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Lipid Requirements for Rhodopsin Regenerability†

Keelung Hong and Wayne L. Hubbell*

ABSTRACT: The regenerability of purified rhodopsin has been determined in phospholipid bilayers, in digalactosyl diglyceride bilayers, and in phospholipid-free solutions of digitonin. A high regenerability in the latter system indicates the lack of an absolute requirement for phospholipid. In lipid bilayers, there is no specific requirement for a particular polar head group or fatty acid species. It is suggested that structural rather than specific chemical requirements are important in maintaining a regenerable configuration of the molecule.

Rhodopsin is an integral component of the disk membranes in vertebrate photoreceptors and plays a central role in generation of the receptor potential. Recent data derived from X-ray diffraction (Blasie, 1972; Blaurock and Wilkins, 1972) and freeze-fracture electron microscopy (Hong and Hubbell, 1972; Chen and Hubbell, 1973) have been interpreted to suggest that an appreciable fraction of the rhodopsin molecule is located within the hydrophobic interior of the membrane and in direct contact with the fatty acid chains of the phospholipids. A study of the interactions between rhodopsin and bilayer phospholipids, and their modulation by light, is a step toward elucidating molecular mechanisms of the early events in phototransduction.

It has previously been shown that purified, delipidated rhodopsin can be incorporated into bilayers of egg phosphatidylcholine (Hong and Hubbell, 1972). Such recombined membranes represent an ideal system for investigating the details of phospholipid–rhodopsin interactions, since the compositional variables may be completely specified. More recently, Chabre *et al.* (1972) have disrupted native rod outer segment (ROS)[1] membranes with Triton X-100 and re-formed bilayers containing rhodopsin by extraction of the detergent with toluene. In the development of such model systems, it is necessary to establish an assay to determine whether or not the protein has maintained its native integrity during the various manipulations involved in the protein purification and recombination with phospholipids. Clearly, functional activity is the ultimate criterion. At present, the *in vivo* function of rhodopsin is not known and until established other criteria must be selected. The first necessary (but not sufficient) criterion that must be met for rhodopsin to be considered native is that the protein have the characteristic visible absorption spectrum. This criterion is met for the protein in many detergent solutions and the recombinant membranes previously described (Hong and Hubbell, 1972). It is possible that rhodopsin may exist in various states of partial denaturation even though the characteristic absorption spectrum is maintained. In this paper the term "rhodopsin" will be used to describe any state of the protein characterized by the native absorption spectrum. A more demanding criterion is that of chemical regenerability. Regeneration yields are dependent on the molecular environment of the protein, and so far have been found to be high only in the native membranes, recombined membranes, and digitonin solution. Rhodopsin solubilized in most detergents is either not regenerable following bleaching or the yields are very low.

Two recent reports in the literature have dealt with the subject of phospholipid requirements for rhodopsin regenerability. Zorn and Futterman (1971) have reported that partially delipidated cattle rhodopsin is not regenerable, and of a variety of phospholipids examined, only phosphatidylethanolamine appeared to be capable of restoring the regenerability of the bleached pigment. Shichi (1971) has reported that the photochemical regenerability of rhodopsin is lost when 20–30% of the phospholipid is extracted from cattle rod outer segment membranes, but is restored upon the addition of phospholipid. Shichi's experiments suggest no specific requirement for the type of phospholipid recombined with the partially delipidated membranes.

High regenerability is a characteristic of native rhodopsin, and the degree of regeneration is, in part, a reflection of the ability of the local environment to maintain a particular, regenerable configuration of the protein. From this point of view, molecular requirements for high regenerability are of interest in defining the nature of rhodopsin–lipid interactions.

The present work is concerned with elucidating such requirements, using the approach of recombining purified, phospholipid-free rhodopsin with chemically defined synthetic and natural lipids.

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[1] Abbreviations used are: ROS, rod outer segments; DTAB, dodecyltrimethylammonium bromide; TrTAB, tridecyltrimethylammonium bromide; TeTAB, tetradecyltrimethylammonium bromide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Materials and Methods

Purification of Rhodopsin. In the following procedures, all buffers were used at a pH of 6.6, and all procedures involving rhodopsin were done under dim red light (Kodak series 1A filter) or in total darkness at 4° unless otherwise specified. ROS membrane fragments were isolated from dark adapted bovine retinas (George Hormel Co., Austin, Minn.) by the following procedure: 100 partially thawed retinas were ground in a cold mortar to a homogeneous slurry, and the total volume was brought to 100 ml with 1.38 M sucrose in 0.1 M phosphate buffer. The suspension was centrifuged at 2445*g* for 15 min, and the supernatant was collected and diluted with an equal volume of 0.1 M phosphate buffer and recentrifuged at 43,500*g* for 15 min. The resulting pellet was suspended in 40 ml of 1.02 M sucrose in 0.1 M phosphate buffer by homogenization in a Teflon–glass homogenizer, and the suspension was centrifuged at 43,500*g* for 45 min. The supernatant with floating material was collected, diluted with an equal volume of buffer, and centrifuged at 43,500*g* for 15 min. The floatation in 1.02 M sucrose was repeated two more times and the final pellet was washed twice in distilled water. The ROS membranes were lyophilized and stored at −20°.

Rhodopsin was extracted from the lyophilized membranes of 100 retinas by homogenization in 3.5 ml of 15 mM sodium phosphate buffer containing 100 mM dodecyltrimethylammonium bromide (DTAB) and 1 mM dithiothreitol. The homogenate was centrifuged at 45,000*g* for 20 min. The optically clear supernatant contained nearly all of the rhodopsin and the light pink pellet was discarded. Further purification of the rhodopsin and removal of phospholipids was accomplished by chromatography on hydroxylapatite (DNA grade, Bio-Gel HTP, Bio-Rad Laboratory, Richmond, Calif.). The column bed was 2.5 × 6.5 cm, and was equilibrated with a 15 mM sodium phosphate buffer containing 100 mM DTAB and 1 mM dithiothreitol. The DTAB solution of crude rhodopsin was loaded on the column and eluted with a 0–0.5 M linear gradient of sodium chloride. During the elution, the concentrations of phosphate, DTAB, and dithiothreitol were constant at the values used in the equilibration buffer. The total gradient volume was 300 ml. The flow rate was controlled at 0.4 ml/min with a peristaltic pump and fractions of 2.4 ml were collected. The absorption spectrum of each fraction was recorded on a Cary 118C spectrophotometer. In some experiments, rhodopsin was extracted and purified in either tridecyltrimethylammonium bromide (TrTAB) or tetradecyltrimethylammonium bromide (TeTAB) according to the same procedure.

Detergent Exchange Procedures. Solid digitonin or egg lysophosphatidylcholine (lyso-PC) was added to the purified rhodopsin in 100 mM DTAB so that the final concentration was 2% for digitonin or 1.5% for lyso-PC. DTAB was removed by dialyzing against 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer containing 1 mM dithiothreitol.

Incorporation of Rhodopsin into Lipid Bilayers. Solid DTAB was added to the 100 mM DTAB solution of purified rhodopsin so that the final concentration of detergent was 300 mM, and solid, solvent-free lipid was added directly to the resulting solution to give a lipid:rhodopsin molar ratio of approximately 100:1. Values of molar concentrations of rhodopsin in DTAB were based on a molar extinction coefficient at 498 nm of 42,700 (Hong and Hubbell, 1972), a value which agrees well with that determined in other detergents (Shichi *et al.*, 1969; Wald and Brown, 1953). The mixture was incubated for at least 5 hr at 4° to obtain a homogeneous solution prior to removal of DTAB. The detergent was then removed by dialyzing against a 5 mM Hepes buffer with the desired content of dithiothreitol and/or EDTA (see Table I). Dialysis was carried out with 500 ml of dialysis buffer for each 1 ml of detergent solution, and changed four times over a period of 48 hr. In order to prevent the oxidation of unsaturated lipids, the dialysis buffer was deoxygenated with nitrogen gas, and a constant stream of nitrogen was bubbled through the solution during the course of dialysis. When rhodopsin was purified with TrTAB or TETAB, the same procedure was followed for recombination. The recombined membranes were harvested by centrifugation at 73,400*g* for 1 hr, and the absorption spectrum of the recovered protein was recorded after solubilization in Hepes buffer containing 80 mM TeTAB.

During the recombination process, some rhodopsin is inevitably denatured, resulting in loss of the 498-nm absorption but with little change in the extinction coefficient at 278 nm (Hubbard, 1969). Thus, the per cent of total protein in the recombined membranes that retains the native absorption spectrum can be estimated as

$$\left(\frac{A_{498}}{A_{278}}\right)\left(\frac{A'_{278}}{A'_{498}}\right) \times 100$$

where A_{498} and A_{278} are the absorbances at 498 and 278 nm of the protein extracted from the recombined membranes, and A'_{498} and A'_{278} are the corresponding absorbances of the rhodopsin before the recombination procedure. In the present work, we refer to this value as the per cent recovery of rhodopsin.

Possible errors involved in the measurement of the recovery are: (1) increased light scattering at 278 nm due to increased micelle size in the presence of phospholipid; (2) absorption at 278 nm due to oxidation products of unsaturated fatty acids on the phospholipids. These errors tend to make the recovery determined from the above considerations *less* than the actual value. Since our recoveries are high, these errors cannot be considered serious.

Bleaching and Regeneration of Rhodopsin. Suspensions of membranes containing rhodopsin were bleached at room temperature in the presence of 0.2 M hydroxylamine (pH 6.6) by exposure either to a 300-W flood lamp provided with a heat filter or with monochromatic light at 498 nm. Irradiation times were usually of the order of 3–5 min. Parallel experiments have been performed in the absence of hydroxylamine, and in these cases a significant absorption, but no maximum, often remained at 498 nm after irradiation. Regeneration was always more complete when hydroxylamine was used in the bleaching procedure, and all results reported here for recombinant and ROS membranes employed this method. Regeneration of rhodopsin in homogeneous solutions of digitonin and lyso-PC was carried out with samples bleached in the absence of hydroxylamine. Complete bleaching was confirmed by recording the visible spectrum of a small portion to which hydroxylamine had been added in the dark.

To remove excess hydroxylamine, the bleached membranes were washed three times by centrifugation with a buffer of the same composition used in the recombination procedure. The spectrum of the protein in the bleached, washed recombinant was obtained after solubilization of a portion, in the dark, in Hepes buffer containing 80 mM TeTAB and 0.2 M hydroxylamine. Regeneration was initiated by the addition of a twofold excess of 11-*cis*-retinal as a concentrated ethanol

solution. The recombinant suspension or the detergent solutions were then incubated for 5–12 hr in the dark at 4°, and finally a sufficient volume of 1 M hydroxylamine was added to make the final concentration 0.2 M. The spectrum of the protein in the regenerated membrane was obtained after solubilization in 5 mM Hepes buffer containing 80 mM TeTAB and 0.2 M hydroxylamine.

The fraction of rhodopsin regenerated was computed as the ratio of the 498-nm absorbance after regeneration to that before bleaching. A difficulty arises because a direct comparison of absorbances relies on solubilizing an identical amount of protein in the membrane suspension before bleaching and after regeneration. This is difficult, since in some cases, the membranes are aggregated, giving a very heterogeneous suspension. To overcome this problem, we again take advantage of the fact that the rhodopsin extinction at 278 nm is an insensitive function of the state of the protein. Thus, this absorption can be used to normalize the protein content in two samples and allow direct comparison of the A_{498} values. Per cent regeneration is then estimated as

$$\left(\frac{A_{498}}{A_{278}}\right)\left(\frac{A'_{278}}{A'_{498}}\right) \times 100$$

where A_{498} and A_{278} are the absorbances of the protein at 498 and 278 nm after regeneration, and A'_{498} and A'_{278} are the corresponding absorbances of the protein before bleaching.

An error involved in this determination arises from the fact that the 365-nm absorption of the retinal oximes present in the solution after regeneration overlaps the 278 nm, but not the 498 nm, absorption of the protein (see Figure 3). The oxime absorption at 278 nm usually amounts to roughly 13% of the protein absorption at that wavelength It is possible to estimate the contribution from the oximes to the 278-nm absorbance in the mixture since the ratio A_{278}/A_{365} for both the pure *all-trans*- and 11-*cis*-retinal oximes is nearly independent of oxime concentration for the conditions employed. The contribution of rhodopsin to the 365-nm absorbance in the mixture can be computed from the 498-nm absorbance and the measured ratio A_{365}/A_{498} for the pure protein. Since only rhodopsin and the oximes contribute significantly to the 365-nm absorbance, that due to the oximes alone can be determined. From this absorbance, and the measured ratio A_{278}/A_{365} for the oximes, their contribution to the 278-nm band can be estimated and the absorbance due to the protein alone determined. In general, a mixture of *all-trans*- and 11-*cis*-retinal oximes of unknown composition is present in the solutions of regenerated rhodopsin. Because of the relatively small value of A_{278}/A_{365} for both oximes, the propagated error in the calculated value of the protein absorbance at 278 nm incurred by assuming the presence of only one isomer is less than *ca.* 3%. We have chosen to use A_{278}/A_{365} values corresponding to the *all-trans*-oxime (0.15) which will give the *minimum* correction for all possible mixtures. We have checked the utility of this procedure by adding mixtures of *all-trans*- and 11-*cis*-retinal to a solution containing 0.2 M hydroxylamine and pure rhodopsin of known absorbances at 498 and 278 nm. From the measured absorbances in the mixture, we were able to determine the correct value for the protein 278-nm absorbance to within about 2%.

Preparation of Detergents. The detergents DTAB, TrTAB, and TeTAB were prepared by the following general procedure: cold, (0°) anhydrous trimethylamine (1.6 mol) was added to a mixture of 0.4 mol of the corresponding alkyl bromide and 100 ml of methanol at 0°, and the reaction mixture was refluxed for 4–6 hr in a round-bottom flask fitted with a Dry Ice–acetone condenser. Methanol and excess trimethylamine were evaporated under reduced pressure, and the white solid was washed exhaustively with diethyl ether. The product was three-times recrystallized from acetone–methanol.

Preparation and Purification of Lipids. Egg phosphatidylcholine (egg PC) was purified according to Singleton *et al.* (1965). The 1-palmitoyl-2-docosahexaenoylphosphatidylcholine (16:0–22:6-PC) was synthesized from 1-palmitoyllysophosphatidylcholine and docosahexaenoic acid by the general method of Cubero Robles and van den Berg (1969).

Synthetic dilinoleoylphosphatidylcholine (18:2–18:2-PC) and natural digalactosyl diglyceride from spinach chloroplasts were obtained from the Hormel Institute (Austin, Minn.). Phosphatidylethanolamine (PE) from *Escherichia coli* was extracted according to Bligh and Dyer (1959) and purified according to Hanahan *et al.* (1958). Whole lipids from purified ROS were isolated according to Folch *et al.* (1957), and neutral lipids (including retinaldehyde) were removed by chromatography on a short column of silica gel, eluting with chloroform. The polar lipids were then eluted with methanol, the solvent was evaporated, and the lipids were extracted into chloroform to remove silica gel.

The 11-*cis*-retinal was a generous gift of Paul Brown, Harvard University. Phosphate was determined according to the method of Bartlett (1959).

Freeze-Fracture Electron Microscopy. Single small droplets of the membrane suspension were mounted on copper disks and frozen rapidly in Freon-22 cooled by a liquid nitrogen bath. Freeze fracturing was performed in a Balzers instrument at −110° with no etching. Replication was done by platinum–carbon shadowing followed by carbon reinforcement. Replicas were cleaned by floatation on commercial bleach, washed in distilled water, and mounted on bare 300 mesh copper grids.

Results

Purification of Rhodopsin and Properties of the Lipid-Free Protein. Rhodopsin extracted from purified ROS membranes in DTAB solution and chromatographed on hydroxylapatite as described above eluted as a sharp peak with a small satellite band (Figure 1). The yield of rhodopsin from the column based on the amount loaded was typically 75%. The concentration of phosphate in the elution buffer was found to be critical; concentrations below about 15 mM caused excessive broadening and retention of the rhodopsin peak while concentrations above 15 mM resulted in coelution of other 278-nm-absorbing substances. Addition of 1 mM dithiothreitol or the use of TrTAB or TeTAB in place of DTAB in the elution buffer did not significantly affect the column behavior. The absorption spectrum of rhodopsin purified in this manner is shown in Figure 2. The spectral ratio A_{278}/A_{498} was typically 1.7, while that for A_{400}/A_{498} was 0.19.

Phosphate analysis of the purified protein after removal of inorganic phosphate by dialysis gave 0.2–0.8 mol of phosphate/mol of rhodopsin based on a rhodopsin extinction coefficient of 42,700 at 498 nm. This is an upper limit to the phospholipid content since the determined phosphate may well contain significant contributions from colloidal hydroxylapatite and/or phosphorylated rhodopsin.

As reported earlier (Hong and Hubbell, 1972), purified rhodopsin in DTAB solutions is stable at 4° and undergoes a rapid thermal denaturation in the dark at room temperature.

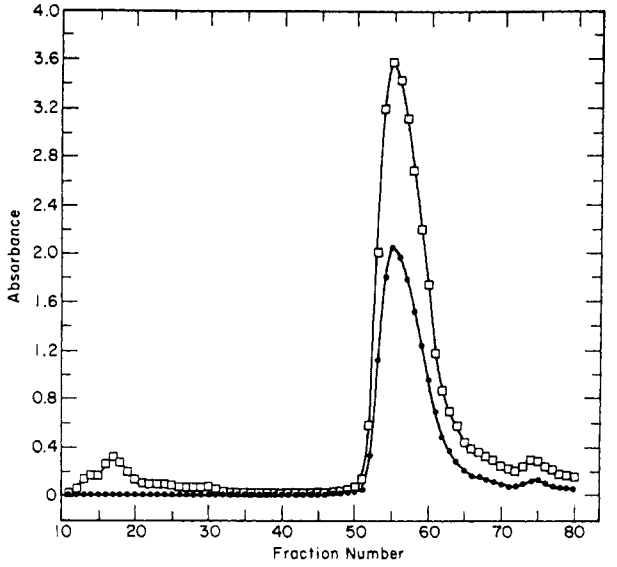

FIGURE 1: The elution profile of rhodopsin from the hydroxylapatite column: (□) 278-nm absorbance and (●) 498-nm absorbance of the fractions at 4°.

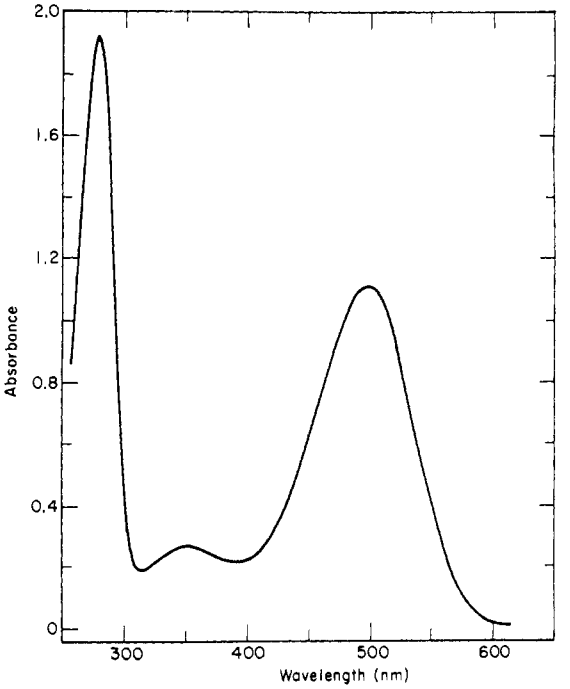

FIGURE 2: The absorption spectrum of purified rhodopsin in 100 mM DTAB–15 mM phosphate buffer (pH 6.6) at 4°.

The thermal stability of rhodopsin in solutions of alkyltrimethylammonium bromide detergents increases with increasing chain length and solutions of rhodopsin in 80 mM TeTAB may be stored at room temperature for at least several hours without a detectable decrease in the 498-nm absorbance. In addition, it is found that the thermal stability of rhodopsin in these detergents increases when the detergent concentration is increased from 100 to 500 mM.

Removal of detergent from rhodopsin solutions by dialysis against 5 mM Hepes buffer at 4° in the absence of added lipid leads to extensive denaturation of the protein with a recovery of rhodopsin less than *ca.* 45%. Addition of 1 mM dithiothreitol and 1 mM EDTA to the dialysis buffer increases the recovery of rhodopsin to 65%, and regeneration yields are as high as 48%.

Regenerability of ROS Membranes and Detergent Solutions of Rhodopsin. Regeneration of photolyzed rhodopsin in the purified ROS membranes by the methods described was 83 ± 5%. This value is presented here as a reference point for comparison with the regenerations obtained in the various rhodopsin systems considered

We have previously shown that DTAB is readily removed from rhodopsin or rhodopsin–lipid mixtures by dialysis (Hong and Hubbell, 1972) Thus it is possible to prepare solutions of phospholipid-free rhodopsin in other detergents by dialyzing a DTAB solution of the protein in the presence of the second detergent. We have used this procedure to prepare solutions of purified, phospholipid-free rhodopsin in lyso-PC and digitonin. Although these detergents dialyze very slowly relative to DTAB, some fraction is undoubtedly lost during dialysis. The recovery of rhodopsin in this procedure is 60–68% for lyso-PC and 93–95% for digitonin. Although rhodopsin is not regenerable in DTAB, the regeneration yields of phospholipid-free rhodopsin in lyso-PC and digitonin were 20–25 and 80–90%, respectively. Rhodopsin was not regenerable after bleaching in a solution containing 0.1 M DTAB and 2% digitonin. In some experiments, dithiothreitol and EDTA were included in the dialysis buffers. This modification had little effect on the recoveries or regeneration yields in these systems

Regenerability and Structure of Rhodopsin–Lipid Recombinants. It has been shown previously that purified, phospholipid-free rhodopsin in DTAB solution can be incorporated into bilayers of egg PC with complete removal of the detergent (Hong and Hubbell, 1972). We have now extended that work and recombined rhodopsin with synthetic 1,2-dilinoleoylphosphatidylcholine (18:2–18:2-PC), 1-palmitoyl-2-docosahexaenoylphosphatidylcholine (16:0–22:6-PC), a natural digalactosyl diglyceride, a mixture (1:1 mole ratio) of *E. coli* PE and egg PC and the mixture of phospholipids extracted from the ROS membranes. The values for the per cent recovery and regeneration after bleaching are given in Table I for different lipids and experimental conditions. Spectra of the bleached and regenerated egg PC recombinants solubilized in 80 mM TeTAB are shown in Figure 3. The spectra are similar in all cases.

Since the overall molecular organization in the recombinants (as opposed to specific noncovalent interactions of the rhodopsin with the lipids) could determine the physical-chemical properties of the protein, it is important to define the recombinant structures. We have employed the method of freeze-fracture electron microscopy to determine whether or not the bilayer configuration characterizes the various rhodopsin–lipid recombinants and to derive information regarding the location and distribution of rhodopsin within these structures. In this technique, frozen membranes and lipid bilayers are split along an interior plane, exposing extensive face views of the hydrophobic interior (Branton, 1971; Deamer *et al.*, 1970). When fracture proceeds along a plane in the center of a bilayer, etching exposes an additional face corresponding to the external bilayer surface (Pinto da Silva and Branton, 1970). Figure 4 shows representative electron micrographs of the freeze-fractured (unetched) digalactosyl diglyceride–rhodopsin recombinant and some of the phospholipid–rhodopsin recombinants. The morphologies of the 18:2–18:2-PC, 16:0–22:6-PC, and digalactosyl diglyceride recombinants in freeze-fracture are very similar and of these only the latter is shown (Figure 4a). In each case the fracture face is studded with particles approximately 110 Å in

TABLE I: Recoveries and Regeneration Yields of Rhodopsin in Recombinant Membranes.

Lipid	Addn to 5 mM Hepes Dialysis Buffer	% Recov	% Regeneration	No. of Determinations[a]
Egg PC	None	80–84	61–76	2
	1 mM Dithiothreitol	77–91	81–89	3
	1 mM EDTA	88–89	84–97	3
	1 mM Dithiothreitol 1 mM EDTA	88–91	82–92	5
18:2–18:2-PC	1 mM Dithiothreitol	<50		2
	1 mM Dithiothreitol 1 mM EDTA	77–83	83–84	2
16:0–22:6-PC	1 mM Dithiothreitol	<50		2
	1 mM Dithiothreitol 1 mM EDTA	83–89	69–72	2
Digalactosyl diglyceride	1 mM Dithiothreitol	<50		2
	1 mN Dithiothreitol 1 mM EDTA	78–84	71–77	2
Egg PC + *E. coli* PE	1 mM Dithiothreitol 1 mM EDTA	72–83	57–59	2
Extracted lipids from ROS	1 mM Dithiothreitol 1 mM EDTA	75–78	74–80	2
ROS[b]	1 mM Dithiothreitol 1 mM EDTA		83–88	3

[a] Each determination of recovery and regeneration was performed on a separate batch of the corresponding recombinant membrane. [b] ROS used for regeneration were not lyophilized, and were suspended in 5 mM Hepes buffer containing 1 mM dithiothreitol and 1 mM EDTA through the bleaching and regeneration treatments

diameter, and is qualitatively similar to that found for native membranes in freeze-fracture electron microscopy (Branton and Deamer, 1972). A second, smooth face is exposed on etching, characteristic of the bilayer fracture mode. Alterations of the fracture face are caused by etching, and micrographs from etching experiments are not shown. A detailed account and interpretation of these effects in rhodopsin–lipid systems will appear elsewhere (Chen and Hubbell, 1973).

The presence of PE in a recombinant results in a nonuniform distribution of particles in the fracture face as shown in Figure 4c,d. The nearly circular, smooth zones devoid of particles suggest the separation of another phase in the plane of the membrane. Freeze-fracture experiments on aqueous suspensions of phospholipid vesicles without incorporated rhodopsin show fracture and etch faces without particles, as reported by Deamer *et al.* (1970). An electron micrograph of a freeze-fractured (unetched) aqueous dispersion of digalactosyl diglyceride without incorporated protein is shown in Figure 4b. The pure lipid apparently forms extensive multilayered structures with closely apposed bilayers and smooth fracture faces.

Cross fractures in these preparations indicate that the rhodopsin-containing bilayers form closed surfaces, as do the lipids without incorporated protein. However, recombinant membranes tend to form small (*ca.* 4000 Å) vesicles bounded by a single bilayer while the parent lipids alone form large, multilayered liposomal structures (Figure 4b).

Discussion

Approximately 80–90% of the protein content of ROS membranes purified from bovine retinas is attributable to rhodopsin (Heitzmann, 1972; Kühn and Dreyer, 1972; Steinemann and Stryer, 1973). Thus, isolation of these membranes provides a preparation of rather high purity with respect to protein. Further purification of rhodopsin has been accomplished by chromatography on calcium phosphate gels (Shichi *et al.*, 1969) or hydroxylapatite (Hong and Hubbell,

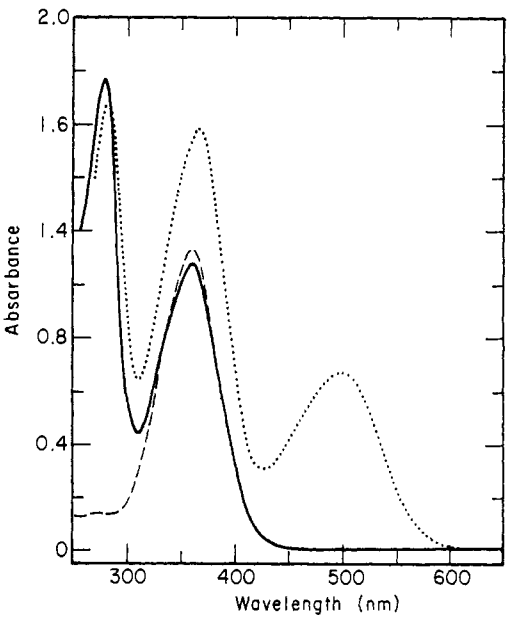

FIGURE 3: The absorption spectra of (a) the egg PC recombinant bleached in the presence of 0.2 M hydroxylamine and solubilized in 80 mM TeTAB–5 mM Hepes buffer containing 0.2 M hydroxylamine (pH 6.6) (solid line); (b) 11-*cis*-retinal in 80 mM TeTAB–5 mM Hepes buffer containing 0.2 M hydroxylamine (pH 6.6) (broken line) and (c) the regenerated egg PC recombinant solubilized in 80 mM TeTAB–5 mM Hepes buffer containing 0.2 M hydroxylamine (pH 6.6) (dotted line). The spectra were recorded at 20°.

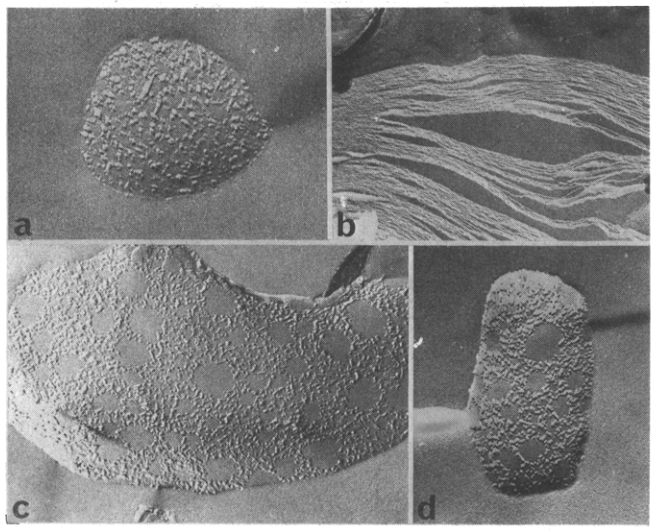

FIGURE 4: Freeze-fracture electron micrographs of (a) the digalactosyl diglyceride–rhodopsin recombinant, × 135,000; (b) fully hydrated digalactosyl diglyceride, × 105,000; (c) egg PC + *E. coli* PE recombinant, × 90,000; (d) ROS lipid recombinant, × 90,000.

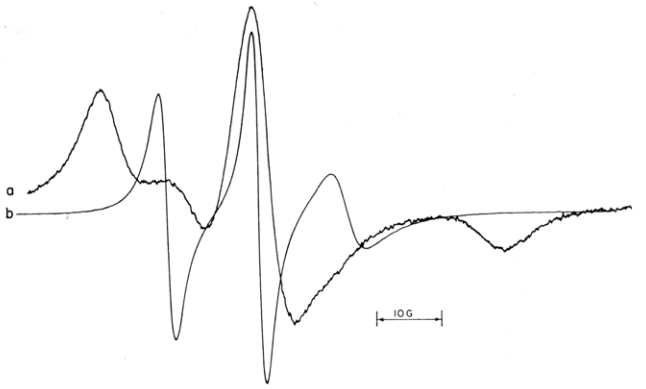

FIGURE 5: The paramagnetic resonance spectra of (7,4) βPC spin label solubilized in (a) digitonin micelles and (b) DTAB micelles. Both spectra were recorded at 20°.

1972; Robinson *et al.*, 1972), as well as differential detergent extraction (Zorn and Futterman, 1971). The usual criterion of purity is the ratio of absorbance at 278 nm to that at 498 nm, the lowest spectral ratio corresponding to the purest preparation. The lowest ratios reported in the literature for rhodopsin purified in different ways lie in the range 1.6–1.8. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of rhodopsin having a spectral ratio in this range indicates a material with a homogeneous monomer molecular weight (Heitzmann, 1972). Using Emulphogene BC 720, Shichi *et al.* (1969) observed a single protein band in acrylamide gel electrophoresis of rhodopsin having a spectral ratio of 1.75. In this system, the protein migration velocity is a function of both molecular size and charge, while migration velocity in sodium dodecyl sulfate is dependent on molecular size only. Considering the purity of the protein in the native membranes, the rather constant spectral ratios obtained for rhodopsin purified in different ways and the uniform migration of such purified protein in different gel systems, preparations having spectral ratios in the range 1.6–1.8 must represent an essentially pure material, and, as other workers, we have adopted the spectral ratio as a useful criterion of purity.

Chromatography on hydroxylapatite as described in the present paper yields rhodopsin with spectral ratios in the range 1.6–1.8, the usual value being 1.75. The purification scheme used here makes it possible to obtain concentrated solutions of purified protein directly from the column without the need for additional concentration steps. Absorbances at 498 nm of 1.3 are routinely obtained while in some experiments values greater than 3.0 have been realized.

An important aspect of the current chromatographic purification is that it yields rhodopsin nearly free of phospholipid, having a maximum content of 0.2–0.8 mol of phospholipid/mol of retinal.

A significant result of the present experiments is that phospholipid (in excess of 0.2–0.8 mol/mol of protein) is apparently not specifically required for high regenerability of rhodopsin. This is clearly demonstrated by the high recovery and regenerability of delipidated rhodopsin in both digitonin micelles and digalactosyl diglyceride bilayers. As far as the authors are aware, the structure of the digitonin micelle is not known. However, the paramagnetic resonance spectra of both a spin-labeled phospholipid (7,4) βPC (Hong and Hubbell, 1972), and a spin-labeled steroid, 5α17OH (Hubbell and McConnell, 1969), solubilized in digitonin indicate an extremely rigid structure, allowing little molecular motion within the micelle (see Figure 5a). In fact, these spectra are similar to those obtained for labels of this type in an isotropic glass at −180° (McConnell and McFarland, 1970). In contrast, these same spin labels exhibit nearly isotropic, unrestricted motion within the micelles of other detergents in which rhodopssin is not regenerable (see Figure 5b). Although the digitonin structure is doubtlessly very different in detail from the phospholipid bilayer, it provides a structurally rigid environment capable of maintaining the configuration of the bleached protein necessary for high regenerability.

The results from freeze-fracture and freeze-etch electron microscopy indicate that the phospholipids and digalactosyl diglycerides in the recombinant membranes are in the bilayer configuration, as anticipated from earlier work (Hong and Hubbell, 1972). The appearance of the particles in the fracture plane is correlated with the presence of incorporated rhodopsin, and suggests that the protein is, to some degree, localized within the hydrocarbon interior of both types of bilayer.

The fact that both the recovery and regeneration of rhodopsin are high in digalactosyl diglyceride as well as in phosphatidylcholine bilayers suggests that specific interactions between the polar head groups of the lipids and the protein are not dominant factors influencing the incorporation of stable, regenerable rhodopsin into lipid bilayers.

Table I presents the recoveries and regenerabilities of rhodopsin in the various membrane systems studied. The experiments with egg PC show that the use of either dithiothreitol or EDTA in the dialysis buffer during recombination results in an increased recovery and regeneration yield. Including both dithiothreitol and EDTA in the dialysis buffer does not produce a significant increase in recovery or regenerability above that for either compound alone. The data in Table I show that for 18:2–18:2-PC, 16:0–22:6-PC, and digalactosyl diglyceride recombinants, the presence of EDTA is required for a high recovery and regenerability. Although the data are not shown, the same is true for the PC + PE and the native lipid recombinants. In contrast with the egg PC, these lipids do not give high regenerability and recovery with dithiothreitol alone. Experiments are in progress to define the role of EDTA in the recombination process.

The kinetics and perhaps the mechanism of the recombi-

nation process itself doubtlessly depend on the structures of the intermediate detergent–lipid–rhodopsin phases and may thus vary with the lipid used. In principle, the detergent, temperature, dialysis rate, etc., may have to be optimized for each lipid in order to achieve maximum recovery and regenerability.

Thus, until these variables are fully investigated we hesitate to attribute the differences reported in Table I to intrinsic abilities of the lipids to maintain a regenerable state of the protein. The main point to be made is that in each case the recoveries and regenerabilities compared under the same conditions of recombination with dithiothreitol and EDTA are high and significantly greater than those obtained by detergent removal in the absence of added lipid.

The current experiments were designated to investigate the phospholipid requirements for rhodopsin stability and regenerability. From the above results, we must conclude that to maintain high regenerability of rhodopsin: (a) phospholipid is not specifically required; (b) when rhodopsin is incorporated into lipid bilayers, there is no specific requirement for a particular polar head group on the lipid; (c) in bilayers of pure phosphatidylcholines, a specific type or particular distribution of hydrocarbon chain is not required.

A common feature of the regenerable rhodopsin–lipid recombinants studied here is that the environment of the protein is a stable, organized lipid bilayer. Rhodopsin is also regenerable in digitonin solutions, a detergent that appears to be unusual in providing a rigid, stable hydrophobic environment for the protein. In other detergents of the type in which rhodopsin is not regenerable, the hydrophobic micelle interior is highly fluid and disordered, and the micelle lifetime is short (Jaycock and Ottewill, 1964; Nakagawa and Inoue, 1964). Since the thermal stability of rhodopsin increases with increasing detergent concentration, and increases with increasing chain length in the series of alkyltrimethylammonium bromides, the lack of regenerability may not be due to an adverse effect of the detergent on the protein, but rather that the micelle does not provide an adequate environment to maintain a regenerable conformation. These results suggest that high regenerability of rhodopsin depends on general structural features of the local environment and not on specific interactions with individual lipid (or detergent) molecules.

Acknowledgment

We thank Dr. Paul K. Brown for the generous gift of 11-*cis*-retinaldehyde, and Dr. Daniel Branton for making his freeze-fracture facilities available for our use.

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