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Estimation of Disk Membrane Lateral Pressure and Molecular Area of Rhodopsin by the Measurement of Its Orientation at the Nitrogen–Water Interface from an Ellipsometric Study[†]

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ABSTRACT: The internal lateral pressure of a bilayer has been estimated by numerous investigators. Most of these measurements were made by using the monolayer technique. In our approach, the disk membrane lateral pressure was estimated by assuming that this value is equal to the surface pressure necessary to maintain the transmembrane orientation of rhodopsin. The orientation of rhodopsin at the nitrogen–water interface was determined by using ellipsometry, which can measure the thickness of the film. By examining surface pressure and ellipsometric isotherms of intact and partially hydrolyzed rhodopsin, we have determined that a lateral pressure of 38 mN/m is necessary to give rhodopsin its natural transmembrane orientation and that surface pressures exceeding 45 mN/m lead to the formation of multilayers in the disk membrane film. At 38 mN/m, pure rhodopsin is found to have a molecular area of 2300 Å^2.

A number of studies have evaluated the lateral pressure in a lipid bilayer by using model systems. Blume (1979) and Albrecht et al. (1978), who compared phase transitions of phospholipids in monolayers and bilayers, reported values of 30 and 12 mN/m, respectively. Hui et al. (1975) found, from electron diffraction studies, that the molecular packing of a bilayer corresponds to that of the precursor monolayer at a surface pressure of 47 mN/m. Demel et al. (1975), who compared the action of various phospholipases on erythrocyte membranes and on phospholipid monolayers, concluded that the lipids of the outer monolayer of the erythrocyte membrane experience a lateral surface pressure between 31 and 34.8 mN/m. Nagle (1980) and Gruen and Wolfe (1982) reported a theoretical value of 50 mN/m. In their calculations of a membrane lateral pressure, Georgallas et al. (1984) considered the interaction between lipid chains in opposite halves of the bilayer and found a value of 30.5 mN/m.

In the disk membrane of rod visual cells, rhodopsin comprises at least 95% of the intrinsic proteins (Krebs & Kühn, 1977; Amis et al., 1981). In these membranes, the bilayer structure maintains the transmembrane orientation of rhodopsin. In order to estimate the lateral pressure needed to preserve this orientation, we have measured rhodopsin orientation in monolayers at the nitrogen–water interface. Analysis of these data gives the possible orientations rhodopsin can take under different conditions, and the minimum lateral pressure required to maintain its natural transmembrane orientation.

Monolayer methodology has the great advantage, as a model membrane system, that packing pressure can be varied at will. Moreover, because of its high sensitivity in the submonolayer region (Bootsma & Meyer, 1969), ellipsometry has already been shown to be a powerful and well-proven method in deriving information regarding the orientation of molecules in monolayers at the air–water interface (Den Engelsen & De Koning, 1974a,b; Ducharme et al., 1985, 1987, 1990). Accordingly, ellipsometry, along with the monolayer technique, was used to investigate the orientation of intact rhodopsin, partially hydrolyzed rhodopsin, and rhodopsin in intact disk

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membrane films at the nitrogen–water interface.

MATERIALS AND METHODS

All manipulations involving the visual pigment were carried out in a dark room under dim red light (λ >650 nm).

Purification of ROS Disk Membranes. Highly purified bovine rod outer segment (ROS)[1] membranes were prepared by using a buffered continuous sucrose concentration gradient (VBG-ROS) as previously described (Salesse et al., 1984). Purification of disk membranes was then achieved by Ficoll flotation of the VBG-ROS (Smith & Litman, 1982). The fatty acid composition and the number of phospholipid molecules per rhodopsin molecule of purified ROS and purified disk membranes were in agreement with values previously reported (Salesse et al., 1984, 1988).

Thermolysin Digestion of Disks. Freshly prepared disks were treated in the dark with thermolysin (Calbiochem, Los Angeles, CA), at room temperature for a short period of time (5 min). As described by Kühn and Hargrave (1981), this treatment leads to the hydrolysis of only the last 12 amino acids of the carboxyl terminus of the polypeptide chain of rhodopsin, which consists of 348 amino acids (Hargrave & Fong, 1977; Hargrave et al., 1980, 1983; Kühn & Hargrave, 1981; Kühn et al., 1982). The extent of proteolysis was estimated by polyacrylamide gel electrophoresis (Laemmli, 1970), and the results agreed with those of Kühn and Hargrave (1981).

Purification of Intact and Partially Hydrolyzed Rhodopsin. Intact and thermolysin-treated disk membranes were regenerated with 11-*cis*-retinal (Salesse et al., 1984), solubilized in 3% (v/v) Ammonyx LO (Onyx Chemical Co., Jersey City, NJ), and centrifuged at 43500*g* for 30 min. Rhodopsin from the supernatant was then purified by affinity chromatography on concanavalin A–Sepharose 4B (Sigma Chemical Co., St. Louis, MO) at 4 °C by a modified method of Litman (1982). After the column was washed with a large volume of 1% (v/v) Ammonyx LO in 50 mM Tris (pH 7.3), rhodopsin was eluted with 0.1 M methyl mannoside (Aldrich Chemical Co., Milwaukee, WI) in 50 mM Tris (pH 7.3) containing 30 mM octyl glucoside (Sigma Chemical Co.). The mannoside concentration was reduced by dialysis at 4 °C against a 20-fold excess of 50 mM Tris buffer (pH 7.3), containing 30 mM octyl glucoside, for 36 h with two buffer changes. The dialysis medium was continuously deoxygenated by bubbling with pure nitrogen.

Polyacrylamide gel electrophoresis (Laemmli, 1970) showed that purified intact rhodopsin and purified partially hydrolyzed rhodopsin comigrated with rhodopsin, respectively, from intact disk membranes and from thermolysin-treated disk membranes. These results are in good agreement with those of Kühn and Hargrave (1981). The $A_{280}/\Delta A_{500}$ ratio of both purified rhodopsins was 1.68 ± 0.02. The intact and partially hydrolyzed rhodopsins were virtually phospholipid-free, having less than 1 phospholipid molecule per 10 rhodopsin molecules, in agreement with the results of Jackson and Litman (1985).

Spreading of Disk Membranes and Rhodopsin. Intact disk membranes as well as intact and partially hydrolyzed rhodopsin were spread at the nitrogen–water interface by using a modification of the method of Trurnit (1960). A cylindrical piece of wet glass (5-mm diameter and 25 mm high) with a flat bottom and a rounded and narrowed top was inserted into a piece of Teflon immersed in the subphase. The top of this glass cylinder protuded about 3 mm above the surface of the water. Next, 100 μL of ca. 1×10^{-5} M intact disk membranes (in terms of regenerated rhodopsin) or 1.5×10^{-5} M intact or partially hydrolyzed rhodopsin (the latter two in 30 mM octyl glucoside) was spread over this device, which was then rinsed with 250 μL of pure water and removed.

Measurement of Isotherms. The water used was deionized and prepurified by filtration (NANO pure-filter system, Barnstead, MA), and twice distilled with a quartz still (Model Bi-18, Amerisil Inc., Sayerville, NJ). Its specific resistivity was 18 MΩ·cm, and its surface tension was ≥71 mN/m.

Immediately following the spreading of the rhodopsin-containing material, compression was initiated, and continued until a 2 mN/m surface pressure was reached. Then, the subphase was agitated 10 times with a specially designed device (Ducharme et al., 1987), to allow those molecules possibly lost in the subphase during spreading to be readsorbed at the surface. The film was then decompressed to the maximum available area in order to leave space at the interface for the readsorbed molecules. This operation increased the spreading yield of the disk membranes while not affecting the rhodopsin film. Further compression, agitation, and decompression did not further increase the spreading yield.

The films were then recompressed until collapse. Surface pressure and ellipsometric isotherms were measured in the same manner as previously described (Ducharme et al., 1985, 1987, 1990).

ELLIPSOMETRIC FORMULAS

In order to consider the optical properties of the monolayers, the following formulas have been used (Den Engelsen & De Koning, 1974a). Absorption of light is represented by the imaginary part of the refractive index as

$$\tilde{n} = n(1 - ik) \tag{1}$$

where k is related to the ordinary absorption coefficient α through

$$k = \alpha\lambda/4\pi n \tag{2}$$

The ellipsometric angles $\delta\Delta$ and $\delta\psi$ are given by

$$\delta\Delta = \Delta - \bar{\Delta} = B\left[n_0^2 + n_2^2 - n_x^2(1 - k_x^2) - \frac{n_0^2 n_2^2(1 - k_z^2)}{n_z^2(1 + k_z^2)^2}\right] \tag{3}$$

$$\delta\psi = \psi - \bar{\psi} = B \sin 2\bar{\psi}\left[\frac{n_0^2 n_2^2 k_z}{n_z^2(1 + k_z^2)^2} - n_x^2 k_x\right] \tag{4}$$

where

$$B = \frac{4\pi d \cos\phi \sin^2\phi \, n_2^2}{\lambda(n_2^2 - n_0^2)(n_0^2 \sin^2\phi - n_2^2 \cos^2\phi)} \tag{5}$$

and n_0, n_2, n_x, n_z, ϕ, and d are, respectively, the refractive index of air, aqueous subphase, film refractive indices, angle of incidence, and film thickness, respectively. Δ and $\bar{\Delta}$ are, respectively, the filmed and bare subphase changes due to reflection whereas ψ and $\bar{\psi}$ refer to changes in relative amplitude.

Upon compression, simple molecules like phospholipids are known to undergo reorientation (Ducharme et al., 1985, 1990). During this reorientation, as the film thickness increases, the value of n_z grows while n_x decreases. This change in anisotropy

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[1] Abbreviations: ROS, rod outer segment(s); DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC_{ROS}, phosphatidylcholine from rod outer segment(s); PE_{ROS}, phosphatidylethanolamine from rod outer segment(s); PS_{ROS}, phosphatidylserine from rod outer segment(s); LE, liquid-expanded state; SC, solid-condensed state.

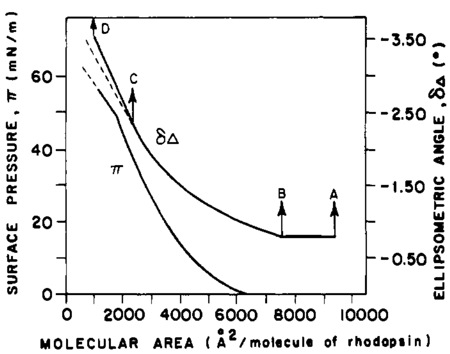

FIGURE 1: Surface pressure (π) and ellipsometric ($\delta\Delta$) isotherms of intact (—) and partially hydrolyzed (---) rhodopsin at the nitrogen–water interface. Isotherms were measured at 17.0 ± 0.5 °C over a subphase of 10^{-3} M phosphate buffer/10^{-1} M NaCl, pH 7.2, ϕ = 60.00 ± 0.05°. Labels ABCD represent the different segments of the $\delta\Delta$ isotherms.

tends to make $\delta\Delta$ more positive. However, this effect is compensated by the increasing value of d, so that the overall effect makes $\delta\Delta$ more negative. The dielectric constants are taken as tensors. During reorientation, the variation of anisotropy, i.e., the increase of n_z at the expense of n_x, is described by the relation (Den Engelsen & De Koning, 1974b):

$$n_x = n_y = \{1/2[(1 + \cos^2\theta)\epsilon_{x'} + \epsilon_{z'}\sin^2\theta]\}^{1/2}$$

$$n_z = (\epsilon_{x'}\sin^2\theta + \epsilon_{z'}\cos^2\theta)^{1/2} \qquad (6)$$

where $\epsilon_{x'}$ and $\epsilon_{z'}$ are, respectively, the parallel and perpendicular (to the surface) dielectric constants as a function of the orientation (θ) of the molecule. Experimental two-zone measurements have shown no substantial deviation from zero in the ellipsometric angle: $r = |P_1 - P_2| - 90 = 0$. These measurements thus indicate that the film shows anisotropic behavior with respect to its optic axis which lies in the plane of incidence.

RESULTS

Intact and Partially Hydrolyzed Rhodopsin. Figure 1 presents the surface pressure (π) and ellipsometric ($\delta\Delta$) isotherms of intact and partially hydrolyzed rhodopsin. The isotherms correspond to an average of eight different measurements from two independent purifications.

The surface pressure isotherm which begins at ~6500 Å²/molecule is highly reproducible (±50 Å²/molecule). This becomes evident when the relative experimental error obtained with the rhodopsin film at 38 mN/m (2300 ± 50 Å²/molecule = ±2.2%) is compared to the one measured in the case of a well-known monolayer-forming molecule (e.g., dioleoylphosphatidylcholine), at the same surface pressure (55 ± 1 Å²/molecule = ± 1.8%; Ducharme et al., 1985). The good agreement between these two values suggests that the rhodopsin molecule, when spread from an octyl glucoside solution, behaves similar to a tensio-active molecule. This property of rhodopsin is further evidenced when the experiment involving the 10 agitations of the subphase is performed. This process is very effective in forcing soluble proteins injected in the subphase of a phospholipid monolayer to adsorb at the interface (Salesse et al., 1989; Lamarche et al., 1988). However, the spreading yield of rhodopsin is not affected by this manipulation. This experimental observation strongly suggests that all the rhodopsin molecules remain at the surface during the spreading process.

Intact and partially hydrolyzed rhodopsins have the same π–A isotherms. However, partially hydrolyzed rhodopsin can be compressed to a smaller molecular area (~750 Å²/molecule) than intact rhodopsin (~1000 Å²/molecule).

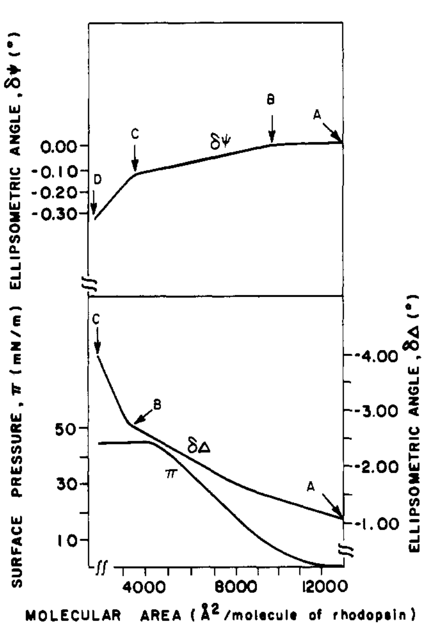

FIGURE 2: Surface pressure (π) and ellipsometric ($\delta\Delta$, $\delta\psi$) isotherms of intact disk membranes at the nitrogen–water interface. Isotherms were measured at 17.5 ± 0.5 °C over a subphase of 10^{-3} M phosphate buffer/10^{-1} M NaCl, pH 7.2, ϕ = 60.00 ± 0.05°. Labels ABCD represent the different segments of the $\delta\Delta$ and $\delta\psi$ isotherms.

The ellipsometric isotherms of intact and partially hydrolyzed rhodopsin are indistinguishable from the beginning (−0.80°) up to −2.30° (38 mN/m) ($\delta\Delta$ curve, segments A–C, Figure 1). Initially, a plateau region (−0.80°) can be observed from 9350 to 7500 Å²/molecule ($\delta\Delta$ curve, segment A–B, Figure 1). Then, slightly before the onset of the surface pressure, the ellipsometric angle begins to decrease (becomes more negative). The relationship between the ellipsometric angle and the molecular area becomes almost exponential from −0.80° to −2.30° (0–38 mN/m) ($\delta\Delta$ curve, segment B–C, Figure 1); i.e., the decrease of the ellipsometric angle is larger than the decrease of the molecular area. From −2.30° to −3.30° (38–56 mN/m) ($\delta\Delta$ curve, segment C–D, Figure 1), both isotherms show a linear relationship between ellipsometric angle and molecular area. However, in this range, intact rhodopsin shows an ellipsometric angle lower (more negative) than that of partially hydrolyzed rhodopsin. The difference between the two angles increases with compression, the difference being −0.10 ± 0.02° at the beginning and −0.20 ± 0.02° at the end.

Equation 4 shows that a change in $\delta\psi$ can be caused by light absorption. However, it is noteworthy that this ellipsometric angle (result not shown in Figure 1) remained unchanged during compression. This indicates that rhodopsin does not absorb light of wavelength 632.8 nm. It is therefore unlikely that the rhodopsin was bleached by the laser light of this wavelength used in our procedures. Furthermore, the fact that $\delta\psi$ remains null throughout compression also means that the film is very thin (Tomar, 1974).

Intact Disk Membranes. Surface pressure (π) and ellipsometric ($\delta\Delta$, $\delta\psi$) isotherms of intact disk membranes are shown in Figure 2. The surface pressure isotherm is the average of eight different measurements from three independent purifications. This isotherm begins at 12 500 Å²/molecule and reaches collapse pressure (44.8 mN/m) at ~4000 Å²/molecule. A slope change is observed at approximately 36 mN/m. This surface pressure isotherm indicates that disk membranes also behave as liquid-expanded monolayers.

This isotherm is also reproducible (±100 Å^2/molecule). However, an increase of the spreading yield is observed after the 10 agitations of the subphase, indicating that some material is lost during the spreading process and that complete recovery of these membranes is uncertain.

The ellipsometric isotherms, $\delta\Delta$ and $\delta\psi$, are the average of 11 different measurements from 3 independent purifications and are again highly reproducible (±0.02°). The ellipsometric jump takes place at ~13 000 Å^2/molecule (-1.12°), slightly before the onset of the surface pressure. Following the jump, a linear relationship between the ellipsometric angle and the molecular area is observed ($\delta\Delta$ curve, segment A–B, Figure 2). This relationship remains constant up to the collapse surface pressure. In this region (~4500 Å^2/molecule), the surface pressure corresponds to 44.8 mN/m (-2.50°). However, at a slightly smaller molecular area (~3250 Å^2/molecule; -2.76°), a large slope change can be seen ($\delta\Delta$ curve, segment B–C, Figure 2). From this point (point B, $\delta\Delta$ curve, Figure 2), the decrease of the ellipsometric angle is very important. At about the same molecular area, as seen for the $\delta\Delta$ isotherm (3250 Å^2/molecule), the $\delta\psi$ isotherm also shows an important slope change (3500 Å^2/molecule) ($\delta\psi$ curve, segment C–D, Figure 2).

DISCUSSION

Intact and Partially Hydrolyzed Rhodopsin. It is very often believed that proteins are denatured by spreading at an air–water interface. However, it is likely that proteins having strong structural bonding such as bacteriorhodopsin (or rhodopsin) are not susceptible to denaturation when spread at the air–water interface (Cadenhead, 1985).

Evidence that the conformation of a protein in a model membrane system is identical with that of the protein in its native membrane has generally been based on the preservation of protein properties in the model system. Korenbrot and Pramik (1977) have used four criteria to ascertain the structural integrity of rhodopsin in interface films. (i) Dark absorption spectrum: rhodopsin in interface films exhibits a visible absorption spectrum in the dark, identical with that found in disk membranes. (ii) Formation of photoproducts: illumination of rhodopsin in interface films results in the formation of Meta I in photo-steady-state equilibrium with rhodopsin and isorhodopsin. Identical results are obtained when the photocycle of rhodopsin is arrested at the Meta I stage. (iii) Photoregeneration: rhodopsin photoregenerates in the interface films, just as it does in disk membranes. (iv) Chemical regeneration: rhodopsin is chemically regenerable in the interface films. This is a particularly indicative criterion since rhodopsin is in a chemically regenerable configuration only under a limited number of conditions. The combination of all criteria suggests that the conformation of rhodopsin in the interface films is indistinguishable from that of rhodopsin in the rod disk membrane.

Moreover, the rhodopsin monolayer is very stable, and a high surface pressure can be reached (56 mN/m) as compared to many other protein films which collapse at much lower pressures (ca. 12–25 mN/m) (Quinn & Dawson, 1970; Graham & Phillips, 1979; Lamarche et al., 1988).

Furthermore, it has also been shown that both the spectroscopic and functional integrity of bacteriorhodopsin, a very similar membrane protein (Ovchinnikov, 1982), are preserved in air–water interface films (Hwang et al., 1977a,b).

These experimental evidences therefore suggest that rhodopsin is not affected by spreading at the air–water interface.

Measurement of surface isotherms of intact or partially hydrolyzed rhodopsin requires a concentration not less than ~2500 molecules of octyl glucoside per molecule of rhodopsin. Although the spreading of rhodopsin in this large excess of detergent results in a positive surface pressure (1.1–3.3 mN/m), this excess is likely solubilized in the subphase during compression and thus does not interfere with the molecular area measurement. The solubilization of octyl glucoside is likely due to (i) its eight-carbon fatty acid chain is only weakly tensio-active, and as a pure compound, it shows no surface pressure upon spreading and compression; (ii) the positive surface pressure noted following the spreading of rhodopsin does not increase upon compression prior to the beginning of the surface pressure isotherm, and during decompression, returns to or close to zero; (iii) the isotherms measured during compression and recompression are highly reproducible if a surface pressure of 38 mN/m is not exceeded (vide infra).

The main objective of this work was to determine at what monolayer surface pressure the orientation of rhodopsin corresponds to its transmembrane orientation in intact disk membranes (z axis of rhodopsin perpendicular to the water surface, see schematic diagram, point C, Figure 3B). In order to reach this goal, the change of orientation of rhodopsin taking place during compression will be deduced from the three segments of its ellipsometric isotherm (segments A–B, B–C, and C–D, Figures 1 and 3B).

The ellipsometric isotherm shows a plateau at surface areas larger than 6500 Å^2 ($\delta\Delta$ curve, segment A–B, Figures 1 and 3B). It indicates a constant value for both film thickness and refractive indices (Ducharme et al., 1985, 1989). It thus may correspond to a rhodopsin orientation with the x and z axes lying along the surface (vide infra and Figure 3B). With a refractive index of 1.410 (Sidman, 1957; Blaurock & Wilkins, 1969; Enoch et al., 1973) and a birefringence of 0.005 (Liebman, 1975), the presumed orientation would result in anisotropic refractive indices of 1.410 for n_x and 1.405 for n_z, since $n_z < n_x$ (Ducharme et al., 1985). By using eq 3 ($k = 0$), we then find a value of 22 Å for the film thickness in the plateau region. This value is in good agreement with the dimension of rhodopsin as measured along its y axis (25 Å) and thus indicates an orientation where its x and z axes are parallel to the surface (vide infra and schematic diagram, point B, Figure 3B).

The values of the above-mentioned optical properties (refractive indices and birefringence), used to calculate the thickness of the rhodopsin interface film, were drawn from intact disk membranes (vide supra). These optical properties, measured in bilayers (intact disk membranes), can also be used to calculate the thickness of monolayers (disk membranes, Figure 2) for the following reasons: (i) The thickness of a monolayer of dioleoylphosphatidylcholine (DOPC) has already been calculated by using literature refractive indices measured in bilayers (black lipid membranes of egg lecithin). The value obtained was in good agreement with the thickness of one monolayer of DOPC (CPK molecular models) (Ducharme et al., 1985). (ii) The theoretical calculations of Huang and Levitt (1977) have shown that there is only a very small difference between the monolayer refractive indices and the bilayer ones. The optical properties measured in intact disk membranes can, therefore, be applied to disk membrane monolayer calculations. In order to show that these quantities can also be used to calculate the thickness of the rhodopsin monolayer, the ellipsometric isotherms of pure rhodopsin (Figure 1), and of disk membranes (Figure 2), must be compared. However, these isotherms are not readily comparable because they cover different molecular areas. Nevertheless, as shown in Figure 3B, the plot of the ellipsometric angle as

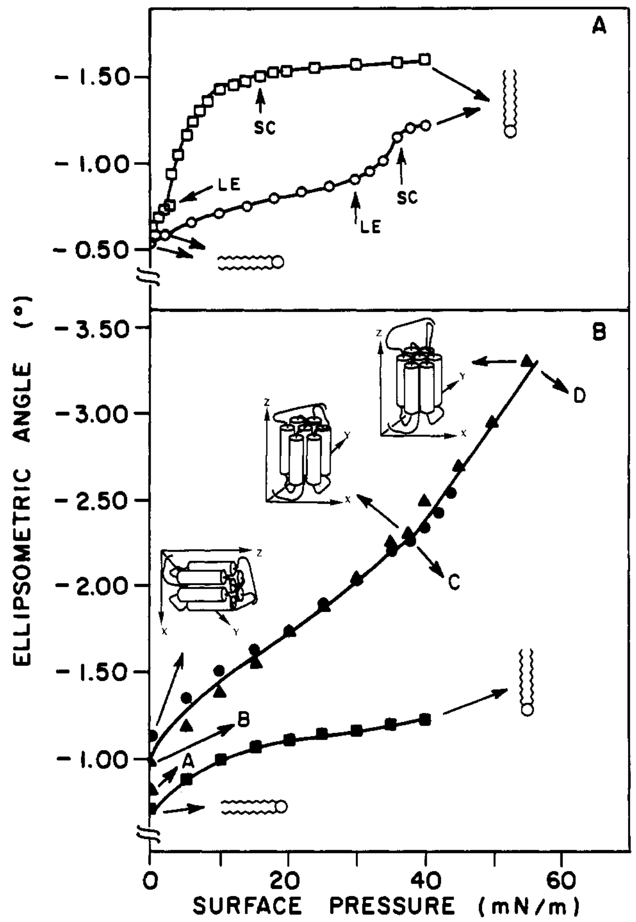

FIGURE 3: Plot of the ellipsometric angle ($\delta\Delta$) against the surface pressure. (A) Ellipsometric isotherms of DMPC (○) and DPPC (□) at the air–water interface. LE and SE indicate, respectively, the end of the liquid-expanded (LE) state and the beginning of the solid-condensed (SC) state. The orientation of these phospholipids at the beginning (main molecular axis parallel to the interface) and at the end (main molecular axis perpendicular to the interface) of the ellipsometric isotherms is also shown. (B) Ellipsometric isotherms of intact rhodopsin (▲), intact disk membranes (●), and PS_{ROS} (■) at the air–water interface. The isotherms of DOPC, PC_{ROS}, and PE_{ROS} are similar to the one of PS_{ROS} (Ducharme et al., 1985). The orientation of PS_{ROS} is shown as in the case of DMPC and DPPC [see (A)]. The postulated orientation of rhodopsin α helices (as cylinders) at the beginning (point B, y axis perpendicular to the interface, z and x axes parallel to the interface), at 38 mN/m (point C, z axis perpendicular to the interface, x and y axes parallel to the interface), and at the end [point D, same orientation as at 38 mN/m (point C) except that the structure is thicker and denser] of the ellipsometric isotherm is also shown. Labels ABCD represent the different segments of the $\delta\Delta$ isotherms of intact rhodopsin and intact disk membrane.

a function of surface pressure allows a direct comparison. It can be seen (Figure 3B) that the ellipsometric isotherms of pure rhodopsin and of disk membranes are almost the same. This means that, at given surface pressures, the thickness and the refractive indices of pure rhodopsin and disk membrane monolayers can be assumed to be the same. Therefore, the optical properties measured in intact disk membranes can also be used to calculate the thickness of the pure rhodopsin film.

In the two other segments of the ellipsometric isotherm of rhodopsin (segments B–C and C–D, Figures 1 and 3B), it can be seen that the ellipsometric angle becomes more and more negative. Due to the strong structural bonding of rhodopsin, this decrease of the ellipsometric angle, $\delta\Delta$, can only be explained as follows: (i) an increase in the thickness, accompanied by an increase of n_z at the expense of n_x; (ii) an increase in thickness, and an increase in both refractive indices (n_z and n_x).

Explanation i can be used to interpret the decrease of the ellipsometric angle in the segment B–C of the ellipsometric isotherm of rhodopsin (−0.80° to −2.30° or 0–38 mN/m, Figure 3B). This explanation implies that an orientation change takes place during compression.

Such a reorientation upon compression has already been shown to take place for phospholipids (vide supra, eq 6). The orientation of the main molecular axis of PS_{ROS}, PE_{ROS}, PC_{ROS}, and DOPC changes from parallel to perpendicular to the interface, from the beginning to the end of the ellipsometric isotherm (Figure 3B) (Ducharme et al., 1985). DMPC and DPPC undergo the same reorientation upon compression; it starts with the onset of the LE state and finishes at the end of the SC state (Figure 3A) (Ducharme et al., 1990).

An orientation change can also be postulated to explain the segment B–C of the ellipsometric isotherm of rhodopsin (0–38 mN/m, Figure 3B). Thus, during compression, the orientation of rhodopsin changes from the z axis being parallel to the interface at 0 mN/m (−0.80°, point B, Figure 3B) to the z axis being perpendicular to the interface at 38 mN/m (−2.30°, point C, Figure 3B). The following arguments support this hypothesis.

(A) The 22-Å thickness calculated at −0.80° (segment A–B, Figures 1 and 3B, vide supra) is in good agreement with the dimension of rhodopsin along its y axis (25 Å, vide infra, Molecular Area of Rhodopsin) and thus with the postulated orientation of rhodopsin at 0 mN/m (z axis parallel to the interface; see schematic diagram, point B, Figure 3B).

(B) The calculated thickness at 38 mN/m is 73 Å, a value in good agreement with the dimension of rhodopsin along its z axis (70 Å; Corless et al., 1982), and thus with the postulated orientation of rhodopsin at this surface pressure (z axis perpendicular to the interface; see schematic diagram, point C, Figure 3B). This thickness value of 73 Å is calculated by using refractive indices taken for the plateau region (segment A–B, Figures 1 and 3B, vide supra) that have changed according to eq 6, making $n_x = 1.4075$ and $n_z = 1.410$.

(C) Additional support comes from comparison between the ellipsometric isotherms of intact and partially hydrolyzed rhodopsin (Figure 1). When only the 12 carboxyl-terminus amino acids of rhodopsin are hydrolyzed, the purity ($A_{280}/\Delta A_{500}$ ratio and the 1:10 ratio of phospholipid to rhodopsin molecules) and the surface pressure isotherms are nearly identical with those of intact rhodopsin. It is thus highly probable that the refractive indices of intact and partially hydrolyzed rhodopsin are the same. Therefore, the difference in $\delta\Delta$ noted between intact and partially hydrolyzed rhodopsin from 38 to 56 mN/m ($\delta\Delta$ curve, segment C–D, Figure 1) can only be due to the increased thickness contributed by the 12 carboxyl-terminus amino acids. Because of the position of these 12 carboxyl-terminus amino acids (see schematic diagrams in Figure 3B), this thickness difference would be almost undetectable if the z axis of rhodopsin was not perpendicular to the interface between 38 and 56 mN/m. In addition, since the difference in $\delta\Delta$ between intact and partially hydrolyzed rhodopsin can only be attributed to the 12 carboxyl-terminus amino acid difference, it follows that the highly hydrophilic oligosaccharide groups of the N-terminal must lie in the aqueous phase.

Orientation change during compression implies that the orientation of rhodopsin in the interface film is determined exclusively by the lateral pressure within the film. In the pure rhodopsin film, i.e., at high protein surface concentration, forces that arise from protein–protein interactions may contribute to the orientation of the protein. However, one may

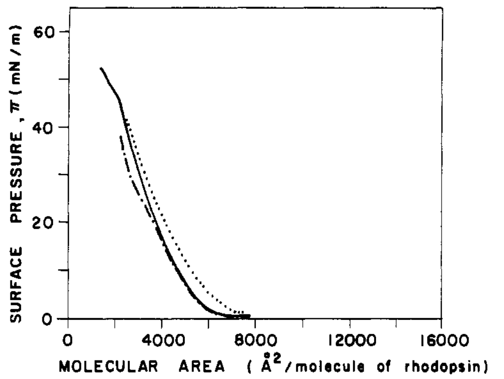

FIGURE 4: Surface pressure isotherms of intact rhodopsin at the nitrogen–water interface: compression to 41 mN/m (···); decompression (–·–); recompression (—). Compression to 35 mN/m and the following decompression and recompression are identical with the one of compression to 41 mN/m (···). Isotherms were measured at 17.0 ± 0.5 °C over a subphase of 10^{-3} M phosphate buffer/10^{-1} M NaCl, pH 7.2.

wonder whether or not the orientation of rhodopsin, at given lateral pressures, is the same in mixed protein–lipid films as in pure protein films. From Figure 3B, it can be seen that the ellipsometric isotherms of pure rhodopsin and disk membranes (lipid/protein ratio is 75/1; Miljanich et al., 1981) are very similar. This means that the thickness, the refractive indices, and thus the orientation of rhodopsin in both films, at given surface pressures, are the same. Therefore, in this segment (segment A–B, Figure 3B) of the ellipsometric isotherm of rhodopsin, protein–protein interactions may be negligible. This suggests also that the remaining detergent is unlikely to produce anomalous behavior in the protein.

Explanation ii can be used to interpret the decrease of the ellipsometric angle in section C–D of the ellipsometric isotherm of rhodopsin (−2.30° to −3.30° or 38–56 mN/m, Figure 3B): an increase in thickness and an increase in both refractive indices of the film (n_z and n_x). This explanation implies that the structure of the film becomes progressively denser and thicker.

From 38 mN/m onward, a significant difference exists between intact and partially hydrolyzed rhodopsin ($\delta\Delta$ curve, segment C–D, Figure 1). Contrary to the behavior observed for phospholipids, a clear phase transition of the LC to SC type (Figure 3A) is unlikely to occur in pure rhodopsin films. However, as seen in Figures 1 and 3B (segment C–D), the slope change observed from 38 mN/m in the ellipsometric isotherm of pure rhodopsin and of disk membranes (from point C, Figure 3B) suggests that some condensation of the protein structure takes place. This change in the behavior of the pure rhodopsin film between 38 and 56 mN/m can be explained by the following mechanism: the z axis of rhodopsin remains perpendicular to the interface, and continued compression constrains the α helices in a manner which decreases the distance between both the α helices and the connecting loops. The result is a denser, thicker structure (see schematic diagram, point D, Figure 3B). This mechanism has two implications: (A) The detergent molecules are progressively excluded, allowing closer packing of rhodopsin molecules, which results in their progressive aggregation. Evidence for this aggregation of rhodopsin molecules, upon compression to surface pressures greater than 38 mN/m, is shown in Figure 4. Although recompression under these conditions results in a decreased molecular area, the recompression isotherm is identical with the initial compression isotherm when 35 mN/m surface pressure is not exceeded. It therefore appears that when compressed to more than 38 mN/m, some rhodopsin aggregates, such that the original organization is irreversibly disturbed. (B) The section of the carboxyl terminus is increasingly pushed away from the aqueous surface by the connecting loops. The data in Figure 1 support this hypothesis. The ellipsometric isotherm of intact rhodopsin differs increasingly from that of partially hydrolyzed rhodopsin between 38 and 56 mN/m (segment C–D, Figure 1). As discussed above, the refractive indices of both rhodopsins are likely to be the same. Thus, the thickness difference between intact and partially hydrolyzed rhodopsin will be enhanced as the section of the carboxyl terminus of intact rhodopsin is pushed away from the surface.

In conclusion, the experimental results suggest that rhodopsin undergoes a reorientation upon compression from 0 to 38 mN/m and that its orientation, at 38 mN/m, corresponds to the one found in disk membranes (z axis perpendicular to the interface; see schematic diagram, point C, Figure 3B). It follows that the surface pressure required to maintain the transmembrane orientation of rhodopsin, under our experimental conditions, is 38 mN/m. The results of Korenbrot and Jones (1979) support this conclusion. They have demonstrated that rhodopsin–phospholipid films transferred onto a hydrophilic glass support at this pressure (38 mN/m) show pigment orientation indistinguishable from that found in native disk membrane when examined by linear dichroism.

Intact Disk Membranes. Pattus et al. (1978a) concluded that spreading probably results in a dramatic change in the phospholipid bilayer structure. It has been shown that DOPC forms a monolayer at the nitrogen–water interface, whether it is spread from an organic solvent (Ducharme et al., 1985) or from DOPC vesicles (Salesse et al., 1987). However, it does not follow that spreading biological membranes containing intrinsic proteins results in the formation of monolayers (Verger & Pattus, 1976; Pattus et al., 1978b, 1981; Kanno et al., 1983).

The formation of a monolayer upon the spreading of intact disk membranes was verified by comparing their surface properties with those of rhodopsin and ROS phospholipids. The ellipsometric angle of disk membranes at the jump (−1.12°) was much lower than that of rhodopsin (−0.80°) or ROS phospholipids (−0.64° to −0.75°; Ducharme et al., 1985).

The thickness of the disk membrane film at the jump can be calculated by using the refractive indices of rhodopsin at the beginning of the ellipsometric isotherm (n_x = 1.410, n_z = 1.405). This results in a calculated thickness of 31 Å, a value much larger than the thickness of rhodopsin, 22 Å, or of ROS phospholipids, 5 Å (CPK molecular model of a phospholipid lying in the plane of the water surface). Using these thickness values, it appears that rhodopsin in a disk membrane film may be surrounded by a shell of phospholipids (vide infra Molecular Area of Rhodopsin).

The similarity of the shapes of the surface pressure isotherms of intact disk membranes (π curve, Figure 2) and ROS phospholipids (Ducharme et al., 1985) suggests that rhodopsin behaves like ROS phospholipids when surrounded by them, and in a different manner when surrounded by detergent (π curve, Figure 1). However, the dramatic slope change in the two ellipsometric isotherms (segment B–C, $\delta\Delta$ curve, and segment C–D, $\delta\psi$ curve, Figure 2) above the collapse pressure of intact disk membranes, in contrast to ROS phospholipids or pure rhodopsin, indicates differences in the behavior of these entities in this region of the isotherm.

The collapse region (close-packed region) is poorly understood but is often regarded as a region where multilayers are formed (Ries, 1979; Ries & Swift, 1982; Ries et al., 1985).

The following observations provide evidence that disk membranes form multilayers in the collapse region and that this property is inherent to this film. Since neither rhodopsin nor intact disks show optical absorption of the 632.8-nm beam used for measurement of ellipsometric angles, the large decrease of the $\delta\psi$ angle from approximately 45 mN/m and onward can only be accounted for by a significant increase in film thickness, i.e., the formation of multilayers. Such a pronounced slope change in the $\delta\psi$ and $\delta\Delta$ isotherms was not observed either for rhodopsin (Figure 1) or for ROS phospholipids (Ducharme et al., 1985). In fact, nonzero k values have an opposite effect on $\delta\psi$ and $\delta\Delta$ (eq 3–5). For a given film thickness, larger k values lower $\delta\psi$ while they increase $\delta\Delta$ (more positive). However, as film thickness increases, for the same k values and refractive indices, $\delta\Delta$ becomes more negative. The fact that, in disk membranes, the $\delta\psi$ angle decreases ($\delta\psi$ curve, Figure 2) while the $\delta\Delta$ values are comparable in magnitude to those of pure rhodopsin (compare $\delta\Delta$ curves, Figures 1 and 2) can only be accounted for by the formation of a much thicker film in the case of disk membranes than in that of pure rhodopsin.

The property of forming multilayers in the collapse region may well be facilitated by the fact that rhodopsin in the disk membrane film is surrounded by a shell of phospholipids at the nitrogen–water interface. The results of Korenbrot and Pramik (1977) support this conclusion. They made electron micrographs of single rhodopsin–egg phosphatidylcholine interface films transferred to glass at their collapse pressure. The micrographs showed that the films consisted of nonoverlapping, randomly distributed vesicle membrane fragments, separated by a lipid monolayer.

At 38 mN/m, we observed ellipsometric angles of −2.25° for disk membranes (Figure 2) and −2.30° for rhodopsin (Figure 1). Using the refractive indices of rhodopsin (n_x = 1.4075, n_z = 1.410), we calculated a thickness of 71 Å for the disk membranes and 73 Å for rhodopsin at 38 mN/m. Therefore, as stated above, the orientation of rhodopsin in the pure rhodopsin or disk membrane monolayers, at this surface pressure, is virtually the same. This conclusion is supported by the close similarity between the ellipsometric isotherms of intact rhodopsin and intact disk membranes (Figure 3B).

Molecular Area of Rhodopsin. At 38 mN/m, the surface pressure isotherm of pure rhodopsin (Figure 1) shows a molecular area of 2300 Å^2. This value is much greater than the 1100–1500 Å^2/molecule previously reported (N'Soukpoé & Leblanc, 1985). However, the higher purity ($A_{280}/\Delta A_{500}$ = 1.68 ± 0.02, phospholipid to rhodopsin ratio = 0.1/1) of the present samples, the increased spreading yield obtained by the addition of 0.1 M NaCl in the subphase, and the absence of loss of rhodopsin in the subphase, assured by the 10 agitations, make the value reported here more reliable.

In two-dimensional lattices of rhodopsin, the repeat distance is 25 Å (Corless et al., 1982). Taking this value as the small axis for an ellipse, with an axial ratio of 2.1/1, formed by the seven α helices of rhodopsin, as proposed by Hargrave et al. (1984), one finds a molecular area of 1030 Å^2/rhodopsin. This value is in agreement with that of 1120–1400 Å^2/molecule which is obtained if we consider only the area of individual α helices estimated at 160–200 Å^2/helix (Batenburg, 1987; Fidelio et al., 1986). However, such an estimation does not take into account the fact that the presence of retinal within the helices and the interconnecting loops (9–29 amino acids long) may prevent close-packing of the helices, which would result in a molecular area greater than 1400 Å^2.

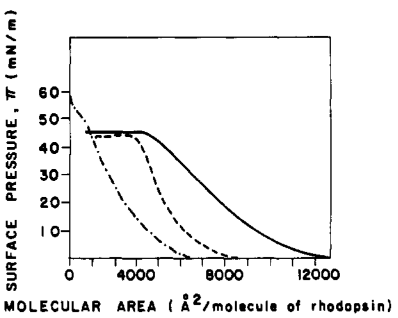

FIGURE 5: Surface pressure isotherm of intact rhodopsin (-•-), total disk membrane lipids (---), and intact disk membranes (—) at the nitrogen–water interface. Isotherms were measured at 17.0 ± 1.0 °C over a subphase of 10^{-3} M phosphate buffer/10^{-1} M NaCl, pH 7.2.

In one procedure, total disk membrane lipids were quantitatively extracted (Salesse et al., 1983), and their surface pressure isotherm was measured. It is represented in Figure 5. Since the rhodopsin concentration of disk membranes was precisely known, isotherms were always expressed in terms of angstroms squared per molecule of rhodopsin. In order to calculate the molecular area of rhodopsin at 38 mN/m, the contribution of total disk lipids at this pressure (4325 Å^2, Figure 5) was subtracted from the value measured for intact disk membranes (5450 Å^2, Figure 5), giving a value of 1125 Å^2 for the area of rhodopsin. It is noteworthy that the lipid contribution of 4325 ± 100 Å^2/rhodopsin obtained here is in agreement with the 4700 ± 200 Å^2 value calculated through combination of the molar ratio of each principal lipid with respect to rhodopsin (Miljanich et al., 1981) with the corresponding area of these individual lipids at 38 mN/m (Ducharme et al., 1985).

While a rhodopsin area of 1125 Å^2 is in good agreement with other predicted values (vide supra), it remains only half that observed experimentally for pure rhodopsin (2300 Å^2 at 38 mN/m, Figure 1). It can be argued that (i) in pure rhodopsin monolayers, some empty space may exist between individual molecules due to the fact that they cannot be perfectly packed at the interface, making the measured molecular area apparently larger than its actual size, or that (ii) in disk membrane films (mixed rhodopsin–lipid films), the lipids which fill spaces between rhodopsin molecules are not all in the monolayer state, and some may be present in the form of bilayers. As a matter of fact, in disk membranes, there exists a shell of 24 phospholipids more or less immobilized around the rhodopsin (Watts et al., 1979; Marsh et al., 1982). If that bilayer shell resisted spreading at the nitrogen–water interface, it would reduce the apparent molecular area of rhodopsin. The decrease of $\delta\psi$ from 0.00 to approximately −0.10° ($\delta\psi$ curve, segment B–C, Figure 2) measured for disk membranes and the calculated thickness of disk membranes at the jump (31 Å, vide supra) suggest that such shells are present at the interface. Assuming they are of the size previously mentioned (24 phospholipids), and correcting the rhodopsin molecular area for that part of the shell which only makes a 50% contribution to the area (because it is in the form of a bilayer), results in a molecular area of 1809 Å^2/molecule for rhodopsin when disk membranes are spread at the interface. This latter value is closer to the 2300 Å^2 measured for pure rhodopsin. In addition, these values would probably be in better agreement if incomplete recovery of the disk membranes lost in the subphase during the spreading process is considered (vide supra) or if the presence of a larger shell than the one assumed above (24 phospholipids/rhodopsin) is taken into account, as suggested by the results of Korenbrot and Pramik (1977).

Thus, the area of 2300 Å^2 measured for pure rhodopsin at 38 mN/m at the nitrogen–water interface is probably correct. However, for the reasons put forward above, the rhodopsin molecular area, in the presence of lipids, is reduced to 1809 Å^2. Taking now the surface of the extracted disk lipids, as a bilayer, where area = 2162 Å^2 (4325 Å^2/2), we find a total area of 3970 Å^2 for the rhodopsin–lipid mixture in a membrane. In the event that, under the same conditions, the rhodopsin alone occupies the 2300 Å^2, as it does in the pure state, the area becomes 4462 Å^2 for the same rhodopsin–lipid mixture. Both values fall within the 4000–5000 Å^2 already predicted by calculation (Amis et al., 1981; Krebs & Kühn, 1977).

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Role of Specific Acidic Lipids on the Reconstitution of Na^+-Dependent Amino Acid Transport in Proteoliposomes Derived from Ehrlich Cell Plasma Membranes[†]

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ABSTRACT: The effect of acidic phospholipids on the activity of a Na^+-dependent amino acid transporter (A system) from Ehrlich ascites cell plasma membranes was examined. Plasma membranes were solubilized in cholate/urea and reconstituted with Ba^{2+}-precipitated asolectin (soybean phospholipid free of anionic phospholipids) replenished with different acidic phospholipids. In the absence of added acidic phospholipids, transport activity was very low. However, three acidic lipids [cardiolipin > phosphatidic acid (PA) > phosphatidylinositol] were capable of restoring transport activity (in the order given) to proteoliposomes made from Ba^{2+}-precipitated asolectin, while other acidic phospholipids (phosphatidylserine and phosphatidylglycerol) were much less active in this respect. For restoration of optimal activity, PA containing at least one unsaturated fatty acyl moiety, particularly in the β position, was required. PA containing only saturated fatty acids in the β and γ positions was largely inactive. No difference in restoration of function was observed on varying the saturated fatty acyl chain length in PA from 10 carbons to 18 carbons. The specific effects of PA on the A-system transporter were not shared by the Na^+-independent amino acid exchange system (L system) or the glucose transport system. Treatment with poly(ethylene glycol) 8000 was shown to reduce the nonspecific permeability of the reconstituted proteoliposomes and to enhance Na^+-dependent amino acid transport.

Any "in vitro" study of solute translocation with isolated membrane proteins is coupled to the need to restore a suitable phospholipid environment. In contrast to binding studies or conventional enzyme assays, the requirements for reconstitution of transport include a need to form a compartment of finite volume and differential permeability, which will sustain ion gradients and/or the accumulation of translocated solutes.

Variability in the restoration of a native (or near native) conformation of the protein, as well as the disposition of the protein in the reconstituted membrane, would substantially affect the rate of transport.

Even if all the inherent catalytic (translocating) activity of the transporter were theoretically recovered, any variability in the size of the vesicles or their nonspecific permeability in different preparations would be reflected in differences in the apparent transport rate per unit amount of reconstituted protein.

Despite these stringent requirements, many investigators have reported the reconstitution of a wide variety of solute transporters from plasma membranes of mammalian cells (Kasahara & Hinkle, 1977; Bardin & Johnstone, 1978; Im & Spector, 1980; McCormick et al., 1984; Fafournoux et al., 1989). The level of reconstituted transport activity, however, rarely approaches that of the original system in the intact cell. It is self-evident that many of the variables in the reconstitution process still require systematic investigation to advance our understanding of how transporters work and the factors required for reconstitution of near-native activity.

One of the unresolved questions in the reconstitution of functional membrane proteins is the role, if any, of specific phospholipids. Studies on reconstitution of many transport activities have shown that heterologous phospholipids can provide an adequate environment for normal functioning of the transporter [for reviews, see Sandermann (1978), McElhaney (1982), and Yeagle (1989)]. While homologous lipid sources have been shown to provide optimal recovery of activity, asolectin (a plant phospholipid mixture) has been successfully used by many investigators to reconstitute transport activity from mammalian as well as bacterial systems (Villegas et al., 1977; Wu et al., 1981; McCormick et al., 1984; Wilson et al., 1985; Maloney & Ambudkar, 1989). Attempts to show highly selective lipid requirements for restoration of specific transport activities have met with only modest success. While acidic lipids have been implicated in the restoration of function of a number of plasma membrane transporters, requirements for specific acidic lipid species for this purpose have been less clearly established (Wheeler & Whittam, 1970; Niggli et al., 1981; Philipson & Nishimoto, 1984). Overall, most of the data from reconstitution studies have been consistent with the conclusion that the lipid environment must fulfill general conditions such as adequate lipid fluidity, and in some cases a suitable bilayer surface charge, with relatively little evidence for stringent requirements for specific lipid species (Knowles, 1976; Dean & Tanford, 1977; George et al., 1989; Yeagle, 1989). Such a conclusion would be in line with current observations that a variety of detergents (Lund et al., 1989) are excellent substitutes for phospholipids in the restoration of Ca^{2+}-ATPase activity.

Our recent attempts to obtain optimal conditions for recovery of Na^+-dependent amino acid transport from a detergent-solubilized membrane fraction from Ehrlich ascites cells have revealed a number of unexpected requirements,

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