellar detergent}]_f$ to $[\text{phospholipid}]_{\text{total}}$ should give a straight line with slope $1/K$ and an intercept equal to the rhodopsin to phospholipid molar ratio of the native disk membrane.

When the amount of bound phospholipid from the fully solubilized region of Figure 3 was plotted as suggested by eq 3 (figure not shown), the intercept of the resulting line corresponded closely to the rhodopsin to phospholipid molar ratio of the disk membrane in agreement with eq 3, and the slope of the line gave a value of $K = 400 \pm 50$ mol of octyl glucoside per mol of rhodopsin for the distribution coefficient in eq 2. The reciprocal of this fitted line is shown as the solid line in Figure 3. The agreement between the fitted line and the data points in Figure 3 shows that the partitioning model gives an adequate description of the amount of phospholipid bound to rhodopsin in fully solubilized disk membranes.

The value derived above for K in eq 2 indicates that, in the presence of 400 mol of free micellar octyl glucoside per mol of rhodopsin, the phospholipid in the solubilized disk sample was equally distributed between the rhodopsin-lipid-detergent complexes and the free lipid-detergent micelles. Figure 1, curve B, suggested that each solubilized rhodopsin molecule bound 270 molecules of octyl glucoside. If this level of detergent binding did not change appreciably at high detergent levels, the above value of K indicates that the rhodopsin-containing complexes had a slightly higher affinity for phospholipid than the rhodopsin-free micelles, arguing against ideal mixing of the detergent and phospholipid in the system.

Discussion

The aim of the present study was to correlate the effects of changes in the composition of the amphipathic microenvironment with changes in the conformational stability of rhodopsin. This report comprises the first half of the study, i.e., measurement of the amounts of detergent and phospholipid associated with rhodopsin as a function of the concentration of disk membranes and detergent. This information allows the opsin denaturation rate measurements presented in the following paper (Stubbs and Litman, 1978) to be interpreted with reference to the lipid-protein structures of defined chemical composition. The results presented here for the solubilization of disk membranes by octyl glucoside are in qualitative agreement with the mechanism of solubilization of other biological membranes by other detergents as summarized in the review by Helenius and Simons (1975). The present study is unique in that we have quantitatively evaluated the amounts of protein and phospholipid solubilized, the amount of phospholipid bound to the solubilized protein, and the effects of solubilization on the stability of the protein over a wide range of detergent to membrane ratios.

For a given concentration of disk membranes, three stages in the solubilization process were apparent depending on the detergent concentration: (1) partitioning of the detergent into the membranes at subsolubilizing detergent concentrations; (2) partial solubilization yielding a mixture of sedimentable membrane fragments and soluble, rhodopsin-lipid-detergent complexes; and (3) complete solubilization, accompanied by the gradual removal of phospholipid from the rhodopsin-lipid-detergent complexes as the detergent concentration was increased. The saturation level for octyl glucoside in disk membranes (defined by the onset of rhodopsin solubilization) was found to be 1.4 mol of octyl glucoside per mol of phospholipid, and the aqueous detergent concentration in equilibrium with the saturated membranes was 15.7 mM. Increasing the detergent concentration beyond the point of membrane saturation resulted in the release of soluble rhodopsin-lipid-

detergent complexes containing approximately 30 mol of phospholipid and 270 mol of octyl glucoside per mol of rhodopsin. Other membrane proteins have been observed to retain similar amounts of phospholipid upon solubilization (Warren et al., 1974). The composition of the phospholipids initially released from the disk membrane with rhodopsin was the same as that of the total disk membrane phospholipids, suggesting that the interaction of rhodopsin with the phospholipids in the membrane is nonspecific. The value of 270 mol of octyl glucoside per mol of rhodopsin determined for the binding of octyl glucoside to the solubilized complex agrees well on a mass basis with the Triton X-100 and dimethyldodecylamine oxide binding reported by others (Osborne et al., 1974; Sardet et al., 1976).

Direct phospholipid binding measurements showed that most of the phospholipid solubilized by octyl glucoside was incorporated into the soluble rhodopsin–lipid–detergent complexes at detergent concentrations where some unsolubilized membrane fragments were still present in the sample. In this range of detergent concentrations the amount of bound phospholipid increased from about 30 mol per mol of rhodopsin in the initial stages of solubilization to 50 mol per mol at the detergent concentration corresponding to complete membrane solubilization. At higher detergent levels the amount of bound phospholipid fell with increasing detergent concentration in a manner which was consistent with a partitioning model for the distribution of phospholipid between the rhodopsin–lipid–detergent complexes and the lipid–detergent micelles.

Two models of the structure of the solubilized rhodopsin–lipid–detergent complex may be imagined. In one case the phospholipid may be considered to interact directly with the protein as it does in the native membrane, while the detergent serves to shield the exposed hydrophobic surfaces from water. Alternatively, the phospholipid and detergent in the complex may mix ideally to form a uniform amphipathic phase which solvates the hydrophobic regions of the protein. Our measurements of the amount of phospholipid associated with the solubilized complexes do not allow a clear distinction to be made between these two models. However, several considerations indirectly favor direct binding of the phospholipid to the protein. The observation that 30 mol of phospholipid per mol of rhodopsin were associated with the initially solubilized protein suggests that the first boundary layer of phospholipid remains intact during solubilization. If this is correct, then the phospholipid likely interacts preferentially with the protein at higher detergent levels as well. In addition, the great difference in length between the acyl side chains of the phospholipids and the eight carbon hydrophobic tail of octyl glucoside makes it intuitively unlikely that these two species would mix ideally in the presence of the hydrophobic surface of the rhodopsin molecule. Finally, the value derived for the distribution coefficient in eq 2 suggests that the phospholipid in solubilized disk membrane samples is not mixed uniformly with the detergent, providing further support for the direct interaction of phospholipid with the rhodopsin. The actual structure of the solubilized complex probably lies somewhere between the two above alternatives. The phospholipid acyl chains likely interact directly with the protein, but the lipid–protein contacts may be weakened relative to the interactions in the native membrane by the presence of the detergent.

In summary, we have demonstrated how the composition of the microenvironment of an integral membrane protein changes as the membrane is exposed to increasing levels of a nonionic detergent. The detergent first partitions into the lipid bilayer regions of the membrane until the membrane becomes saturated. Additional detergent releases protein–lipid–detergent complexes which contain sufficient phospholipid to form a complete boundary layer around the protein. After enough detergent has been added to fully solubilize the membranes, free detergent micelles begin to form, and the phospholipid in the solution is distributed between these detergent micelles and the soluble protein–lipid–detergent complexes. The following paper (Stubbs and Litman, 1978) describes the changes in conformational stability of opsin which accompany these changes in the protein's amphipathic microenvironment.

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