# Effects of Lipid Environment on the Light-Induced Conformational Changes of Rhodopsin. 1. Absence of Metarhodopsin II Production in Dimyristoylphosphatidylcholine Recombinant Membranes†

Patricia A. Baldwin‡ and Wayne L. Hubbell*

*Department of Chemistry, University of California, Berkeley, California 94720*

*Received June 12, 1984*

ABSTRACT: Photolysis of bovine rhodopsin in dimyristoylphosphatidylcholine recombinant membranes results in the production of a relatively stable metarhodopsin I like photointermediate that decays slowly to a species with a broad absorbance maximum centered at about 380 nm [O'Brien, D. F., Costa, L. F., & Ott, R. A. (1977) *Biochemistry 16*, 1295–1303]. On the basis of the results of a variety of chemical and spectroscopic tests, we show that this process corresponds to the production of free retinal plus opsin and not to the slow production of metarhodopsin II. Electron spin resonance studies using a novel disulfide spin-label that is covalently linked to rhodopsin indicate that the apparent arrest of the protein at the metarhodopsin I stage is not due to simple aggregation of the protein in this short-chain, saturated lipid bilayer but must be understood in terms of the effect of the lipid host on the conformational energies of individual protein molecules. Limited production of metarhodopsin II is observed under acidic conditions. Thus, the rhodopsin–dimyristoylphosphatidylcholine recombinants offer a unique system for the study of the effect of the phospholipid bilayer environment on the conformation of an intrinsic membrane protein.

Rhodopsin is an integral protein of the disk membrane of the vertebrate rod outer segment (ROS).[1] It is the site of light absorption and is responsible for the initial event in signal transduction in the visual system. Data from proteolysis and chemical labeling (Fung & Hubbell, 1978) together with the recently determined amino acid sequence (Ovchinikov et al., 1982; Hargrave et al., 1982) suggest that rhodopsin spans the membrane with seven hydrophobic stretches of polypeptide that are separated by hydrophilic domains. Thus, rhodopsin is in intimate contact with the hydrophobic acyl chains of the bilayer membrane in which it is embedded, and its properties should be dependent on the physical and chemical nature of these phospholipids.

There are several lines of evidence that suggest that interaction with surrounding lipid may be particularly important for rhodopsin in the visual system. First, the acyl chain composition of the lipids in the retina is unique, containing a much larger percentage of long-chain, highly unsaturated fatty acids (particularly 22:6) than any other body tissue (Futterman et al., 1971). In addition, when rats are placed on a diet that contains no essential fatty acids, the percentage of these long-chain, unsaturated fatty acids remains high in the retina, apparently at the expense of other tissues (Futterman et al., 1971). Finally, in rats whose photoreceptor acyl chain composition was modified by dietary manipulation, the electroretinogram is found to be a function of the position and total number of double bonds in the dietary supplements (Wheeler et al., 1974). Thus, it seems likely that there are functionally significant features of the rhodopsin–phospholipid interaction.

Absorption of a photon causes rhodopsin to undergo a series of conformational changes through a sequence of intermediates that have been identified by their characteristic absorption spectra (Matthews et al., 1963; Yoshizawa & Wald, 1963; Busch et al., 1972). The interconversion from meta I (which has a $\lambda_{max}$ of 478 nm) to meta II ($\lambda_{max}$ of 380 nm) is the last step that occurs quickly enough to be involved in the triggering mechanism for visual excitation. The ability of this conformational change to take place is known to be affected by the environment of the protein. For example, when lipid- and detergent-free rhodopsin is illuminated, the normal meta I intermediate appears and then decays slowly not to meta II but to opsin plus free *all-trans*-retinal (Van Breugel et al., 1978). This sequence of events is also seen in photoreceptor membranes that have been treated with phospholipase C to create lipid-depleted membranous structures (Van Breugel et al., 1978). Rhodopsin has been recombined with a number of synthetic phospholipids, and in many cases a relatively normal meta I to meta II transition is observed (O'Brien et al., 1977; Applebury et al., 1974). Although there is no specific lipid head-group requirement for the occurrence of a normal transition, O'Brien et al. (1977) have shown that rhodopsin in recombinant membranes made with short-chain, saturated lipids (dilaurylphosphatidylcholine, dimyristoylphosphatidylcholine) appears to be arrested at the meta I stage after illumination and then slowly decays to a species that has an absorbance maximum at 380 nm.

This latter observation is a particularly striking example of the influence of bilayer structure on protein conformational states, and its further characterization is the subject of this

† This investigation was supported by National Institutes of Health Grants EY00729 and EY05216 and the Jules Stein Professorship Endowment.

* Address correspondence to this author at the Jules Stein Eye Institute and the Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024.

‡ Present address: Cancer Research Institute, School of Medicine, University of California, San Francisco, CA 94143.

[1] Abbreviations: ROS, rod outer segment; meta I, metarhodopsin I; meta II, metarhodopsin II; di-14:0-PC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DTAB, dodecyltrimethylammonium bromide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; DMF, dimethylformamide; ESR, electron spin resonance; meta III, metarhodopsin III.

0006-2960/85/0424-2624$01.50/0 

study. In particular, we present evidence suggesting that the arrest of the protein conformation at the meta I stage may be a thermodynamic rather than kinetic effect and that the 380-nm absorbing species slowly produced from meta I is free retinal rather than meta II. In addition, the thermodynamic arrest at meta I is not the result of mass protein aggregation (limited solubility) in the di-14:0-PC bilayer but rather a result of direct phospholipid effects on protein conformation.

In the following paper (Baldwin & Hubbell, 1985), we elucidate the features of the phospholipid bilayer that are required for the occurrence of the normal meta I to meta II transition.

These studies are important not only in terms of establishing a well-defined model system for the study of protein–lipid interaction but also to provide systems in which one can isolate particular rhodopsin conformational states for the investigation of rhodopsin–peripheral protein interaction.

## Experimental Procedures

*Materials.* Di-14:0-PC (A grade) was purchased from Calbiochem-Behring Corp. Phospholipase C from *B. cereus* and BSA (essentially fatty acid free) were obtained from Sigma Chemical Co. Frozen bovine retinas were obtained from American Stores Packing Co. (Lincoln, NE). Hydroxylapatite (DNA grade, Bio-Gel HTP) was purchased from Bio-Rad Laboratories, Richmond, CA. DTAB was prepared from anhydrous trimethylamine and dodecyl bromide (Eastman Organic Chemicals) by the procedure of Hong & Hubbell (1973). Egg phosphatidylcholine was purified according to Singleton et al. (1965).

*Preparation of Rod Outer Segment Membranes.* ROS disk membranes were isolated from frozen dark-adapted bovine retinas by modification of a method given previously (deGrip et al., 1980). All sucrose solutions were made with a buffer containing 20 mM MOPS, 1 mM $CaCl_2$, and 0.2 mM EDTA, pH 7.4, and all procedures involving rhodopsin were carried out under dim red light (Kodak series 1A filter) or in total darkness at 4 °C unless otherwise specified. Twenty-five milliliters of buffer containing 600 mM sucrose was added to 50 bovine retinas that had been thawed at room temperature. Rod outer segments were broken off by shaking in a Vortex mixer for 30 s, and the crude ROS fraction was separated from larger fragments by filtration through a Teflon screen (149-$\mu$m mesh). The Teflon screen was rinsed with 600 mM sucrose in buffer, and the total volume of the filtrate was brought to 70 mL. Four linear sucrose gradients were prepared from equal volumes of filtrate and 40% (w/w) sucrose in buffer, and the gradients were developed at 120000$g$ for 2 h. The ROS were collected from the uppermost dense band, diluted with an equal or greater volume of 600 mM sucrose in buffer, and then sedimented for 20 min at 17300$g$. The ROS were then lysed by two washes with dilute buffer (10 mM MOPS, pH 6.8). The resulting pellet either was then used directly for purification of rhodopsin or was washed once and suspended in 100 mM KCl–10 mM MOPS, pH 6.5, to give a concentrated stock suspension, which was used for absorption spectroscopy without further treatment. Absorbance ratios $A_{278}/A_{498}$ of 2.3–2.5 were typically obtained.

*Preparation of Recombinant Membranes.* Purification of rhodopsin and removal of phospholipids were accomplished by chromatography on hydroxylapatite according to the procedure of Hong & Hubbell (1973). In order to form the recombinant membranes, 2–3 mL of a solution containing di-14:0-PC, 100 mM DTAB, 15 mM sodium phosphate, and 1 mM dithiothreitol at pH 6.6 was combined with the purified, delipidated rhodopsin in detergent to give a lipid:rhodopsin molar ratio of 100:1. The solution of rhodopsin and lipid in detergent was mixed gently and then allowed to equilibrate for several hours at 4 °C. Detergent was removed by dialysis under a nitrogen atmosphere at 4 °C against 10 mM MOPS, pH 6.8. Dialysis was carried out for 4 days with 10 changes of buffer, and the resulting recombinant membranes were harvested by centrifugation at 48200$g$ for 30 min. Phosphate analysis (Bartlett, 1959) of the recombinant membranes was consistent with the initial lipid to protein ratio. In some cases, the pellet was resuspended in deoxygenated 10 mM MOPS, pH 6.8, and 2-mL aliquots were flash frozen in liquid nitrogen and stored at −20 °C for later use. No differences were detected between samples that had been frozen and those that were used immediately.

*UV–Visible Spectroscopy and Photolysis.* Di-14:0-PC–rhodopsin recombinant membranes were prepared for absorption spectroscopy by two washes with 100 mM KCl–10 mM MOPS, pH 6.5, and resuspension in this same medium to form a concentrated stock solution. Samples at pH 5.5 were prepared in the same way except that 10 mM MES buffer was used in place of MOPS. The recombinant membrane suspensions were annealed at 35 °C for 15 min to remove structural defects (Lawaczeck et al., 1976), slowly cooled to 4 °C, and sonicated (Sonifer Heat Systems-Ultrasonics, Inc., Plainview, NY) under nitrogen by using a microtip for cycles of 5 s separated by 25-s intervals. Total sonication time was 2 min. The samples were then annealed once more at 35 °C for 15 min and slowly cooled to 4 °C. Recombinant membranes were stored at 4 °C until use.

Spectral measurements of membrane suspensions were performed in an Aminco DW-2 UV–visible spectrophotometer with buffer as a reference. This instrument is particularly suited for measurement of turbid solutions since the cuvettes are placed immediately in front of the photomultiplier tube. No additional compensation for scattering was made. However, the apparent absorbance at 700 nm due to light scattering was adjusted to an arbitrary value near zero. For photolysis, samples were illuminated for 30 s with a 75-W high-pressure xenon arc lamp equipped with a heat filter (4-cm path of 0.5 M $CuSO_4$) and a 450-nm long-wave pass interference filter (03 LWP 001, Melles Griot) or for 10 s with a 60-W white light equipped with a heat filter; both methods gave similar results. Spectra were recorded from short to long wavelength at a scanning speed of 5 nm/s. The bandwidth of the measuring beam was set at 1.5 nm in order to limit regeneration and bleaching phenomena as much as possible. The total amount of rhodopsin plus isorhodopsin remaining after photolysis was determined by the recording of the absorption spectrum following decomposition of spectral intermediates with hydroxylamine (Van Breugel et al., 1979).

In some experiments, the pH of the sample was changed during the dark reactions following photolysis. Samples were made acidic by addition with gentle stirring of a solution containing 1.5 M glacial acetic acid and 0.5 M potassium acetate at pH 4.3, which lowered the pH of the sample to ≈5.5. Other samples were made basic by addition with gentle stirring of a solution containing 1.5 M Tris base and 0.5 M Tris-HCl at pH.8, which raised the pH of the sample to ≈7.5. Volume changes of the samples were less than 0.5% in all cases.

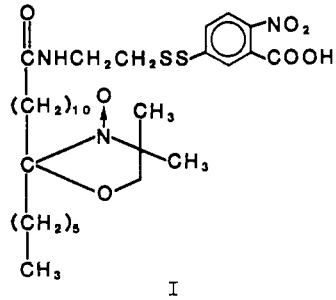
*Estimation of Protonation Changes of Rhodopsin in Response to Illumination.* Measurements of the light-induced pH changes of suspensions of di-14:0-PC recombinant membranes or ROS disk membranes were performed by the method of Bennett (1978). The samples had been prepared essentially as described for absorption spectroscopy; however, different

buffers were used (see Figure 3 legend), and the final annealing step was eliminated for the di-14:0-PC recombinants. The number of rhodopsin molecules bleached was determined from the change in the $A_{278}/A_{498}$ ratio of aliquots of the bleached and unbleached suspensions solubilized in 120 mM tridecyltrimethylammonium bromide, 250 mM hydroxylamine, and 10 mM MOPS, pH 6.8. The molar extinction coefficient at 498 nm for rhodopsin in this detergent is 42 700 (Hong & Hubbell, 1972).

*Bovine Serum Albumin Extraction of Retinal.* In order to determine whether the chromophore of the 380 nm absorbing species that was produced after bleaching could be extracted by BSA, the following procedure was employed. An aliquot of concentrated membrane suspension was diluted by addition of 10 mL of 2% (w/v) BSA in 100 mM KCl–10 mM MOPS, pH 6.5, and incubated for 15 min at 35 °C to extract free retinal that was present (Katre et al., 1981). The membranes were then pelleted, and the pellet was resuspended in 2% BSA in 100 mM KCl–10 mM MOPS, pH 6.5. The absorption spectrum of the sample was then taken, the sample was bleached, and several postillumination spectra were recorded vs. a reference solution of 2% BSA in buffer. The sample was then removed from the cuvette and centrifuged at 4 °C to separate the membranes and the BSA. A spectrum of the BSA-containing supernatant vs. 2% BSA in buffer was taken to determine the amount of retinal that had been extracted by the BSA. The membranes were resuspended in 2% BSA in buffer and a spectrum was taken. Hydroxylamine was then added as described above.

*Preparation of 1-(2-Aminoethyl)-2-(3-carboxy-4-nitrophenyl) Disulfide.* 5,5′-Dithiobis(2-nitrobenzoic acid) (1.98 g, 5 mmol) and 2-aminoethanethiol hydrochloride (0.568 g, 5 mmol) were combined in 50 ml of $H_2O$ with stirring at room temperature. Anhydrous $Na_2CO_3$ was added to raise the pH to 8. The flask was placed on ice for 1 h, and the solid was collected by filtration, washed with a small amount of cold $H_2O$, methanol, $CHCl_3$, and finally cold $H_2O$ again. The product was dried at 110 °C for 4 h and used without further purification.

*Preparation of Disulfide Spin-Label (I).* 12-Doxylstearic




I

acid [0.78 mmol; synthesized according to Waggoner et al. (1969)] was placed in a 10-mL pear-shaped flask. To this was added, with stirring at 0 °C, approximately 0.5 mL of dry dimethylformamide (DMF) and 0.78 mmol (109 μL) of triethylamine. Isobutyl chloroformate (0.78 mmol, 101 μL) in 0.5 mL of DMF was added, and the reaction was allowed to proceed for 20 min at 0 °C. This solution was added with stirring at 0 °C to 0.78 mmol (0.214 g) of 1-(2-aminoethyl)-2-(3-carboxy-4-nitrophenyl) disulfide in 0.5 mL of DMF containing 0.78 mmol of triethylamine. The reaction mixture was warmed to room temperature and allowed to stand in the dark with stirring for 16 h. The solids were removed by centrifugation. Distilled water (10 mL) was added to the supernatant, and the solution was acidified to between pH 1 and pH 2 with concentrated HCl, resulting in the separation of a yellow oil.

The yellow oil was purified by preparative TLC on 2 mm thick silica gel G plates (Analtech, Inc), which had been activated for 2 h at 110 °C. The plate was developed with $CHCl_3$–methanol–acetic acid (10:1:0.1) under dim red light in order to prevent light-induced decomposition of the disulfide spin-label ($R_f$ 0.49). The pure product had a negative reaction to ninhydrin and was quantitatively cleaved by dithiothreitol to yield 2-nitro-5-thiobenzoic acid as one of two products. The IR spectrum (film) showed the expected amide I absorption at 6.1 μm and strong bands at 3.30, 5.95, and 7.8 μm due to the hydrogen-bonded aryl carboxylic acid group and strong bands at 6.60 and 11.5 μm due to the aryl nitro group. The isotropic ESR spectrum in $CCl_4$ was indistinguishable from the 5,10-doxyl fatty acid. Spin-label concentrations were determined from the absorbance at 412 nm after cleavage of the spin-label with dithiothreitol at pH 8.0. Concentrations were calculated on the basis of an extinction coefficient of 13 600 (Ellman, 1959).

*Transmembrane Transport of the Disulfide Spin-Label (I).* In order to determine whether the spin-label (I) has access to both the inner and the outer surfaces of phospholipid vesicles when added externally, the procedure used by Chen & Hubbell (1978) to estimate the permeability of Ellman's reagent was employed. Briefly, the ability of the disulfide spin-label (I) (final concentration $2.4 \times 10^{-5}$ M) to react with glutathione (50 mM) trapped within the interior volume of sonicated egg phosphatidylcholine vesicles (3.4 mM lipid) was determined. In our experiments, the buffer used was 100 mM sodium phosphate–2 mM EDTA, pH 7.0. Reaction of the disulfide spin-label (I) with glutathione produces 2-nitro-5-thiobenzoic acid, and the progress of the reaction was determined on the basis of an extinction coefficient of 13 400 at 412 nm for 2-nitro-5-thiobenzoic acid in this buffer at pH 7.0 (Baldwin, 1983). This value differs from that given by Ellman (1959) because these experiments were performed at a lower pH.

*Electron Spin Resonance Experiments.* The disulfide spin-label in methanol was dried onto the wall of a centrifuge tube by removal of the methanol with a stream of nitrogen. To this tube was added a suspension of rhodopsin–di-14:0-PC recombinant membranes, which had been prepared as described for absorption spectroscopy except that 0.1 M Tris–maleate buffer (pH 7.0) was used. The spin-label was present in 10-fold excess over the amount of rhodopsin. After a 30-min incubation at room temperature, the sample was washed 3 times with 2% BSA in 0.1 M Tris–maleate (pH 7.0) and then once with buffer alone to remove all of the unreacted spin-label. Electron spin resonance (ESR) spectra of labeled rhodopsin in the recombinant membranes were taken on a Varian Model E-109 spectrometer operated in the X-band frequency range. The temperature was controlled to within ±0.1 °C with a stream of temperature-regulated nitrogen flowing past the sample cell. These experiments were conducted under dim red light, although photolysis of the di-14:0-PC recombinants had no effect on the observed ESR spectra at 15 or 30 °C.

*Phospholipase C Treatment.* Rhodopsin–di-14:0-PC recombinant membranes, prepared as described for absorption spectroscopy except for the use of 0.1 M Tris–maleate buffer (pH 7.0), were incubated with 0.25 unit of phospholipase C/nmol of rhodopsin at 30 °C (Olive et al., 1978). The extent of phospholipid hydrolysis was measured by phosphate determination of the supernatant (Bartlett, 1959).

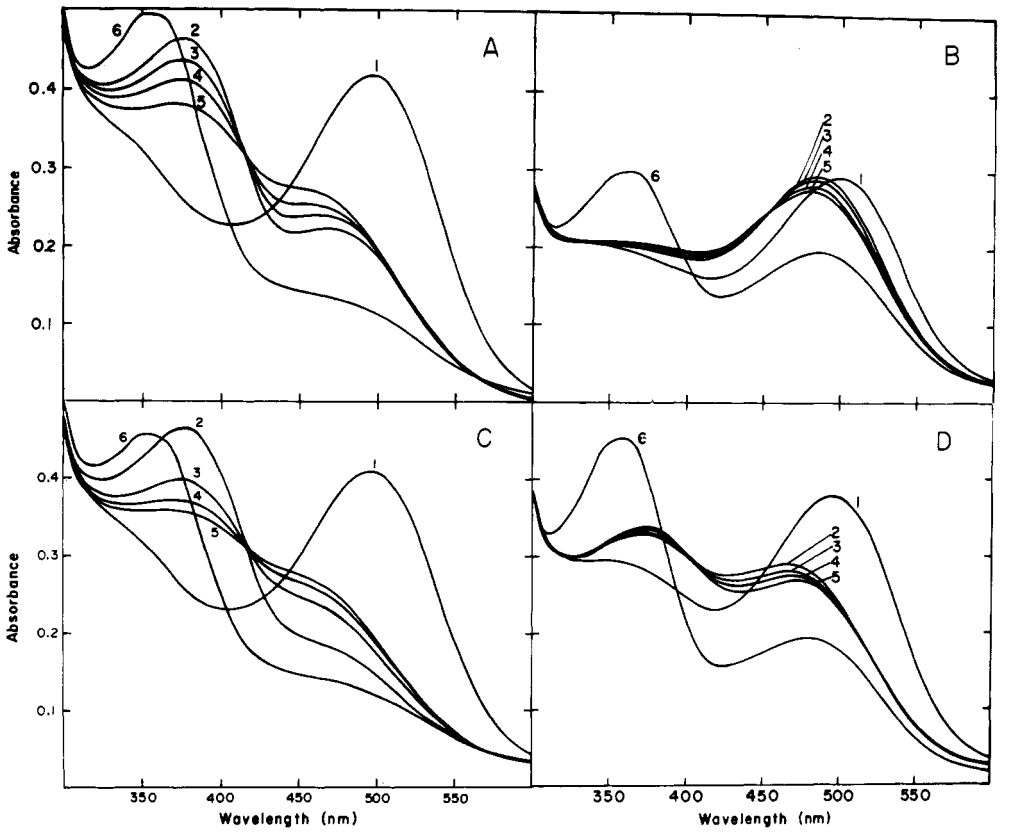



FIGURE 1: Absorption spectra of membrane suspensions in 100 mM KCl–10 mM MOPS, pH 6.5: (A) ROS disk membranes, 15 °C; (B) di-14:0-PC recombinant membranes, 15 °C; (C) ROS disk membranes, 30 °C; (D) di-14:0-PC recombinant membranes, 30 °C. Curve 1 is taken before illumination (see Experimental Procedures for illumination conditions). Curves 2–5 are scanned starting at about 1, 7 (4 min for C and D), 15 (7 min for C and D), and 30 min (10 min for C and D) after illumination, respectively. Curve 6, scanned in the presence of 50 mM hydroxylamine added after illumination, shows the amount of rhodopsin plus isorhodopsin (Van Breugel et al., 1978, Williams et al., 1973) remaining in the sample.

For ESR studies, rhodopsin–di-14:0-PC recombinant membranes spin-labeled as described above were incubated for 30 min with 0.25 unit of phospholipase C/nmol of rhodopsin in the ESR cavity at 30 °C, and then a spectrum was taken. The temperature of the sample was then lowered to 15 °C, and another spectrum was taken.

## Results

*Spectral Transitions Observed after Bleaching in Native and Recombinant Membranes.* The changes in absorption spectra observed upon illumination at 15 and 30 °C are shown in Figure 1 for ROS disk vesicles and for rhodopsin–di-14:0-PC recombinant membranes. For rhodopsin in native ROS membranes at 15 °C (Figure 1A), the first recording (ca. 1 min) after illumination shows the expected meta I and meta II populations. Between 1 and 30 min after illumination, the dominant process is the decay of meta II to meta III (Van Breugal et al., 1979). Thus, the characteristic spectral changes in the native system under these conditions show a *decrease* in absorbance at $\lambda \approx 380$ nm and increase at $\lambda \approx 465$ nm, the absorption maximum of meta III. However, a quite different picture emerges in the rhodopsin–di-14:0-PC recombinant membranes, which, at 15 °C, are below their 26 °C phase-transition temperature (Chen & Hubbell, 1973). As shown in Figure 1B, the predominant intermediate seen after illumination has a $\lambda_{max} \approx 480$ nm, and this decays very slowly to a species with a broad absorption maximum at $\lambda \approx 380$ nm. Thus, the recombinant membranes show an *increase* in absorbance at $\lambda \approx 380$ nm and a *decrease* at $\lambda \approx 480$ nm. To the extent that they can be compared, these findings are in agreement with the data of O'Brien et al. (1977), which showed a rapid (millisecond) increase in absorbance at 470 nm after illumination followed by its decay over several minutes with a concurrent rise in 380-nm absorbance.

Parts C and D of Figure 1 show the changes in absorption spectra observed upon illumination of ROS disk vesicles and rhodopsin–di-14:0-PC recombinant membranes at 30 °C, above the phase-transition temperature of the recombinant membranes (Chen & Hubbell, 1973). In the native system, the decay from meta II to meta III is again observed, although it occurs at a more rapid rate than at 15 °C. In the recombinant membranes at 30 °C, substantial amounts of intermediates absorbing at approximately 380 and 475 nm are seen in the first minute after illumination. The 475 nm absorbing species decays slowly to the species with a $\lambda_{max}$ at 380 nm. Thus, the spectral transition pattern for the di-14:0-PC recombinants is similar above and below the thermal phase transition, although the long wavelength absorbing pigment blue shifts with increasing temperature (see below).

We will follow the precedent of O'Brien et al. (1977) and refer to the pigment in these recombinant membranes that absorbs in the region 470–480 nm as meta I. This seems reasonable since the absorption maximum and rate of appearance (O'Brien et al., 1977) both match rather well those for meta I in native membranes and digitonin and these are the characteristics that serve to define the pigment. It must be remembered, however, that meta I is simply a "spectral intermediate", and we have no way of telling at this point whether or not the global protein conformation is the same in the meta I state in native and recombinant membranes. An interesting feature of meta I in the recombinant membranes is the rather large temperature dependence of the absorption

maximum, which blue shifts by about 5 nm when the temperature is changed from 15 to 30 °C. This is the temperature range in which di-14:0-PC undergoes a thermal phase transition and, hence, rather dramatic changes in lipid structural and dynamic characteristics, and this likely is the origin of the temperature-dependent changes in $\lambda_{max}$ in these membranes. The initial amount and rate of appearance of the 380 nm absorbing species are also quite temperature dependent in this same range.

Another interesting feature of the bleaching process in the recombinant membranes is the large proportion of rhodopsin that remains unbleached after photolysis compared to that seen in the native membranes (spectrum 6, Figure 1A–D). This is an expected consequence of the presence of a longer lived meta I species in the recombinant membranes that can be photoregenerated to rhodopsin and isorhodopsin (Hubbard & Kropf, 1958). Under our illumination conditions ($\lambda \geq 450$ nm), meta II is not photoreversible, and thus, its formation allows almost complete bleaching in the native system.

*Identification of the 380 nm Absorbing Species Observed after Illumination of Rhodopsin–Di-14:0-PC Recombinant Membranes.* In native ROS membranes or digitonin extracts of rhodopsin, the presence of the meta II species after illumination is confirmed by three distinctive characteristics in addition to the $\lambda_{max}$ at 380 nm. First, meta II is in pH-dependent equilibrium with meta I (Matthews et al., 1963), and in conjunction with this pH-dependent behavior, protons are taken up from solution during the meta I to meta II transition (Wong & Ostroy, 1973; Emrich & Reich, 1974; Bennett, 1978, 1980). Second, irradiation of meta II with ultraviolet light causes formation of meta III, rhodopsin, and isorhodopsin (Matthews et al., 1963). Third, the retinal chromophore of meta II is covalently attached to the protein through a Schiff base linkage (Doukas et al., 1978). In order to determine the identity of the 380 nm absorbing species seen in the recombinant membranes, the experiments described below were performed to see whether it exhibited these known characteristics of meta II.

As a point of comparison with the recombinant membranes, Figure 2A illustrates the well-known pH dependence of the meta I and meta II populations as observed in native ROS membranes, in this case, at 3 °C. When the recombinant membranes are illuminated at 3 °C, pH 6.5, most of the absorbance seen initially is at 480 nm (Figure 2B). In contrast to the native system, addition of acid results in only a slight increase in 380-nm absorbance. This slight increase is due, at least in part, to the decay of the 480-nm species over the time that it took to add the acid. When rhodopsin–di-14:0-PC recombinant membranes are illuminated at 30 °C, pH 6.5, both meta I and the 380 nm absorbing species are present within 1 min after illumination. Addition of base does not result in an increase in the absorbance due to meta I (Figure 2C) as it does in the native membranes. Instead, the decay to the 380 nm absorbing species continues as before, and the decrease in the meta I absorbance after base addition is as predicted by the decay kinetics calculated from Figure 1C for the recombinant at 30 °C. These results suggest that the 380 nm absorbing species is not meta II and that meta II is not detected in the equilibrium mixture within the experimental error of this assay (±5%).

Consistent with this lack of pH-dependent behavior is the fact that protons are not taken up from solution during the formation of the 380 nm absorbing pigment as they are in the native system (Bennett, 1978, 1980). In fact, as shown in Figure 3A, at pH 6.5 and 35 °C, di-14:0-PC recombinant

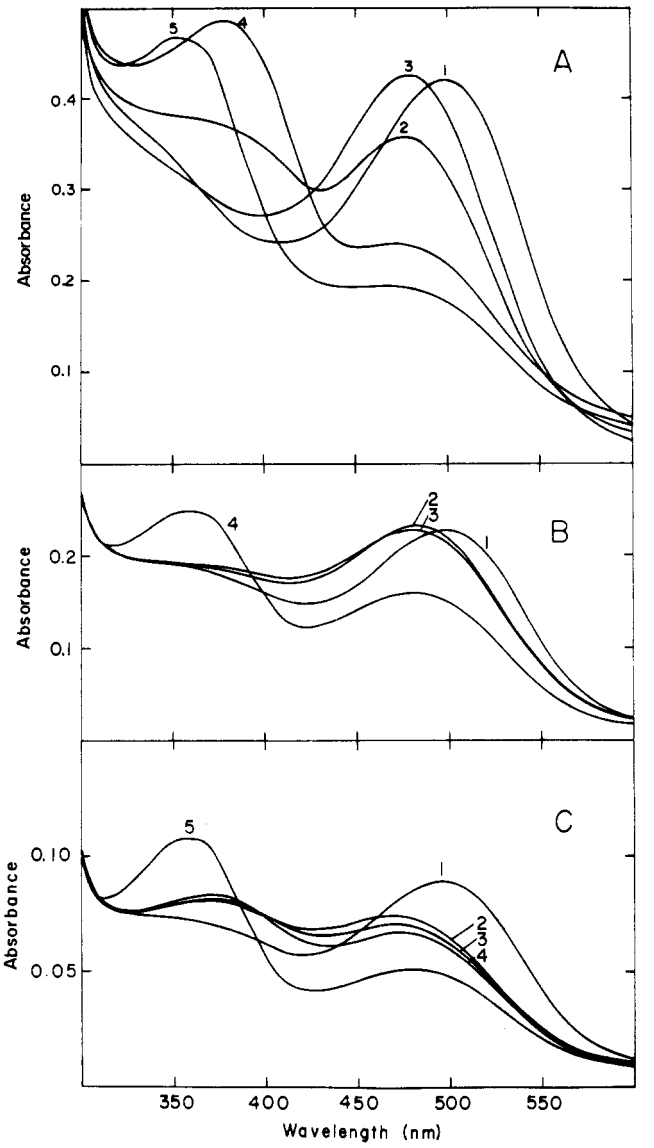



FIGURE 2: Effect of pH changes on the spectral intermediates observed after illumination of membrane suspensions that were initially at pH 6.5. In each case, curve 1 is recorded before illumination, and curve 2 is scanned starting 1 min after illumination started. The final curve in each series is in the presence of 50 mM hydroxylamine added after illumination. (A) ROS disk membranes at 3 °C. After curve 2 was recorded, the pH was raised to ≈7.5, and curve 3 was recorded. Curve 4 was recorded after the pH was lowered to ≈5.5 in a separate experiment. (B) Di-14:0-PC recombinants at 3 °C. After the recording of curve 2, the pH was lowered to 5.5, and curve 3 was recorded. (C) Di-14:0-PC recombinant membranes at 30 °C. Curve 3 was taken at about 12 min after illumination. After recording of curve 3, the pH was raised to ≈7.5, and curve 4 was taken 17 min after illumination.

membranes *release* approximately 0.75 proton per rhodopsin bleached. In the native system under these same conditions, approximately 0.8 proton per bleached rhodopsin is *taken up* (Bennett, 1978). This proton release increases in magnitude and rate with increasing temperature and thus may be related to the appearance of the 380 nm absorbing species.

The photochemistry of the 380 nm absorbing species also supports the hypothesis that it is not meta II. For example, when rhodopsin–di-14:0-PC recombinant membranes are bleached at 30 °C to produce some of the 380 nm absorbing species and then illuminated with ultraviolet light, the overall absorbance simply decreases due to photodestruction as shown in Figure 4A. Such photodestruction is well-known for free but not for protein-bound retinals (Chen & Hubbell, 1978).

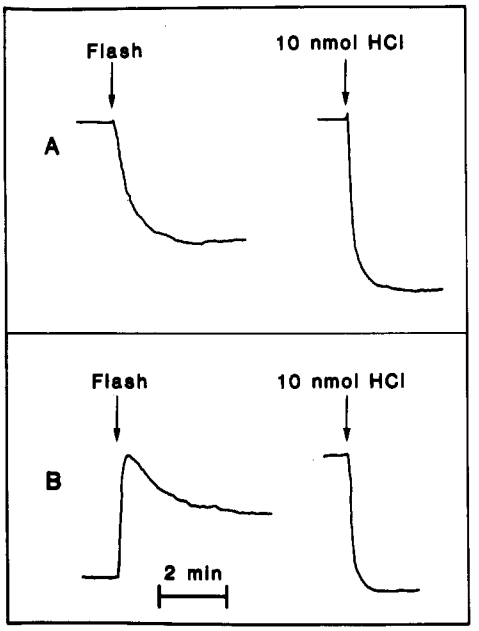



FIGURE 3: pH meter recording of the light-induced changes of protonation state of membrane suspensions at 35 °C. Samples were illuminated by a 600-$\mu$s flash from a xenon flashlamp, which bleached 50% of the rhodopsin. After illumination, injections of 10 nmol of HCl (10 $\mu$L of a 1 mM solution) were made in order to calibrate the signal. (A) Di-14:0-PC recombinant containing 23.4 nmol of rhodopsin in 150 mM NaCl–1 mM MOPS, pH 6.5. (B) Di-14:0-PC recombinant containing 18.6 nmol of rhodopsin in 150 mM NaCl–1 mM MES, pH 5.5.

No increase in longer wavelength species is observed, and the amount of rhodopsin plus isorhodopsin observed after the addition of hydroxylamine is actually less in this case (compare Figures 4A and 1D). For comparison, Figure 4B illustrates the photoreaction of meta II in native ROS membranes upon exposure to ultraviolet light, with the resulting formation of meta III, rhodopsin, and isorhodopsin. This process was originally observed by Matthews et al. (1963) for digitonin extracts of rhodopsin.

Clearly, by these criteria, the 380 nm absorbing species seen after bleaching of rhodopsin–di-14:0-PC recombinant membranes does not display the expected characteristics of meta II. Thus, it seems likely that the absorbance is due to retinal that has detached from the protein. If the chromophore has, in fact, detached from opsin, then it should be possible to extract it from these membranes with bovine serum albumin. BSA has previously been used to remove excess retinal from bacteriorhodopsin-containing membranes after regeneration (Katre et al., 1981). Control experiments using native ROS disk membranes demonstrate that BSA is not capable of extracting retinal when it is covalently bound to the protein in the meta I and meta II states. However, when di-14:0-PC recombinant membranes that are suspended in 2% BSA are bleached and the membranes are removed by centrifugation, almost all of the 380-nm absorbance that had appeared after illumination is found in the BSA-containing supernatant. As expected, the meta I absorbance, which is not affected by the BSA treatment, is observed in a spectrum taken of the membrane pellet. Thus, a major portion of the 380 nm absorbing species that results from the decay of meta I is not meta II but rather is retinal that is no longer covalently bound to the protein.

*Production of Meta II in Di-14:0-PC Recombinant Membranes at Low pH.* After illumination at 30 °C of rhodopsin–di-14:0-PC recombinant membranes at pH 5.5, the spectra shown in Figure 5 are obtained. At this lower pH, a slight

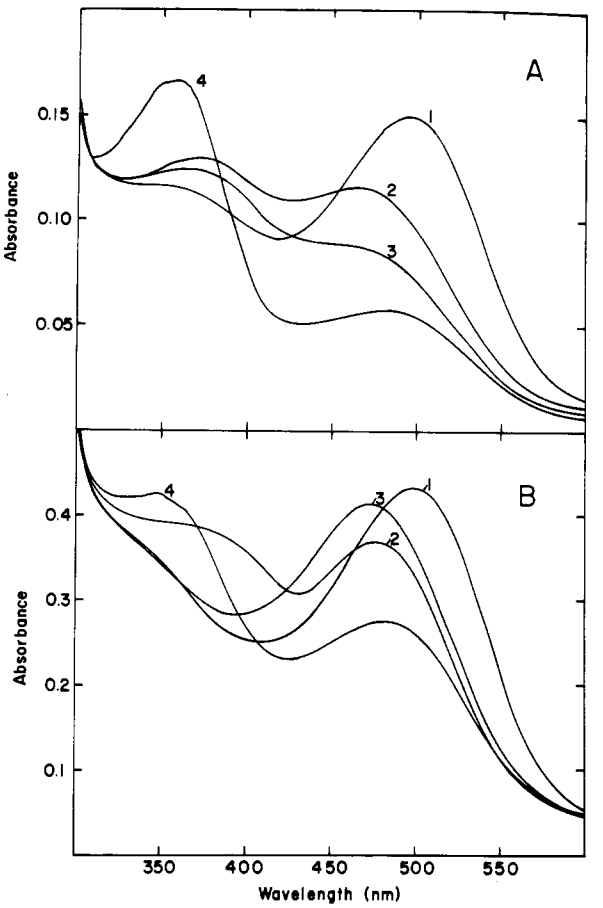



FIGURE 4: Effect of ultraviolet illumination on the spectral intermediates observed after photolysis. Curve 1 is recorded before illumination. Curve 2 is scanned starting 1 min after illumination with visible light. After curve 2 was recorded, the samples were illuminated a second time for 1 min with a 75-W high-pressure xenon arc lamp fitted with a band-pass filter centered at 360 nm (Schott Glass, UG 1) and a $CuSO_4$ heat filter. Curve 3 was taken immediately following this illumination. Curve 4 is in the presence of 50 mM hydroxylamine added after illumination. (A) Suspension of di-14:0-PC recombinant membranes at 30 °C. The decrease in absorbance in the 380-nm region seen in the recombinant membranes is due to the conversion of free *all-trans*-retinal to cis isomers (Matthews et al., 1963) and photodestruction of retinal (Chen & Hubbell, 1978). The absorbance decrease seen at longer wavelengths probably occurs as a result of bleaching of the $\beta$-bands of meta I and the residual rhodopsin and isorhodopsin. (B) Suspension of ROS disk membranes at 3 °C.

*decay* at 380 nm and a concurrent *increase* in absorbance at $\lambda \approx 465$ nm are seen during the first 3–4 min after illumination. The initial production of $\lambda_{max} \approx 465$ nm pigment at the expense of a $\lambda_{max} \approx 380$ nm pigment is a sequence observed in the native system corresponding to the meta II to meta III transition and suggests the possibility that at acidic pHs some meta II is being produced in the recombinant membranes. It is unlikely that the decay at 380 nm could be due to retinal, since free retinal itself is never observed to decay to a species absorbing at longer wavelengths. At later times, a decay at the longer wavelength is observed concurrent with an increase in absorbance at 380 nm, perhaps as a result of the decay of the meta III species.

Data on proton uptake in di-14:0-PC recombinant membranes at pH 5.5 is also consistent with the apparent production of meta II. Figure 3B shows that a rapid uptake of 0.75 proton per bleached rhodopsin is observed at 35 °C and pH 5.5. This uptake is followed by a release of 0.35 proton per rhodopsin, perhaps due to the decay of meta II (Bennett, 1980). Unlike the proton uptake in the native system, which

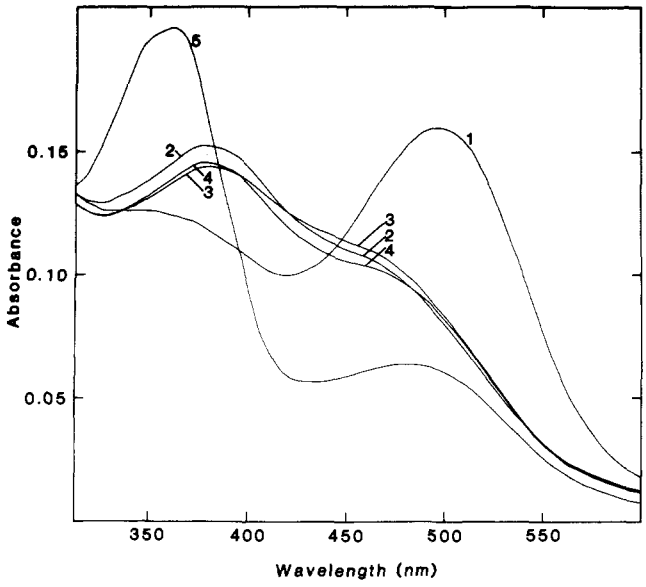



FIGURE 5: Absorption spectra of di-14:0-PC recombinant membranes in 100 mM KCl–10 mM MES, pH 5.5, at 30 °C. Curve 1 is taken before illumination. Curves 2–4 are scanned starting about 1, 4, and 10 min after illumination, respectively. Curve 5 is in the presence of 50 mM hydroxylamine added after illumination.

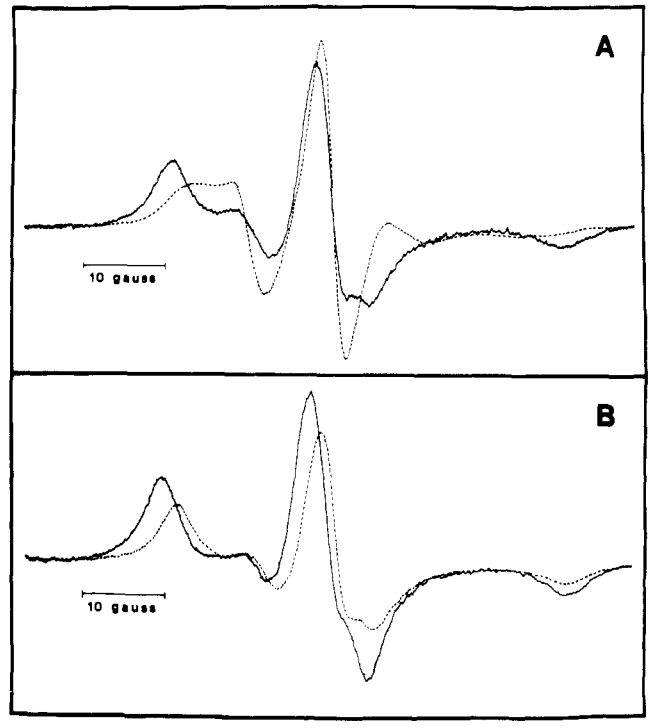



FIGURE 6: Electron spin resonance spectra of the disulfide spin-label (I) after reaction with rhodopsin. Approximately two sulfhydryl groups per opsin are spin-labeled. (A) 30 °C; (B) 15 °C. In each panel, the spectrum represented by the dashed line was taken in di-14:0-PC recombinant membranes, and the spectrum represented by the solid line was taken after 75% of the lipids were hydrolyzed by phospholipase C digestion, resulting in two-dimensional lateral aggregates of rhodopsin. In (A) the two spectra are aligned at the center line zero crossing point. In (B) they are aligned with respect to the high-field extrema to emphasize the difference in the overall separation of the outer hyperfine extrema.

is relatively temperature independent (Bennett, 1978), the proton uptake observed in the recombinants at pH 5.5 increases as a function of temperature. For example, at 15 °C, fewer than 0.1 proton per bleached rhodopsin is taken up, and a series of spectra similar to those observed at pH 6.5 are seen after illumination (data not shown).

*Determination of Aggregation State of Rhodopsin in Di-14:0-PC Recombinant Membranes.* The slow decay of meta I to free retinal plus opsin after photolysis has previously been observed in lipid- and detergent-free preparations of rhodopsin where the protein is highly aggregated (Van Breugel et al., 1978). This raises the question as to whether or not aggregation of rhodopsin in the recombinant bilayer is responsible for the stabilization of meta I and its consequent decay to free retinal. Earlier freeze–fracture (Chen & Hubbell, 1978) and saturation-transfer ESR studies of spin-labeled rhodopsin in recombinant membranes (Davoust et al., 1980; Kusumi et al., 1980; Kusumi & Hyde, 1982) suggest that this is not the case above the thermal phase transition temperature of the membrane. In this work, we adopt an approach to investigate the degree of rhodopsin aggregation similar to that of Favre et al. (1979) but employing the new disulfide spin-label (I), which reacts with the rhodopsin sulfhydryl groups with high selectivity. The reactive rhodopsin sulfhydryls are located close to the bilayer surface (Rousselet & Devaux, 1978), and the long axis of I is constrained to lie near the rhodopsin surface within the hydrocarbon region of the membrane. The mobility of this molecule, assayed by the ESR spectrum, is a direct reflection of molecular motion at the protein surface. This motion is dramatically influenced by protein aggregation [Favre et al. (1979) and below].

Since the rhodopsin in the recombinant membranes employed here is oriented symmetrically in the membrane, sulfhydryl groups capable of reacting with the disulfide spin-label are found on both the inner and outer surfaces of the bilayer (Fung & Hubbell, 1978). In order to determine whether the disulfide spin-label (I) has access to both orientational populations of sulfhydryls, we employed the method of Chen & Hubbell (1978) to investigate the reaction of externally added disulfide spin-label (I) with glutathione trapped in the internal space of phosphatidylcholine vesicles. Reaction between the spin-label and the trapped glutathione is observed (90% complete within 15 min), demonstrating that the spin-label is capable of diffusion across the bilayer to the internal space of the vesicles. A control experiment using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)], which has been shown to be incapable of permeating sonicated phospholipid vesicles (Chen & Hubbell, 1978), confirms that the glutathione is trapped inside the vesicles. These experiments demonstrate the high membrane permeability of the disulfide spin-label in phosphatidylcholine vesicles. The permeability of the rhodopsin-containing phosphatidylcholine vesicles is expected to be even higher due to the presence of protein, and we therefore expect this spin-label to react with both orientational populations of rhodopsin. Under the labeling conditions used for the ESR experiments, two sulfhydryl groups per rhodopsin molecule were labeled as determined from the absorbance of the reaction product 2-nitro-5-thiobenzoic acid.

The ESR spectrum of spin-labeled rhodopsin in di-14:0-PC recombinant membranes at 30 °C, shown by the dashed line in Figure 6A, demonstrates that the spin-label is in a mobile environment (Berliner, 1976). In order to compare this spectrum to that which would be obtained from aggregated rhodopsin, recombinant membranes were digested with phospholipase C, which results in the formation of extensive two-dimensional lateral aggregates of rhodopsin molecules (Olive et al., 1978). The spectrum of spin-labeled rhodopsin in di-14:0-PC obtained after 30 min of phospholipase C treatment (≥75% lipid hydrolysis) is shown by the solid line in Figure 6A and indicates highly restricted motion due to the aggregation. Comparison of the two ESR spectra affords the

conclusion that the disulfide spin-label attached to rhodopsin in di-14:0-PC recombinant membranes that are above their phase transition temperature is in a much more mobile environment than the spin-label attached to aggregated rhodopsin molecules. This more mobile environment must be provided by lipids surrounding the protein.

In the ESR spectrum of spin-labeled rhodopsin in di-14:0-PC recombinant membranes at 15 °C (below their phase transition temperature), the splitting between the outer hyperfine extrema is 61.6 G (Figure 6B). By contrast, after phospholipase C treatment, the splitting of the outer hyperfine extrema has increased to 63.6 G. This significant difference demonstrates that rhodopsin is not extensively aggregated even below the phase transition, although the motion of the spin-label does decrease considerably at the lower temperature. This result is consistent with the saturation-transfer ESR data of Davoust et al. (1980) on rhodopsin in di-14:0-PC, which shows only a progressive change in rotational correlation time of the protein through the phase transition.

## Discussion

A principle conclusion based on the results presented above is that the $\lambda \approx 380$ nm absorbing species appearing in the rhodopsin–di-14:0-PC recombinant membranes after photolysis at pH 6.5 is predominantly, if not entirely, free retinal rather than the usual meta II species. This conclusion apparently holds both above and below the phase-transition temperature of the recombinant membranes at pH 6.5. The primary evidence for this conclusion is the extractability of the 380 nm absorbing chromophore with BSA, the lack of photoreversibility upon UV illumination, and the absence of a detectable equilibrium between meta I, the 380 nm absorbing species, and solution protons in the physiological pH range. Consistent with this latter observation is the absence of proton uptake during the production of the 380 nm absorbing species in the physiological pH range. In fact, at pH 6.5, proton release is observed in the same time domain as the appearance of the 380 nm absorbing species. Although we do not understand the mechanism for this proton release and how it relates to the production of retinal, it seems unlikely that it is due to protein denaturation given that the rhodopsin in these recombinant membranes is highly regenerable (Chen & Hubbell, 1973). On the basis of the kinetic data of O'Brien et al. (1977) and the rapidity of the proton release, it also seems unlikely that this proton release is due to production of meta III.

The mechanism by which free retinal is produced at physiological pH is unclear. Direct hydrolysis of the protonated Schiff base in meta I (Doukas et al., 1978) has not been previously reported, although a recent study has suggested that the chromophore in the meta I state is accessible to hydroxylamine in native membranes (Ratner et al., 1981), and if so, it might also be accessible to water. Note however that simple hydrolysis of a protonated Schiff base at pH 6.5 is not expected to evolve protons as is observed experimentally. Thus, if this mechanism is to operate, the protons must be evolved as the result of concomitant protein conformational changes. Another possible mechanism for retinal production is through a small amount of meta II, which is undetectable in our experiments (<5% of the total bleached rhodopsin). Free retinal has previously been reported as one of the products of meta II decay in both bovine and frog rods (Van Breugel et al., 1979; Blazynski & Ostroy, 1981; Chabre & Breton, 1979).

At higher temperatures a significant peak rapidly appears at 380 nm after photolysis followed by a relatively slow increase in absorbance at this wavelength. This is suggestive of more than one kinetic population of rhodopsin molecules as has been previously postulated (Hoffmann et al., 1978).

The results presented above make it very clear that the meta I/meta II ratio in di-14:0-PC recombinant membranes is extremely high compared to that of native membranes. That is, the standard free energy of meta II in di-14:0-PC membranes is high relative to that of meta I in either gel or liquid-crystalline phases and high relative to that of meta II in native membranes. This fact along with the result that the meta I molecule in fluid di-14:0-PC bilayers is not phase separated leads to the important conclusion that the relative free energies of meta I and meta II are modulated by interaction with the bilayer.

It is of interest to consider the general nature of protein–bilayer interactions that might give rise to such conformational control. We discuss here only the simplest model based on the increase in partial molar volume of opsin in the meta I to meta II transition. As a result of the positive volume change, the conformation equilibrium will be pressure dependent; as pressure increases, the meta II population decreases. Now consider a cylindrical hydrophobic protein of radius $r$ imbedded in and passing through a lipid bilayer. The interface between the protein and liquid hydrocarbon can be characterized in terms of an interfacial free energy (interfacial tension) $\gamma_{pl}$. For a finite interfacial tension at a curved surface it is well-known that there exists an equilibrium pressure differential across the interface (Guggenheim, 1967). For a cylindrical surface, $P_p = P_l + \gamma/r$, where $P_p$ and $P_l$ are the pressures within the protein and lipid phase, respectively. Due to the very small radius of curvature of the protein, quite modest interfacial tensions produce extremely large effective pressures on the protein molecule. For example, for $\gamma_{Pl}$ of 20 dyn/cm, $P_p - P_l = 100$ atm. Now the free-energy difference between meta II and meta I is pressure dependent for the reason discussed above, and the free-energy difference between meta II and meta I, $\Delta G = (G_{\mathrm{MII}} - G_{\mathrm{MI}})$, is just

$$\Delta G = \Delta G' + N_0 \pi \delta \gamma_{pl} \Delta r$$

where $\Delta G'$ is the free-energy difference when $\gamma_{pl} = 0$, $N_0$ is Avogadro's number, $\delta$ is the length of the cylindrical protein, and $\Delta r = r_{\mathrm{MII}} - r_{\mathrm{MI}}$; where $r_{\mathrm{MII}}$ and $r_{\mathrm{MI}}$ are the radii of meta II and meta I, respectively. Energy shifts ($\Delta G - \Delta G'$) of the order of a few kilocalories are certainly sufficient to make meta II low enough in concentration to be undetectable in our experiments. Values of the parameter $\gamma_{pl}\Delta r \geq 10^{-7}$ dyn are sufficient to achieve this condition. For example, if $\delta = 50$ Å for rhodopsin and if $r$ increased by just 2 Å in the meta I to meta II transition, the energy shift would amount to 2.3 kcal for a relatively low $\gamma_{pl}$ of 10 dyn/cm. It is unlikely that the conformational changes could exceed a radius change of more than an angstrom or two, since this corresponds to a change in circumference equivalent to one or two phospholipid molecules and bleaching of rhodopsin does not seem to be accompanied by a significant change in the number of boundary lipids. Such effects are in the correct range to account for the observed behavior. In this simple thermodynamic model, $\gamma_{pl}$ is a measure of the solvation energy of the protein surface by phospholipids in the bilayer. A poor solvation leads to a high $\gamma_{pl}$ and a high internal protein pressure. In the present case, the result is an arrest at the meta I conformer.

Decreasing the pH of the medium from 6.5 to 5 appears to increase the population of meta II to detectable levels. This interesting result suggests that the free-energy shift of meta II due to the lipid bilayer is being compensated for by the increase in proton free energy due to the concentration increase (corresponding to about 2 kcal). This implies that the free-

energy shift of meta II relative to meta I due to di-14:0-PC is of the order of a few kilocalories at most, depending on what one assumes for the proton uptake stoichiometry during the meta I to meta II transition.

To go beyond the simple thermodynamic picture, that is, to interpret the molecular origin of $\gamma_{pl}$ on a molecular level, requires a systematic investigation of the behavior of rhodopsin in bilayers of different composition. This is the subject of the following paper (Baldwin & Hubbell, 1985).

**Registry No.** I, 95799-87-4; di-14:0-PC, 18194-24-6; 1-(2-aminoethyl)-2-(3-carboxy-4-nitrophenyl) disulfide, 71899-86-0; 5,5′-dithiobis(2-nitrobenzoic acid), 69-78-3; 2-aminoethanethiol hydrochloride, 156-57-0; 12-doxylstearic acid, 29545-47-9; triethylamine, 121-44-8; isobutyl chloroformate, 543-27-1; *all-trans*-retinal, 116-31-4.

## REFERENCES


Applebury, M. L., Zuckerman, D. M., Lamola, A. A., & Jovin, T. M. (1974) *Biochemistry 13*, 3448–3458.

Baldwin, P. A. (1983) Ph.D. Thesis, University of California, Berkeley, CA.

Baldwin, P. A., & Hubbell, W. L. (1985) *Biochemistry* (following paper in this issue).

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bennett, N. (1978) *Biochem. Biophys. Res. Commun. 83*, 457–465.

Bennett, N. (1980) *Eur. J. Biochem. 111*, 99–103.

Berliner, L., Ed. (1976) *Spin Labeling: Theory and Applications*, Academic Press, New York.

Blazynski, C., & Ostroy, S. E. (1981) *Vision Res. 21*, 833–481.

Busch, G. E., Applebury, M. L., Lamola, A. A., & Rentzepis, P. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2802–2806.

Chabre, M., & Breton, J. (1979) *Vision Res. 19*, 1005–1018.

Chen, Y. S., & Hubbell, W. L. (1973) *Exp. Eye Res. 17*, 517–532.

Chen, Y. S., & Hubbell, W. L. (1978) *Membr. Biochem. 1*, 107–130.

Davoust, J., Bienvenue, A., Fellmann, P., & Devaux, P. F. (1980) *Biochim. Biophys. Acta 596*, 28–42.

DeGrip, W. J., Daemen, F. J. M., & Bonting, S. L. (1980) *Methods Enzymol. 67*, 301–320.

Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Favre, E., Baroin, A., Bienvenue, A., & Devaux, P. F. (1979) *Biochemistry 18*, 1156–1162.

Fung, B. K.-K., & Hubbell, W. L. (1978) *Biochemistry 17*, 4403–4410.

Futterman, S., Downer, J. L., & Hendrickson, A. (1971) *Invest. Ophthalmol. 10*, 151–156.

Guggenheim, E. A. (1967) *Thermodynamics*, p 52, North-Holland, Amsterdam.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Mohana Rao, J. K., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.

Hoffmann, W., Siebert, F., Hofmann, K.-P., & Kreutz, W. (1978) *Biochim. Biophys. Acta 503*, 450–461.

Hong, K., & Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2617–2621.

Hong, K., & Hubbell, W. L. (1973) *Biochemistry 12*, 4517–4523.

Hubbard, R., & Kropf, A. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 130–139.

Katre, N. V., Wolber, P. K., Stoecknius, W., & Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4068–4072.

Kusumi, A., & Hyde, J. S. (1982) *Biochemistry 21*, 5978–5983.

Kusumi, A., Sadadi, T., Yoshizawa, T., & Ohnishi, S.-I. (1980) *J. Biochem. (Tokyo) 88*, 1103–1111.

Lamola, A. A., Yamane, T., & Zipp, A. (1974a) *Biochemistry 13*, 738–745.

Lamola, A. A., Yamane, T., & Zipp, A. (1974b) *Exp. Eye Res. 18*, 19–27.

Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313–330.

Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.

O'Brien, D. F., Costa, L. F., & Ott, R. A. (1977) *Biochemistry 16*, 1295–1303.

Olive, J., Benedetti, E. L., Van Breugel, P. J. G. M., Daemen, F. J. M., & Bonting, S. L. (1978) *Biochim. Biophys. Acta 509*, 129–135.

Ovchinikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Artamonov, I. D., Zolotarev, A. S., Kostina, M. B., Bogachuk, A. S., Moroshnikov, A. I., Martinov, V. I., & Kudelin, A. B. (1982) *Bioorg. Khim. 8*, 1011–1014.

Ratner, V. L., Bagirov, I. G., & Fesenko, E. E. (1981) *Vision Res. 21*, 251–253.

Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.

Van Breugel, P. J. G. M., Geurts, P. H. M., Daemen, F. J. M., & Bonting, S. L. (1978) *Biochim. Biophys. Acta 509*, 136–147.

Van Breugel, P. J. G. M., Bovee-Geurts, P. H. M., Bonting, S. L., & Daemen, F. J. M. (1979) *Biochim. Biophys. Acta 557*, 188–198.

Waggoner, A. S., Kingzell, T. J., Rottschaefer, S., Griffith, O. H., & Keith, A. D. (1969) *Chem. Phys. Lipids 3*, 245–253.

Wheeler, T. G., Benolken, R. M., & Anderson, R. E. (1975) *Science (Washington, D.C.) 188*, 1312–1314.

Williams, T. P., Baker, B. N., & Eder, D. J. (1973) in *Biochemistry and Physiology of Visual Pigments* (Langer, H., Ed.) pp 83–88, Springer-Verlag, New York.

Wong, J. K., & Ostroy, S. E. (1973) *Arch. Biochem. Biophys. 154*, 1–7.

Yoshizawa, T., & Wald, G. (1963) *Nature (London) 197*, 1279–1286.