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# Modulation of rhodopsin function by properties of the membrane bilayer

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**Abstract**

A prevalent model for the function of rhodopsin centers on the metarhodopsin I (MI) to metarhodopsin II (MII) conformational transition as the triggering event for the visual process. Flash photolysis techniques enable one to determine the [MII]/[MI] ratio for rhodopsin in various recombinant membranes, and thus investigate the roles of the phospholipid head groups and the lipid acyl chains systematically. The results obtained to date clearly show that the p$K$ for the acid-base MI-MII equilibrium of rhodopsin is modulated by the lipid environment. In bilayers of phosphatidylcholines the MI-MII equilibrium is shifted to the left; whereas in the native rod outer segment membranes it is shifted to the right, i.e., at neutral pH near physiological temperature. The lipid mixtures sufficient to yield full photochemical function of rhodopsin include a native-like head group composition, viz, comprising phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), in combination with polyunsaturated docosahexaenoic acid (DHA; 22:6$\omega$3) chains. Yet such a native-like lipid mixture is not necessary for the MI-MII conformational transition of rhodopsin; one can substitute other lipid compositions having similar properties. The MI-MII transition is favored by relatively small head groups which produce a condensed bilayer surface, viz, a comparatively small interfacial area as in the case of PE, together with bulky acyl chains such as DHA which prefer a relatively large cross sectional area. The resulting force imbalance across the layer gives rise to a curvature elastic stress of the lipid/water interface, such that the lipid mixtures yielding native-like behavior form reverse hexagonal ($H_{II}$) phases at slightly higher temperatures. A relatively unstable membrane is needed; lipids tending to form the lamellar phase do not support full native-like photochemical function of rhodopsin. Thus chemically specific properties of the various lipids are not required, but rather average or material properties of the entire assembly, which may involve the curvature free energy of the membrane-lipid water interface. These findings reveal that the membrane lipid bilayer has a direct influence on the energetics of the conformational states of rhodopsin in visual excitation.

*Keywords:* Flash photolysis; Lipid; Membrane; Rhodopsin; Signal transduction; Vision

## 1. Introduction

Current knowledge emphasizes the central role of biological membranes in carrying out the distinctive functions of life, including oxidative phosphorylation and photosynthesis, the generation of nerve impulses, signal transduction by hormone receptors and photoreceptors, the processes of viral infection, and other biological phenomena [1]. It is known that membranes are typically fluid, liquid crystalline assemblies containing mainly lipids and proteins. To formulate a comprehen-

*Abbreviations*: $A_\lambda$, absorbance at wavelength $\lambda$; DHA, docosahexaenoic acid; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; MI, metarhodopsin I; MII, metarhodopsin II; PC, phosphatidylcholine or phosphocholine; PE, phosphatidylethanolamine or phosphoethanolamine; PMT, photomultiplier tube; PS, phosphatidylserine or phosphoserine; ROS, rod outer segment; $V$, voltage; $\lambda$, wavelength; $\lambda_{max}$, wavelength of maximum absorbance. The lipid notation used is di($X$:$Y$)$Z$, where $X$ is the number of carbon atoms and $Y$ the number of double bonds of the fatty acyl chains at the *sn*-1 and *sn*-2 positions of the glycerol backbone, and $Z$ denotes the polar head group, viz, PC, PE, or PS.


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sive understanding of their involvement in the processes of life at the molecular level, one must combine present biological insight with knowledge of the liquid crystalline state of matter. One viewpoint is that the membrane lipid bilayer comprises a relatively inert barrier to the passage of ions and polar molecules; it is mainly an insulator or surface necessary for the functions of membrane proteins. Such a perspective, which we shall refer to as the Standard Model, has become the accepted paradigm by many workers, particularly with the advent of modern molecular biological techniques and genetic engineering methodologies. In this article it is shown that the Standard Model may be in serious need of revision. The alternate point of view, namely that the membrane lipid bilayer represents a unique biological material whose properties are closely associated with the functioning of proteins, has been regarded with skepticism or even disdain by many structural biologists. Although the reasons are perhaps largely historical, it is important to recognize that the function of the tightly regulated lipid compositions of biological membranes is a major unsolved problem in biochemistry. The question of lipid diversity in membranes is perhaps akin in significance to the long standing problem of protein folding [2,3]. In what follows, we shall present the arguments for this radical departure from the widely accepted Standard Model in the case of the visual photoreceptor protein rhodopsin.

How are lipid-protein interactions related to biological functions carried out by rhodopsin and other membrane proteins? In this regard, the photoreceptor cells of the vertebrate retina constitute one of the most experimentally tractable systems for investigating the involvement of membranes in biological functions [4–11]. Because the retina is a part of the brain, the rod and cone cells can be viewed as highly specialized neurons, which are particularly amenable to study at the molecular level. The rod cells contain a single light-sensitive photopigment, rhodopsin ($M_r = 40$ kDa); whereas the cone cells contain one of three photopigments, each having a different absorption maximum. At the molecular level, rhodopsin is by far the most thoroughly studied of these visual receptors, as well as the growing superfamily of seven-helix membrane receptors whose mechanisms of action are coupled to heterotrimeric GTP-binding proteins [12–15]. Fig. 1 shows a schematic illustration of a retinal rod cell, together with the folding of rhodopsin in the rod outer segment (ROS) disk membranes and its association with the membrane lipid bilayer [5]. Rhodopsin comprises a bundle of seven helices and can be divided into three domains, viz, the intradiskal, transmembrane, and cytoplasmic domains, each associated with different functions [16]. The chromophore of rhodopsin is 11-*cis* retinal; the sole function of light is to isomerize retinal from 11-*cis* to all-*trans* followed by a series of structural changes within the membrane. However, the three-dimensional structure of rhodopsin at atomic level resolution is presently unknown [17].

In addition to the visual protein rhodopsin, the lipids of the retinal rod outer segment (ROS) disk membranes comprise the major structural constituents [18]. The native ROS membranes include mainly *phosphatidylcholine* (PC), *phosphatidylethanolamine* (PE), *phosphatidylserine* (PS), and *phosphatidylinositol* (PI) in a molar ratio of approximately 39:42:16:2. The PE and PS are on the outside cytoplasmic surface of the disk membrane, whereas PC is largely on the intradiskal surface [19,20]. The ROS membranes are exceptionally rich in *polyunsaturated fatty acid* content, with *docosahexaenoic acid* (DHA, 22:6$\omega$3) representing approximately 47 mol% of the fatty acyl chains [21]. DHA is also found in high concentrations in cerebral gray matter [22–24] and synaptic plasma membranes [25]. Depletion of phospholipids containing 22:6 acyl chains arising from essential $\omega$3 fatty acid deficiency yields loss of visual function in rats [26–28], in Rhesus monkeys [29], and possibly in humans [30]. Several types of visual diseases are characterized by low levels of DHA [31–36]. Consequently, it is plausible that long chain polyunsaturated fatty acids such as docosahexaenoic acid play an essential role in the visual process [37].

According to Fig. 1 there are essentially three

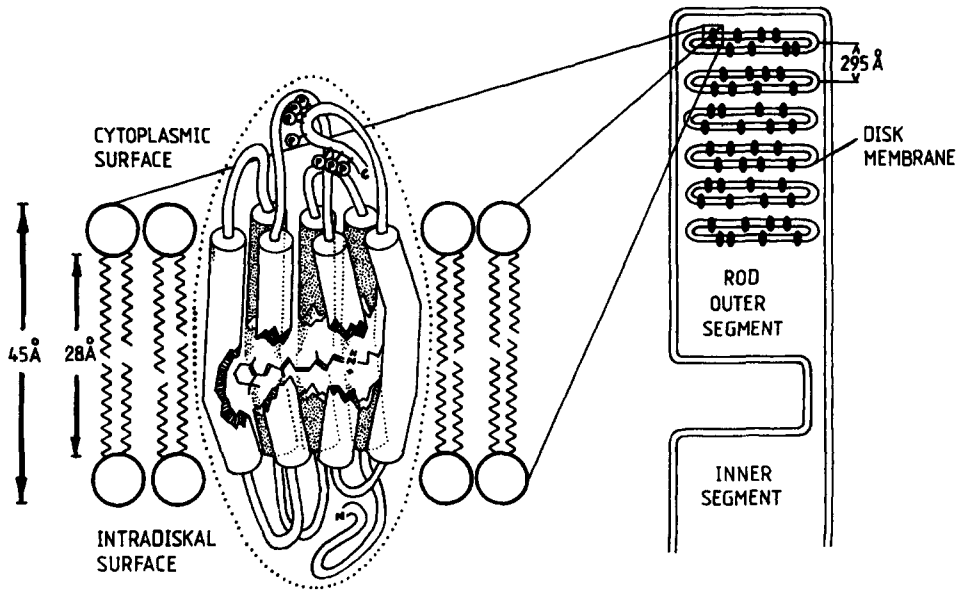


Fig. 1. Schematic drawing of a rod cell indicating the folding of rhodopsin within the disk membranes. Rhodopsin comprises seven transmembrane helices and includes an intradiskal domain, a transmembrane domain, and a cytoplasmic domain. The chromphore is 11-*cis* retinal which is bound via a protonated Schiff base linkage to the $\epsilon$-$NH_2$ group of $Lys^{296}$ in the case of bovine rhodopsin. [Reprinted with permission of Elsevier Science Publishers from E.A. Dratz and P.A. Hargrave (1983) Trends Biochem. Sci. 8, 128–131 (pending).]

approaches to the investigation of structure-activity relationships involving the supramolecular assembly of rhodopsin within the ROS disk membranes: (i) the influences of structurally altered retinals can be studied [38,39]; (ii) site-directed mutagenesis can be utilized to alter the amino acid residues in each of the domains of rhodopsin [40]; and (iii) membrane reconstitution techniques can be used to alter the membrane lipid environment of rhodopsin [41,42]. Each of these strategies has been shown to yield significant alteration of various photochemical and functional parameters of rhodopsin. In this article the role of the membrane lipids is described with regard to the conformational energetics of rhodopsin in the visual process. By combining the above avenues, one can achieve a comprehensive understanding of the relationship of structure to function in the case of the retinal rod disk membranes.

## 2. The process of visual transduction

Photolysis of rhodopsin occurs by a series of intermediates [6,43] which is depicted in Fig. 2. The nature of the changes at the molecular level is largely unknown due to lack of knowledge of the three-dimensional structure of rhodopsin [17]. In the dark, 11-*cis* retinal is bound by a protonated Schiff base linkage to the $\epsilon$-amino group of $Lys^{296}$ in the case of bovine rhodopsin, which is stabilized by the carboxylate of $Glu^{113}$ to form an intramembranous ion-pair [44,45]. Absorption of a photon of visible light yields an 11-*cis* to all-*trans* isomerization of the retinylidene moiety ($\lambda_{max}$ = 498 nm), resulting in the formation within picoconds of bathorhodopsin ($\lambda_{max}$ = 540 nm), a relatively high energy form of the protein [10]. A series of thermal reactions then occurs [43] in which lumirhodopsin ($\lambda_{max}$ = 497 nm) appears followed by metarhodopsin I (MI) ($\lambda_{max}$ = 478 nm) and metarhodopsin II (MII) ($\lambda_{max}$ = 380 nm); the different intermediates have progressively longer decay times (Fig. 2). According to present knowledge the key triggering event in the visual process is the MI-MII transition of photolysed rhodopsin [9,46]. In native rod disk membranes near physiological temperature, MI and MII coex-

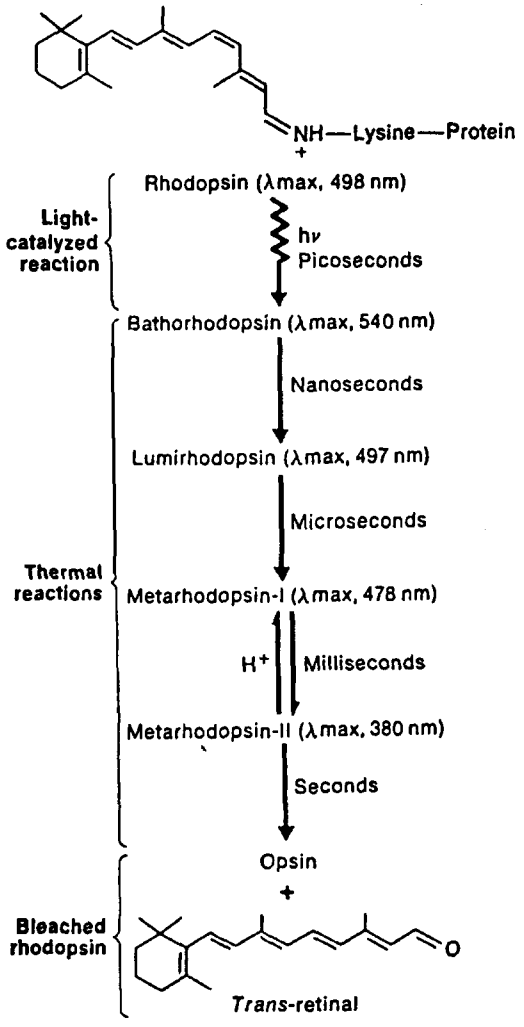


Fig. 2. Sequence of intermediates in the photolysis of rhodopsin, together with their absorption maxima and approximate decay times. The only light catalysed reaction is the formation of bathrorhodopsin; subsequent intermediates involving thermal dark reactions lead ultimately to hydrolysis of retinal from the apoprotein, opsin. [Adapted with permission of the American Chemical Society from P.S. Zurer (1983) Chem. Eng. News 61, 24–35 (pending).]

ist in a pH-dependent, acid-base equilibrium on the millisecond time scale [47–49],

$$\mathrm{MI} + \nu \mathrm{H_3O^+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MII} \qquad (1)$$

Here $K_{eq} = k_1/k_{-1}$ and $\nu$ is the stoichiometric coefficient for $H_3O^+$. It should be noted that the retinal Schiff base becomes concomitantly *deprotonated* at the MII stage of photolysis [9]. The MI-MII transition is influenced by a number of factors, including pH, temperature, pressure, and cholesterol [42,47,48,50–58]. A conformational change of rhodopsin is associated with the MI-MII transition [50,59–68], which exposes recognition sites on the cytoplasmic domain for a signal transducing *G* protein (known also as transducin) [69–74]. Binding of the *G* protein to photoactivated rhodopsin leads to the amplified exchange of GTP for GDP, followed by release of the $G_\alpha$-GTP subunit which activates a cyclic GMP phosphodiesterase; two amplification steps are involved. The hydrolysis of cGMP and subsequent closure of cGMP-gated plasma membrane sodium channels then cause hyperpolarization of the rod, yielding transmission of a visual nerve impulse [7,9,75,76]. Finally, MII decays by processes that are poorly understood at present, resulting in hydrolysis of the Schiff base linkage to produce free all-*trans* retinal plus the apoprotein, opsin. It is noteworthy that the bleaching of rhodopsin does not involve a reversible photocycle as in the case of bacteriorhodopsin; regeneration of opsin occurs in vivo by enzymatic processes. One can conclude that the ratio of MII/MI following an actinic flash provides a measure of the *photochemical function* of rhodopsin, and is linked directly to the molecular mechanism of visual excitation.

This research has tested the hypothesis that the lipid composition of the ROS membranes is tightly regulated due to lipid-rhodopsin interactions that are associated with the visual process [41,77–79]. Polyunsaturated lipids are very important in the nervous system, including the brain and the retina in particular, and may play an essential, albeit largely unknown role in neural functions [37,80]. Among the major unanswered questions are the following: is MII the activated form of photolysed rhodopsin? Are the recognition sites for the *G* protein influenced by the membrane lipid bilayer? What is the contribution of the membrane lipid environment to the energetics of signal transduction? One proposal is that the lipid influences are not specific to the polyunsaturated retinal lipids,

i.e., chemically specific, but rather involve average or material properties of the bilayer [41]. The *free energy of curvature* of the membrane lipid/water interface may be important [41,79]; a large curvature energy can provide a source of work for the MI-MII transition, which for a given amount of phosphatidylethanolamine (PE) is increased by the presence of bulky polyunsaturated DHA chains [41]. Moreover, changes in the membrane lipid composition may be associated with the biological effects of lipids and essential fatty acids [26,28,29,81], including visual diseases such as retinitis pigmentosa [31–36].

## 3. Influences of membrane lipids on the photochemical function of rhodopsin

From the above a general strategy with regard to the investigation of structure-activity relationships in the visual system is the following. The various photochemical parameters and activities implicated in the mechanism of visual signal transduction include: (i) the MI-MII transition of rhodopsin; (ii) activation of the G protein; and finally (iii) the phosphodiesterase activity and hydrolysis of cGMP. In the work described here, the first of the above processes has been investigated, viz, the MI-MII transition, utilizing flash photolysis methods [48,82]. The chemical and functional aspects of rhodopsin have been documented extensively in the native ROS disk membranes, in detergent solutions, and in recombinant membranes. Rhodopsin can be recombined with various phospholipids by detergent dialysis [83–85], and the dependence of the energetic and kinetic parameters of the MI-MII transition on the membrane lipid composition can be investigated. Fig. 3 shows the structures of the lipids utilized in the studies described in this article; both the head groups and acyl chains of the glycerophospholipids have been investigated in a systematic manner.

### 3.1. The MI-MII equilibrium of rhodopsin is reversible in membrane recombinants

First one needs to demonstrate that a *reversible equilibrium* occurs between the MI and MII states

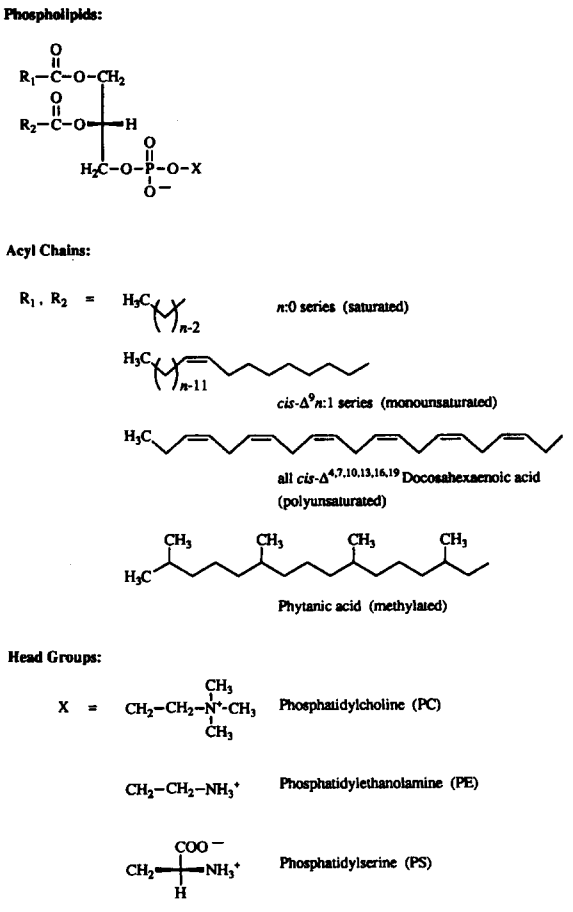


Fig. 3. Chemical structures of phospholipids used to investigate the photochemistry of rhodopsin in recombinant membranes.

of photolysed rhodopsin in the various native and recombinant membrane systems. Representative flash photolysis transients taken from the work of Gibson and Brown [82] are shown in Fig. 4 for rhodopsin in the native ROS membranes and in egg PC and DMPC recombinants at pH 5.0 and 7.0 and a temperature of 28°C. The samples were briefly sonicated to reduce the amount of light scattering; controls for the amount of rhodopsin bleached, the degree of light scattering, and various biochemical characterizations were carried out [41,42]. Whereas the voltage response of rhodopsin in the native ROS membrane vesicles, part (a), is slightly affected by the change in pH, there is a substantial difference for rhodopsin in the

egg PC vesicles, part (b). In parts (a) and (b) the *increase* in the photomultiplier (PMT) voltage (decrease in absorbance) following the actinic flash represents the transition from MI to MII; the phototransient magnitude is due to the [MII]/[MI] ratio present on the millisecond time scale. One should note that an initial *decrease* in the PMT voltage at 478 nm (increase in absorbance) is expected due to formation of MI ($\lambda_{max}$ = 478 nm) from rhodopsin ($\lambda_{max}$ = 498 nm). Hence the observed voltage increase in the case of the ROS membranes and egg PC recombinant reveals that MII ($\lambda_{max}$ = 380 nm) appears within the dead time of $\approx 30$ $\mu$s at 28°C in the former two systems. It is also evident that the apparent forward rate constant $k_{app}$ for the MI-MII transition is greater for the ROS membranes and egg PC recombinants at pH 5.0 than at pH 7.0.

However, the most striking difference among the various membrane recombinants involves the *amplitudes* of the phototransients. A possible explanation involves variations in the amount of rhodopsin bleached by the actinic flash, denaturation of rhodopsin, or various artifacts brought about by the recombination procedure. For the egg PC recombinant, a reduction in pH leads to an increase in the phototransient magnitude, such that nearly the full amount of MII is obtained as in the case of the native ROS membranes, cf. part (b) of Fig. 4. Hence the membrane lipid influences are reversible in the case of egg PC, corresponding to a shift in the equilibrium between MI and MII on the millisecond timescale. At neutral pH the equilibrium lies well to the left in the egg PC recombinant; whereas it lies well to the right in the native ROS membranes near physiological temperature. The case of the DMPC recombinant having relatively short saturated acyl chains is different from either the ROS membranes or the unsaturated egg PC recombinant, as shown in part (c) of Fig. 4. At pH 7.0 there may be an accumulation of MI, as suggested by the observed voltage decrease at 478 nm (increase in absorbance). The slightly higher postflash voltage at pH 5.0 for the DMPC recombinant may indicate the formation of a small amount of MII, or the decay of MI to other products. Consequently the MI-MII transition appears to be largely blocked in accord with the results of Baldwin and Hubbell [86].

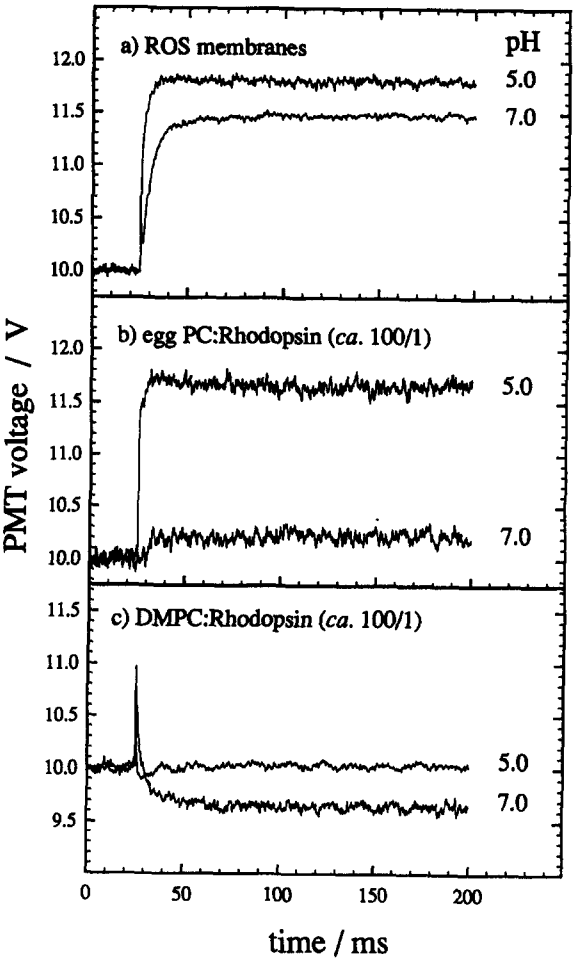


Fig. 4. Flash photolysis transients obtained at $\lambda$ = 478 nm for rhodopsin in native and recombinant membrane vesicles showing influence of the membrane lipid environment. Data were obtained at pH values of 5.0 and 7.0 and a temperature of 28°C (10 mM sodium phosphate buffer). The lipid:rhodopsin ratio was approximately 65/1 for the ROS membranes and 100/1 for the recombinant membranes. (a) Native ROS membrane vesicles; (b) egg PC:rhodopsin vesicles; and (c) DMPC:rhodopsin vesicles. The phototransients at different pH values indicate that the MI-MII transition depends on the membrane lipid environment. [Reprinted with permission of Pergamon Press from N.J. Gibson and M.F. Brown (1991) Photochem. Photobiol. 54, 985–992.] (pending).]

### 3.2. Membrane lipid influences on the MI-MII transition of photolysed rhodopsin

Fig. 5 depicts flash photolysis transients for rhodopsin recombined with a series of saturated and unsaturated phosphatidylcholines, together with rhodopsin in the native ROS membranes [87]. The data illustrate the influences of both the unsaturation and length of the acyl chains. Results are included for the disaturated phosphatidylcholines, di(14:0)PC and di(15:0)PC, as well as a homologous series of unsaturated phosphatidylcholines, di($n$:0)PC, where $n = 12$ to 18. Part (a) shows flash photolysis transients obtained at $\lambda = 485$ nm; an increase in the PMT voltage is seen as noted above. Likewise part (b) depicts results at $\lambda = 390$ nm for rhodopsin in the same series of membranes; a decrease in the PMT voltage is found corresponding to the appearance of an absorbing species, e.g., MII. Several further aspects are worthy of note: (i) the absolute magnitudes of the phototransients at both $\lambda = 485$ and 390 nm are markedly reduced in the phosphatidylcholine recombinants compared to rhodopsin in the native ROS membranes; (ii) for the recombinants having short saturated acyl chains, di(14:0)PC and di(15:0)PC, the MI-MII transition appears essentially blocked, which may indicate that the chains are too short rather than an effect of unsaturation; similar behavior is observed for the di(14:1)PC and di(15:1)PC recombinants; (iii) in the recombinants having unsaturated chains, di($n$:1)PC, there is an increase in the amplitude of the phototransients as the chain length is increased from $n = 12$–18 in the fluid state. (Such investigations are not possible for the homologous series of disaturated phosphatidylcholines on account of the increase in the order-disorder phase transition with the acyl length.) In general, the influence of the acyl chain length indicates that an alteration in the hydrophobic thickness of rhodopsin may occur in the MII state versus the MI state.

Moreover investigations of the combined influences of the polar head groups and polyunsaturation of the acyl chains have been carried out

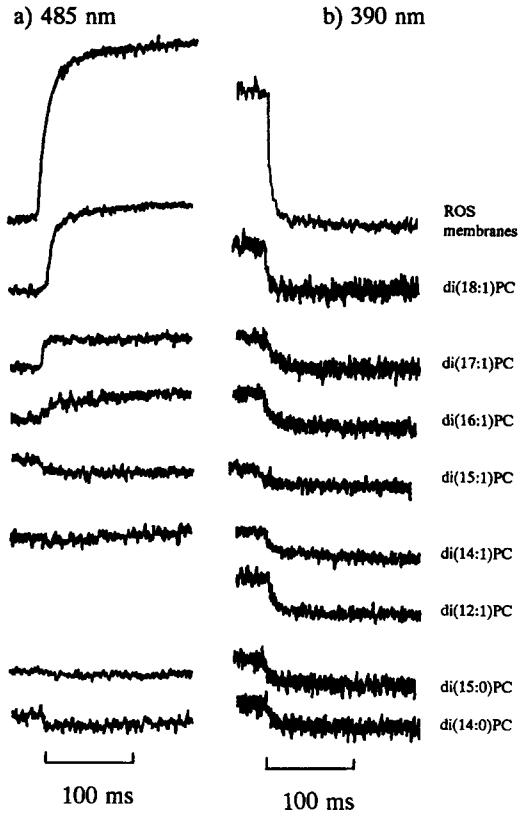


Fig. 5. Flash photolysis transients illustrating the effect of the acyl length for recombinants of rhodopsin with a series of phosphatidylcholines. Data were acquired at pH 7.0 and 29°C (10 mM sodium phosphate buffer); the lipid:rhodopsin ratio was approximately 100/1 in all cases. (a) PMT voltage at $\lambda = 485$ nm, where the increase in transmittance (loss of absorbance) is due to formation of MII from MI. (b) Corresponding flash photolysis transients obtained at $\lambda = 390$ nm, in which the decrease in transmittance (increase in absorbance) indicates formation of MII. The phototransients are very small or undetectable in the recombinants with disaturated phosphatidylcholines having short acyl chains; whereas in the unsaturated phosphatidylcholines an increase is observed for the longer chain lengths.

[41] as depicted in Fig. 6. Phototransients (a) and (b) show that very similar photochemical behavior is obtained for rhodopsin in the native ROS disk membranes and recombined with its total extracted phospholipids as a control. Hence one can conclude that rhodopsin retains its photochemical activity with the recombination procedure em-

ployed [84], and that transbilayer asymmetry of the protein or lipid distribution [19,20,88,89] does not alter this aspect of its function. The inability of rhodopsin to undergo a native-like MI-MII transition in the recombinants with either the saturated or unsaturated phosphatidylcholines (cf. Fig. 5) could be due to a requirement for: (i) polyunsaturation of the acyl chains as found in the native ROS lipids; (ii) the presence of other phospholipid head groups such as PE and/or PS; or (iii) the need for a mixture of phospholipids having different head groups and acyl chains. Wiedmann et al. [41] investigated each of these variables in turn as summarized in Fig. 6. Relative to the native ROS membranes, trace (a), a much smaller phototransient is seen for the recombinant of rhodopsin with egg PC, trace (g), indicating that acyl chain unsaturation is not sufficient for full photochemical activity in the case of phosphatidylcholines. Recombinants with phosphatidylcholines containing 22:6$\omega$3 fatty acyl chains, traces (d) and (e), or with egg PE having unsaturated chains, trace (f), reveal significantly larger phototransients; the magnitudes are less than the native ROS membranes. The recombinant with phospholipids containing both 22:6$\omega$3 acyl chains and PE head groups shows the highest level of MII production among the non-native recombinants, trace (c). Hence one can conclude that 22:6$\omega$3 acyl chains or phosphoethanolamine head groups favor the formation of MII; yet neither alone is sufficient for native-like photochemical behavior. However, when both PE head groups together with polyunsaturated chains are present in recombinants with phosphatidylcholine, the behavior of the native ROS membranes is closely approximated.

One can also convert the flash photolysis results to the change in the absorbance of rhodopsin, $\Delta A$, at $\lambda = 478$ nm, and plot the values at

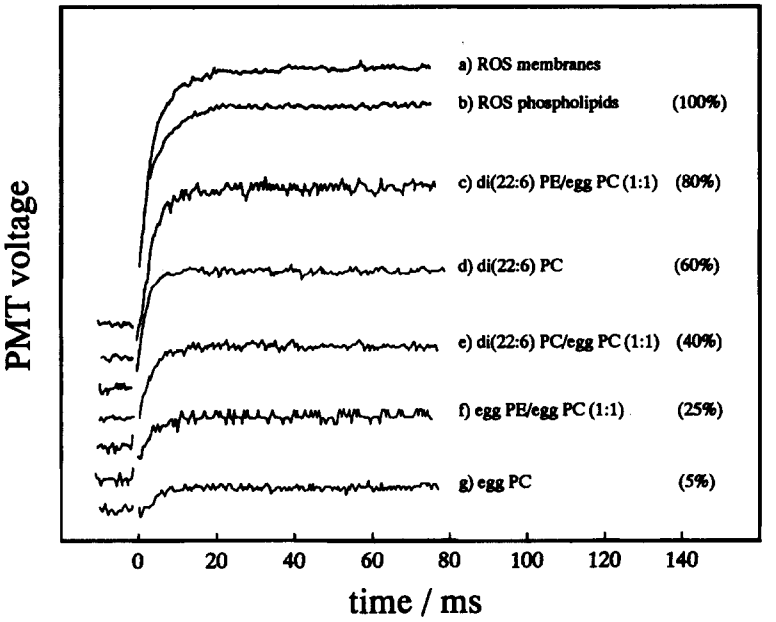


Fig. 6. Flash photolysis transients obtained at $\lambda = 478$ nm demonstrating influences of the polar head group composition and polyunsaturation of the acyl chains. Experiments were conducted at pH 7.0 and 28°C (10 mM sodium phosphate buffer). The lipid:rhodopsin ratio for the recombinants was approximately 100/1, except for the total ROS lipids:rhodopsin recombinant which was 65/1. The numbers in parentheses denote the postflash voltage change as a percentage of that obtained for the native membranes. (Note that the various phototransients are offset vertically relative to one another.) The results indicate that phospholipids containing phosphoethanolamine (PE) head groups and docosahexaenoyl (DHA; 22:6$\omega$3) chains play an important role in the photochemical activity of rhodopsin. [Redrawn from T.S. Wiedmann et al. (1988) Biochemistry 27, 6469–6474.]

different pH values to obtain titration curves for the MI-MII acid-base equibrium on the millisecond time scale, as shown by Gibson and Brown [90,91]. Such titration curves illustrating the effects of the membrane lipid head groups and polyunsaturated acyl chains are presented in Fig. 7. The results for rhodopsin in the ROS membranes and unsaturated recombinants can be explained to a first approximation in terms of a simple acid-base equilibrium, Eq. (1), in which the p$K$ value depends on the membrane lipid environment. For illustrative purposes only, curves for a single proton ionization equilibrium ($\nu = 1$) were generated for rhodopsin in the various membrane lipid recombinants. (The actual titration process for an amphoteric membrane surface may be more complex because the electrostatic potential is a function of the fraction of titratable sites occupied, which can lead to broadening of the titration curve [92].) Fig. 7 shows flash photolysis studies of a control recombinant of rhodopsin with the total ROS lipids, i.e., including a native head group composition together and polyunsaturated acyl chains, which yields a titration curve very similar to that for the MI-MII transition of the native ROS membranes. The data in Fig. 7 can be fit with an apparent p$K$ of $\approx 7.8$ for the native ROS membranes and total ROS lipids recombinant, and an apparent p$K$ value of $\approx 6.3$ in the case of the egg PC recombinant. It follows that the major result of substituting egg phosphatidylcholine for the native ROS phospholipids is a downward shift in the apparent p$K$ for the acid-base MI-MII equilibrium of photolysed rhodopsin by about 1.5 p$K$ units. In the case of the recombinant with PE and PS together with PC,

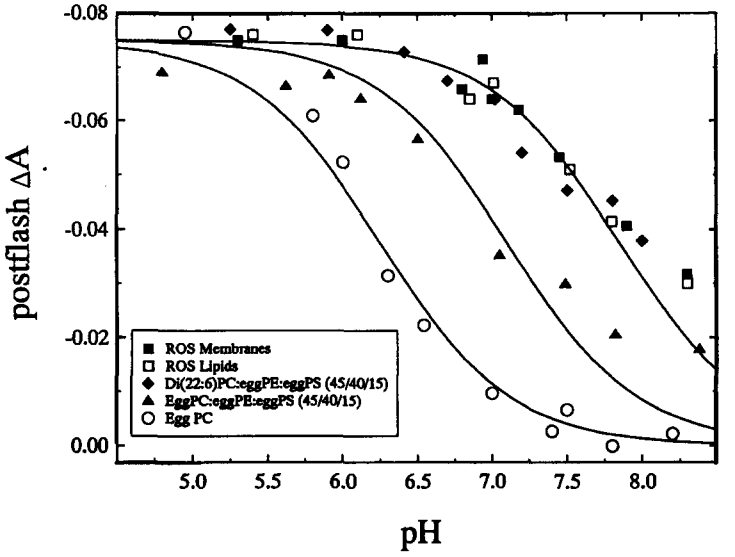


Fig. 7. Postflash change in absorbance of rhodopsin as a function of pH depicting influence of the membrane lipid head groups and polyunsaturated acyl chains. The change in absorption, $\Delta A$, at $\lambda = 478$ nm following an actinic flash is indicated versus pH at 28°C (10 mM sodium phosphate buffer). The lipid:rhodopsin ratio for the recombinants was approximately 100/1, except for the total ROS lipids:rhodopsin recombinant which was 65/1. (■) Rhodopsin in native ROS membranes and (□) recombined with the total ROS lipids; (◆) recombinant of rhodopsin with di(22:6)PC:egg PE:egg PS (45/40/15); (▲) recombinant of rhodopsin with egg PC:egg PE:egg PS (45/40/15); and (○) recombinant of rhodopsin with egg PC. Values in parentheses denote mol percentages of the corresponding phospholipids. Lines are to guide the eye and are derived from different fits of the experimental data to a simple titration equilibrium, depending on how the contribution from MI is treated. (The actual titration behavior of an amphoteric membrane surface may be more complex.) To a first approximation, the recombinants display fairly straightforward acid-base titration behavior, in which the apparent p$K$ depends on both the membrane lipid head group and polyunsaturated acyl chain composition.

but having egg PC-derived acyl chains, the apparent p$K$ value for the MI-MII equilibrium falls between these limiting values (Fig. 7). Thus a native-like head group composition, comprising PC:PE:PS (45/40/15) with unsaturated acyl chains, does not yield native-like behavior [90,91]. Fig. 7 shows that a recombinant of rhodopsin with di(22:6)PC:egg PE:egg PS (45/40/15), having a native-like head group composition together with polyunsaturated acyl chains, yields a titration curve that closely approximates that for the native ROS membranes and the total ROS lipids recombinant. It is noteworthy that the signal transducing event in visual excitation has been successfully reconstituted for the first time in this work.

### *3.3. Temperature studies yield further evidence for a reversible MI-MII equilibrium in membrane recombinants*

The above investigations of the influences of pH on the flash photolysis behavior of rhodopsin in the various unsaturated lipid recombinants are generally consistent with an equilibrium between MI and MII on the millisecond time scale. Likewise, the effects of temperature suggest that a reversible equilibrium occurs between the two metarhodopsin species, e.g., as opposed to a variable degree of inactivation of rhodopsin. The results of a preliminary thermodynamic investigation provide knowledge of the energetics of the MI-MII transition of rhodopsin in the various membrane recombinants [42]. In all cases, the MI-MII transition is accompanied by an increase in both enthalpy and entropy; enthalpy-entropy compensation is observed. The MI-MII transition is favored entropically but is opposed by enthalpic considerations (an endothermic process), leading to a decrease in the apparent equilibrium constant $K_{eq}$ for rhodopsin in the various recombinant membranes. The changes in standard thermodynamic state variables are consistent with a partial unfolding of rhodopsin in the MII conformation, leading to exposure of recognition sites for the signal transducing $G$ protein [42]; further research is needed.

### *3.4. Lipid substitution experiments reveal that membrane bilayer properties are involved*

At this juncture one can summarize the flash photolysis studies of the influences of lipid-protein interactions on the photochemical function of rhodopsin as follows. According to a current model for visual excitation, photolysis of rhodopsin leads to a conformational change, the MI-MII transition, which exposes recognition sites for a $G$ protein leading to transduction of the visual signal. The membrane lipid composition modulates the acid-base MI-MII equilibrium of photolysed rhodopsin, and furthermore mixtures of lipids that are sufficient for this photochemical function have been identified. As a result, the signal-transducing event in visual excitation is governed by the membrane lipid environment. The synthetic recombinant found to most closely duplicate the native ROS membrane behavior contains di(22:6)PC/egg PE/egg PS (45/40/15), indicating that phosphocholine, phosphoethanolamine, and phosphoserine head groups and DHA chains are all important components of the membrane. Thus one can conclude that the presence of native-like lipids in recombinant membranes is *sufficient* to yield native-like photchemical behavior of rhodopsin.

Let us now turn to the central question of whether such a native-like lipid mixture is *necessary* for the full photochemical function of rhodopsin. Are chemically specific interactions of rhodopsin with the lipids important, or rather average or material properties of the bilayer? As a rule the lipid mixtures giving rise to an enhancement of the MII/MI ratio of photolysed rhodopsin tend to form non-lamellar phases, e.g., the reverse hexagonal ($H_{II}$) phase, at slightly higher temperatures. Moreover, it is known that the composition of the native ROS membrane lipids is such that they are near a lamellar ($L\alpha$) to reverse hexagonal ($H_{II}$) phase boundary in the absence of protein [78,79]. In what follows, lipid substitution experiments are described which reveal that chemical specificity is not involved in producing native-like photochemical function of rhodopsin [58]. These experiments have tested the

proposal that non-lamellar forming lipids give rise to native-like photochemical function of rhodopsin, in which the small head group size relative to the chain cross-section yields a curvature elastic stress due to the imbalance of the lateral forces across the layer [41]. The combination of phosphoethanolamine head groups together with bulky 22:6$\omega$3 acyl chains [93] results in lipids having a conical or wedge-like average shape [94], which may influence the lipid/water interfacial curvature free energy of the bilayer, and thus better enable the membrane to accommodate the MI-MII conformational change [41,78,79].

Fig. 8 shows flash photolysis transients for rhodopsin in a series of recombinants having different mol ratios of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC), i.e., di(18:1)PE and di(18:1)PC, revealing the effect of phosphoethanolamine head groups [95]. The phototransient for a recombinant of rhodopsin with DOPC is shown in part (a) of Fig. 8, the amplitude of the photoresponse is similar to that already included in Fig. 5, and is comparable to the results for egg PC in Figs. 4 and 6. As indicated above, a markedly diminished phototransient is seen for recombinants of rhodopsin with saturated or unsaturated phosphatidylcholines, comprising either a single type of acyl chain, or a mixture of acyl chains as in the case of egg PC. Parts (b)–(d) of Fig. 8 show the effect of increasing progressively the molar fraction of DOPE in the recombinants with DOPC. A corresponding increase is seen in the magnitude of the phototransient, viz, the [MII]/[MI] ratio, until at 75 mol% DOPE, part (d), essentially the full amount of MII is obtained as in the native ROS membranes. Fig. 9 shows the full pH titration curves obtained for the MI-MII transition of recombinants of rhodopsin which summarize the influences of DOPE [95]. As can be seen from parts (a)-(d) of Fig. 9, a progressive shift of the apparent p$K$ for the MI-MII transition is found,

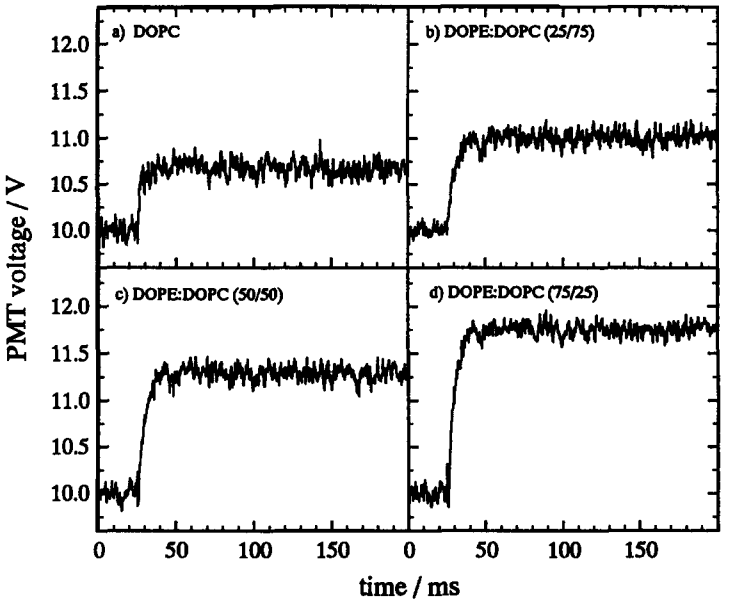


Fig. 8. Flash photolysis transients at $\lambda = 478$ nm illustrating result of increasing the mol fraction of DOPE, i.e., di(18:1)PE, in mixtures with DOPC, i.e., di(18:1)PC. Experiments were conducted at pH 7.0 and 28°C (10 mM sodium phosphate buffer); the lipid:rhodopsin ratios were approximately 100/1. As the mol% of DOPE is increased, parts (a)–(c), a progressive increase in the magnitude of the phototransient occurs, i.e., the amount of MII produced, until nearly the full amount is obtained, part (d), as in the native ROS membranes. Note that DOPE forms the reverse hexagonal phase ($H_{II}$) in the absence of protein under the experimental conditions.

such that at 75 mol% DOPE the full native behavior is reconstituted. Thus by essentially doubling the mole fraction of PE compared to the native level (42 mol%), one can reconstitute the full native MI-MII transition in a synthetic two component mixture of phospholipids, i.e., *in the absence of polyunsaturated acyl chains.*

Further investigations have addressed the possibility that the key characteristic of the polyunsaturated acyl chains is a preference for a relatively large average cross-sectional area [93]. Studies were carried out to further explore the role of the bulky acyl chains, and to ascertain whether substitution of other bulky non-native acyl chains for docosahexanoic acid (22:6$\omega$3) was possible. It was decided to investigate whether phytanic acid, a branched chain fatty acid having methyl side groups, which is not found to any appreciable extent in the ROS membranes, could replace DHA in the various lipid mixtures [58]. A summary of the partial titration curves obtained to date is included in Fig. 10. As noted in Fig. 6, if a native-like head group composition is included together with polyunsaturated chains, e.g., the recombinant of rhodopsin with di(22:6)PC/egg PE/egg PS (45/40/15), then the behavior is essentially the same as the native ROS membranes. The presence of bulky acyl chains in diphytanoyl PC:rhodopsin recombinant membranes is not sufficient for native-like MII production, as in the case of the di(22:6)PC:egg PC (1/1) recombinant (Fig. 10). However, a recombinant of rhodopsin with diphytanoyl PC:egg PE:egg PS (45/40/15) exhibits photochemical behavior that is essentially identical to rhodopsin in the native ROS membranes and the di(22:6)PC:egg PE:egg PS

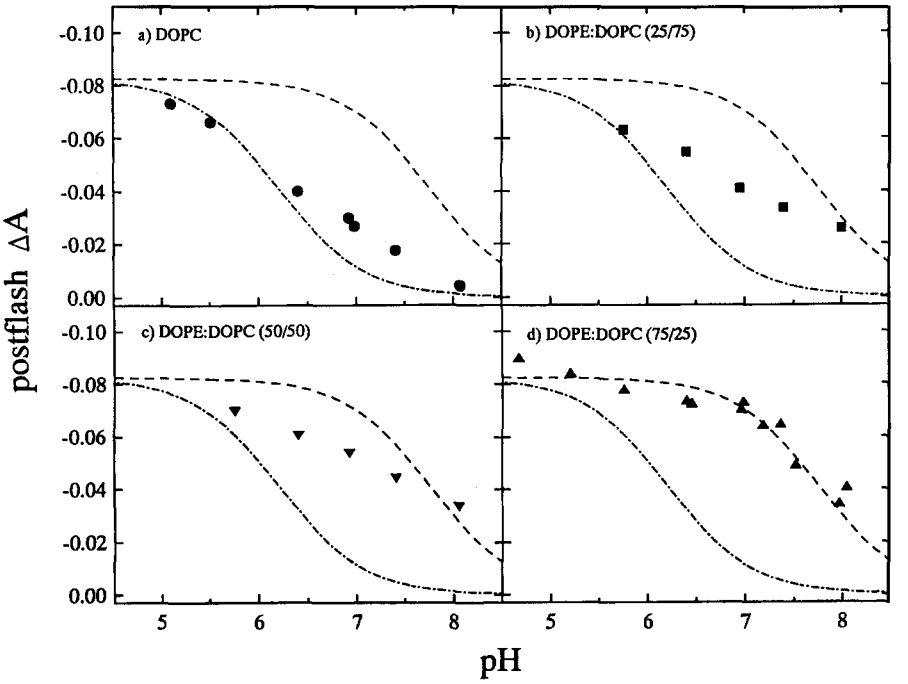


Fig. 9. Postflash change in absorbance at $\lambda = 478$ nm for rhodopsin as a function of pH showing effect of DOPE in recombinants with DOPC at 28°C (10 mM phosphate buffer). The lipid:rhodopsin ratio for the recombinants was approximately 100/1. Reference curves correspond to rhodopsin in the egg PC recombinant and the native ROS membranes. A progressive shift of the titration curve for the MI-MII transition is seen as the mole percentage of DOPE is increased from 0 to 75%, at which the native behavior is reached. Thus it is possible to reconstitute fully the signal transducing event in visual excitation by employing a mixture of synthetic phospholipids.

(45/40/15) recombinant membranes. Hence bulky non-native phytanic acid chains are able to substitute for the polyunsaturated DHA chains; although the latter are sufficient they are not necessary for native-like function [58].

## 4. Membrane bilayer properties in relation to lipid-rhodopsin interactions

The results of the lipid substitution experiments imply that a major biological function of the ROS membrane lipids is to govern the free energies of the conformational states of rhodopsin through modulation of average membrane properties [41,79]. Moreover, evidence has been obtained that the lipid mixtures giving rise to native-like function tend to form non-lamellar phases such as the reverse hexagonal ($H_{II}$) phase near the experimental conditions [79]. Consequently, it is reasonable to propose that chemical specificity of the lipids is not involved in modulation of the photochemistry of rhodopsin linked to the visual process, but rather physical or material properties of the bilayer. What are the properties of the membrane lipid bilayer that may be important in governing the MI-MII conformational equilibrium of photolysed rhodopsin?

### 4.1. Influences of electrostatics

A possible hypothesis is that the properties due to the membranous lipid head groups are of a simple longer-range electrostatic nature [96–98]. The importance of maintaining an optimal membrane surface potential, $\psi_0$, may be reflected in the fact that the native ROS membranes and recombinants containing PS display a significant increase in MII production relative to egg PC or recombinants containing egg PE [42,90]. A negative membrane surface potential, due to the pres-

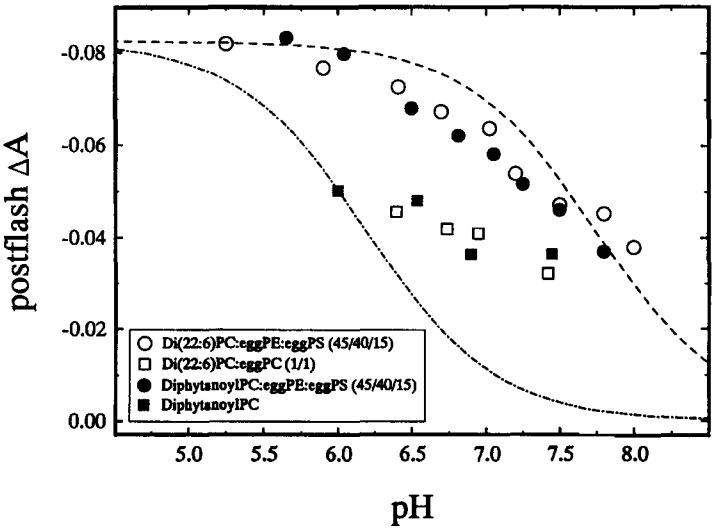


Fig. 10. Postflash absorbance change of rhodopsin as a function of pH in recombinants containing di(22:6)PC and diphytanoyl PC illustrating results of acyl chain substitutions. The absorption change, $\Delta A$, at $\lambda = 478$ nm is plotted versus pH at 28°C (10 mM sodium phosphate buffer). The lipid:rhodopsin ratio for the recombinants was approximately 100/1. Reference curves are for the egg PC recombinant and native ROS membranes. (○) Rhodopsin recombined with di(22:6)PC:egg PE:egg PS (45/40/15); (□) recombinant of rhodopsin with di(22:6)PC:eggPC (1/1); (●) recombinant of rhodopsin with diphytanoyl PC:egg PE:egg PS (45/40/15); and (■) recombinant of rhodopsin with diphytanoyl PC. Values in parentheses indicate mol percentages of the corresponding phospholipids. Note that non-native phytanic acid chains can substitute for docosahexaenoic acid (22:6$\omega$3) chains; native-like photochemical behavior of rhodopsin is obtained in combination with phosphocholine, phosphoethanolamine, and phosphoserine head groups.

ence of acidic PS head groups, yields an accumulation of protons and other cations within the diffuse electrical double layer thereby driving the MI-MII transition to the right [96]. However, the significant effects of neutral phosphoethanolamine head groups together with docosahexaenoic acid chains point towards the role of bilayer properties other than electrostatics. Phosphatidylethanolamine exerts influences on the MI-MII transition similar to PS, yet the phosphoethanolamine head groups are uncharged over the pH range investigated [99]; similar effects of neutral polyunsaturated DHA chains are found [41]. Moreover, lipid substitution experiments evince that it is possible to recover full photochemical function with neutral lipids alone, i.e., in the absence of a surface potential. Hence besides direct electrostatic influences of the lipid polar head groups, membrane properties involving the bilayer hydrocarbon region may be important [41]. Among these properties are: (i) the average bilayer thickness and/or mean interfacial area per lipid molecule; (ii) the lateral compressibility of the lipid bilayer matrix; (iii) lateral or curvature stresses involving the membrane lipid/water and protein/lipid interfaces; and (iv) a combination of the above together with influences of the membrane surface potential which may help drive the MI-MII transition.

### *4.2. The bilayer thickness and interfacial area per molecule*

One variable that has received attention with regard to lipid-protein interactions is the bilayer thickness or length of the phospholipid acyl chains [87,100–104]. The above investigations of the influence of the phospholipid acyl chain length [87] on the MI-MII transition suggest that in the liquid-crystalline state a minimum thickness (corresponding to about 16–18 acyl chain carbons) is needed for appreciable formation of MII (Fig. 5). Similar studies have been carried out in the case of the $Ca^{2+}$-ATPase from muscle sarcoplasmic reticulum [100]. Moreover, the presence of phosphatidylethanolamine leads to a modest enhancement of the amount of MII produced [41]; PE is known to occupy a smaller mean interfacial area per molecule than PC thus leading to an increase in the average projected acyl chain length and bilayer thickness [105]. Yet in each case a substantial reduction in photochemical activity of rhodopsin is found versus the native ROS membranes, suggesting that the bilayer thickness is not the only property involved.

### *4.3. The protein / lipid interface*

It is also possible to formulate the influences of the bilayer thickness and other properties in terms of the *protein / lipid interfacial tension* which governs the energetics of the MI and MII states of photolysed rhodopsin. That is to say, the protein is sealed into the bilayer by the tension due to the protein/lipid interface, which is a measure of the solvation energy of the protein due to the lipids. Equilibrium thermodynamics teaches us that a pressure *differential* exists across a curved interface, the so-called Laplace pressure, which is responsible for capillary action and other interfacial phenomena [106]. If the protein is modeled as a transmembrane cylinder, then a significant Laplace pressure can exist across the interface with the hydrocarbon region of the lipid bilayer, on account of the very small protein radius of curvature [42,86]. The pressure differential due to the curved interface is given by [106]

$$\Delta P \equiv P_P - P_L = \gamma_{P/L}(1/r_1 + 1/r_2) \qquad (2)$$

in which $r_1 = 1/c_1$ and $r_2 = 1/c_2$ are the two principal radii of curvature, and $c_1$ and $c_2$ are the associated curvatures. The protein/lipid interfacial tension, $\gamma P/L$, is a measure of the energy of solvating the protein intramembranous surface by the hydrocarbon region of the bilayer. For the case of an *intramembranous cylinder*, one of the radii of curvature becomes infinite and the Laplace pressure is given by

$$\Delta P = \gamma_{P/L}/r \qquad (3)$$

The *surface work* due to a change in the radius of curvature of a cylindrical protein of length $h$ yields a contribution to the free energy change,

corresponding to a change in the protein/lipid interfacial area, given by

$$\Delta\mu = 2\pi N_A h \gamma_{P/L} \Delta r \quad (4)$$

Taking a value of $\gamma P/L = 10$ mJ m$^{-2}$, as typical of organic liquid interfaces [106], $h = 50$ Å, and $\Delta r = 1$ Å yields $\Delta\mu = +19$ kJ mol$^{-1}$, which is appreciable in relation to the free energy shifts due to the membrane lipids. Thus it can be concluded that rather substantial shifts in free energy are possible due to properties of the protein/lipid interface [42,86]. It is possible that saturated acyl chains, as in the case of the DMPC recombinant, are relatively poor at solvating the intramembranous surface of rhodopsin, leading to a substantial tension $\gamma P/L$; whereas long chain polyunsaturated 22:6 chains are more effective in this regard. Although the above is a plausible explanation of the influences of the lipid acyl chains, how does one account for the additional effects of the polar head groups?

### 4.4. The lipid / water interface

As an alternative one can apply concepts from surface and colloid chemistry to the interface between the lipid bilayer and water. Let us consider the possible role of the lipid/water interface. In the absence of proteins, the molecular organization within a membrane lipid aggregate can be understood in terms of a balance of attractive interactions and repulsive forces, acting at the level of the lipid polar head groups and nonpolar acyl chains [94,107]. Within the head group region there is an effectively attractive interfacial tension, which arises from the unfavorable contact of the hydrocarbon chains with water, viz, the hydrophobic effect; attractive contributions from hydrogen-bonding, e.g., in the case of phosphatidylethanolamines, may be present. The repulsive head group pressure is the result of hydration, steric, and electrostatic contributions. For the acyl chain region the attractive van der Waals interactions among the $CH_2$, CH=CH, and $CH_3$ groups are compensated by the repulsive lateral pressure due to thermally activated dihedral angle isomerizations. Now because the membrane bilayer is at equilibrium, the various lateral pressures are balanced in the absence of external stresses. Yet this may not be the case if a protein conformational change occurs, e.g., involving a change in the cross-sectional membrane area or the hydrophobic thickness of rhodopsin.

*Lateral or interfacial area stress*

One avenue is to consider the balance of forces in terms of lateral or area stresses (area frustration), i.e., which are similar at various depths within the planar bilayer. The lipid/water interfacial free energy can be approximated by an expression of the form

$$\mu(a) = \gamma_{L/W}\, a + K/a \quad (5)$$

where attractive interactions are effectively modeled in terms of an interfacial tension $\gamma L/W$, the parameter $K$ describes repulsive interactions, and $a$ is the interfacial area per molecule [94]. A change in cross-sectional area of photolysed rhodopsin due to the MI-MII transition can provide a mechanism for coupling of the protein conformational change to longer-range lateral forces involving the membrane lipid constituents [108], e.g., due to changes in the exposure of protein functional groups or tilting of the transmembrane helices. Such a conformational change can lead to a relaxation or weakening of the lateral stresses, such that the lipid/water interfacial free energy goes downhill yielding a driving force for the MI-MII transition. However, as noted above the combined influences of the lipid polar head groups and the docosahexaenoyl chains point towards a possible role of other bilayer properties.

*Curvature stress*

A related approach is to formulate the balance of forces in terms of the lateral stress profile across the bilayer. In this regard it is noteworthy that the lipid mixtures favoring the MI-MII transition of rhodopsin tend to form non-lamellar phases in the absence of protein. Similar influences of non-lamellar forming lipids on functional parameters have been observed for the $Ca^{2+}$-

ATPase of sarcoplasmic reticulum in recombinant membranes [109,110]. The formation of non-lamellar phases of membrane lipids originates from an interplay of the curvature free energy of the membrane lipid/water interface together with chain packing forces [111]. On the one hand, the smaller phosphoethanolamine head groups together with their propensity for hydrogen-bonding favor a *smaller* interfacial area per molecule [105]. On the other, the presence of bulky acyl groups, e.g., polyunsaturated fatty acyl chains with vinylic groups that can restrict rotational isomerism, favors a somewhat *larger* cross-sectional molecular area [93]. Within a given lipid monolayer, the imbalance of attractive and repulsive forces at the level of the head group and acyl chains produces a thermodynamic or spontaneous (mean) curvature. Clearly the two monolayers cannot simultaneously be at a free energy minimum with regard to their intrinsic or spontaneous curvature. It follows that a symmetric bilayer composed of non-lamellar forming lipids, i.e., in the planar state, is under a condition of curvature elastic stress [41,79,111]; thus $H_{II}$ and cubic phases are formed as the temperature is increased [111,112].

Fig. 11 provides an illustrative representation of the above ideas [107]. The top part of Fig. 11 shows that for the case of head groups that are small or favor hydrogen bonding, e.g., phosphoethanolamine head groups, a relatively small equilibrium separation is favored. If the chains favor a larger separation, e.g., bulky polyunsaturated or phytanoyl chains, then there is a significant imbalance of the forces across the layer yielding a curvature stress, i.e., producing a *negative* spontaneous curvature. It follows that in the absence of protein the reverse hexagonal ($H_{II}$) phase is favored as the temperature is increased. The middle part of Fig. 11 shows that if the forces within the head group region favor an equilibrium interfacial area similar to the chain cross sectional area, e.g., in the case of disaturated phosphatidylcholines, then the spontaneous curvature is *zero*. Because the attractive and repulsive forces within the head group and acyl chains regions are balanced at a similar equilibrium area, there is no curvature stress and the lamellar phase is favored

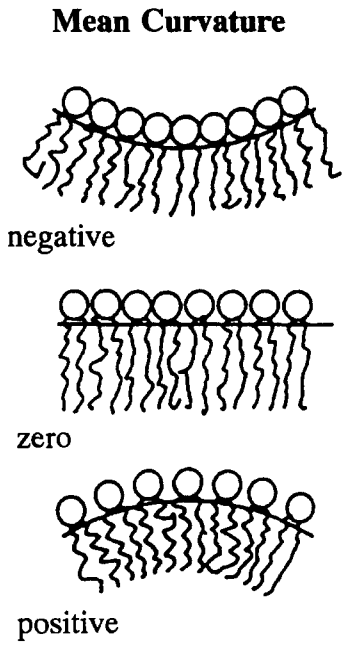


Fig. 11. Depiction of the spontaneous curvature of an individual monolayer arising from the distribution of lateral forces within the head group and acyl chain regions. The spontaneous or mean curvature is a thermodynamic property, and is not necessarily related to the actual interfacial curvature of an assembly of amphiphiles. [Adapted with permission of Elsevier Science Publishers from J.M. Seddon (1990) Biochim. Biophys. Acta 1031, 1–69 (pending).]

by such lipids. Finally, the bottom of Fig. 11 illustrates that when the head groups favor a larger equilibrium separation than in the case of the acyl chains e.g., for gangliosides and detergents, there is a *positive* spontaneous curvature. In this case there is a tendency to form small micelles and the normal hexagonal ($H_I$) phase.

Let us now consider the role of the *curvature elastic stress*, i.e., the interfacial curvature free energy on the conformational energetics of rhodopsin (curvature frustration). It is assumed the attractive and repulsive forces within the polar head group and hydrocarbon regions of the planar bilayer are balanced at different equilibrium areas. The photolysis of rhodopsin alters the force balance and is accompanied by a change in the curvature free energy of the membrane lipid/water interface [41,78,79]. According to current knowledge, the free energy due to *elastic*

*curvature stress* within the bilayer can be formulated in terms of the spontaneous curvature, $H_0$, of the membrane lipid/water interface, together with the elastic constants for curvature deformation. The curvature free energy per unit interfacial area, $g_c$, can be written as [113,114]

$$g_c = \kappa(H - H_0)^2 + \bar{\kappa}K \quad (6)$$

Here $H \equiv (c_1 + c_2)/2$ is the mean curvature, $H_0$ is the spontaneous curvature, $K \equiv c_1 c_2$, in which $c_1$ and $c_2$ are the two principal curvatures of the lipid/water interface ($c_1 = c_2 = 0$ for a planar bilayer), and $\kappa$ and $\bar{\kappa}$ are the bending rigidity and the elastic modulus of Gaussian curvature, respectively. We shall assume for illustrative purposes a value for the bending rigidity of $\kappa = 2k_c = 4 \times 10^{-19}$ J [113,115] and neglect the Gaussian curvature for simplicity. Given a spontaneous curvature for an individual monolayer of $H_0 = 1/(40$ Å) [115,116], an interfacial lipid area of 70 Å$^2$, and 100 lipids per rhodopsin then leads to $\Delta\mu = +530$ kJ mol$^{-1}$. Hence the contribution to the free energy balance from the curvature elastic stress of the bilayer is rather appreciable, e.g., in relation to the much smaller free energy shifts for the MI-MII transition among the membrane recombinants.

It follows that a relief of the curvature stress (frustration) stored within the bilayer may accompany the MI-MII transition of photolysed rhodopsin [41]; this may occur due to alteration of helical or other secondary structure elements. The combination of phosphoethanolamine head groups together with docosahexaenoyl (22:6$\omega$3) acyl chains favors a *negative interfacial curvature*. By enabling a given monolayer of the bilayer to approach its spontaneous curvature, an energetically downhill process, the free energy released can provide a source of work for the MI-MII transition, which is energetically uphill in planar lamellar phases of diacylphosphatidylcholines [41,86,117]. An increase in the absolute value of the lipid spontaneous curvature $H_0$ and/or bending rigidity $\kappa$, due to the presence of polyunsaturated DHA chains in combination with PE head groups, can yield a substantial driving force for the MI-MII transition of the protein [41]. In fact, energies on the order of only 10 kJ mol$^{-1}$ are sufficient to shift the MI-MII transition from being essentially blocked, i.e., undetectable, to completion at the temperatures considered. Such a material science viewpoint is closely related on some length scale to molecular properties of the polyunsaturated phospholipids. These may include a preference of the polyunsaturated chains to form helical or spring-like preferred configurations, on account of the polyallylic motif of alternating vinylic and allylic groups [108,118–122].

A general observation that arises from the above studies is that the balance of opposing attractive and repulsive forces within the head group and acyl chain regions of a lipid bilayer may play an important role in governing the conformational energetics of rhodopsin. A simple working hypothesis for the MI-MI transition of rhodopsin that leads to testable predictions is shown in Fig. 12 [41,42]. The results for the homologous series of diacyl phosphatidylcholines, di($n$:1)PC, suggest that an increase in the protein hydrophobic thickness occurs when MII is formed from MI (Fig. 5). Clearly the chain length cannot be matched simultaneously to the hydrophobic dimensions of rhodopsin in both the MI and MII states; rather a *curvature elastic stress or frustration* exists in one or the other conformational state of the protein. The data for the recombinants with phosphatidylethanolamines having unsaturated and polyunsaturated acyl chains suggest the MI-MII transition may involve a change in the membrane lipid/water interfacial curvature free energy (Figs. 7–9). Let us neglect the Gaussian or saddle curvature for simplicity and only consider a single modulus of curvature [113,114]. For heuristic purposes, we shall assume that an increase in the hydrophobic thickness of photolysed rhodopsin occurs in the transition from MI to MII, and that the acyl chain length *in the planar state* is matched to rhodopsin in the MI conformation (Fig. 11). It follows that a change in the interfacial curvature occurs during the MI-MII transition. In the case of phosphatidylcholines having zero spontaneous curvature the MI state is favored; whereas the MII state is under curvature stress and is higher in energy. By contrast, in lipid

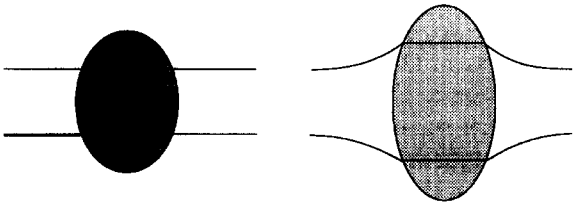


Fig. 12. Schematic illustration of the membrane changes accompanying the MI-MII transition of rhodopsin. The hydrophobic surface of rhodopsin is depicted by the cross-hatched regions; an elongation or protrusion of the protein at the MII stage may occur yielding exposure of recognition sites for the *G* protein, accompanied by curvature of the membrane lipid/water interface. The MI state is favored by lipids tending to form the lamellar phase having a planar lipid/water interface, e.g., phosphatidylcholines. By contrast, the MII state is favored by lipids tending to form the reverse hexagonal ($H_{II}$) phase with a negative interfacial curvature, e.g., unsaturated phosphatidylethanolamines.

mixtures having phosphoethanolamine head groups together with polyunsaturated chains, i.e., which have a tendency to form the reverse hexagonal ($H_{II}$) phase, it is the MI state that is under curvature stress and is disfavored; MII has a curved lipid/water interface in the vicinity of the protein and is relatively low in energy. It follows that a relief of the curvature frustration of photolysed rhodopsin in the MI state occurs which can provide a reservoir of work for the MI-MII transition [41]. In terms of the above picture, material properties associated with the bending energy of the lipid/water interface may govern the MI-MII transition; other interactions can be considered.

## 5. Emerging biophysical principles

The research described above has tested the hypothesis [41,79] that the native lipid composition of the rod disk membranes is associated with characteristic physicochemical properties of the bilayer that influence the photochemical function of rhodopsin. Alternatively, the lipid influences may be chemically more specific, or the presence of specific metabolites of the polyunsaturated docosahexaenoic acid (DHA) chains, e.g., products of cyclooxygenase or lipoxygenase activity, may be linked to the visual mechanism [37]. The data currently available indicate that the lipid polar head groups [42,90,91] as well as the acyl chains of the retinal rod membrane lipids [41,58] influence the energetics of the MI and MII conformational states of photolysed rhodopsin. Based on these observations one must significantly revise the Standard Model for lipid-protein interactions in the case of rhodopsin. The neutral phosphoethanolamine head groups have a smaller size and propensity for hydrogen bonding between the ammonium nitrogen and the phosphate oxygen [123], which yields a more condensed bilayer surface. The effects of acidic phosphoserine head groups may indicate that the membrane surface potential contributes to formation of MII by increasing the local concentration of $H_3O^+$ (Le Châtelier's Principle), thereby raising the chemical potential and driving the process toward completion [42,90,91]. Yet native-like lipids are not necessary for the photochemical function of rhodopsin; rather properties of the lipid bilayer appear to be involved [41]. The MI-MII transition is promoted by non-lamellar forming lipids, viz, lipids close to a $L_\alpha$–$H_{II}$ phase boundary [79], in which case there may be a requirement for membrane instability. A membrane comprising phosphoethanolamine head groups together with bulky docosahexaenoyl (22:6$\omega$3) chains may be *elastically stressed or frustrated* on account of the spontaneous curvatures of the two monolayers [41,79]. Coupling of the MI-MII transition to the lateral and/or curvature stresses within the bilayer can provide a source of work, and thus contribute a thermodynamic driving force for the conformational change. One can further propose that twisting or tilting of the seven-helix bundle comprising the transmembrane domain of rhodopsin may occur, yielding a change in surface free energy as discussed above. A microscopic picture then involves the polyunsaturated 22:6 chains in spring-like or helical configurations of variable pitch [108], which may be associated with changes in the protein/lipid or lipid/water stresses.

In conclusion, one can ask what new structural principles have emerged regarding the relationship of membrane properties to biological activity in the case of visual membranes. The evidence at

present indicates that structure-activity relationships exist for biological membranes which are analogous to those of other biologically important macromolecules, viz, the globular proteins and nucleic acids. These structure-activity correlations involving liquid crystalline supramolecular assemblies appear to be rather subtle, and may not involve the more readily seen van der Waals surfaces of the molecules, as abundantly depicted in standard biochemistry texts and scientific journals. The primary observations and conclusions derived from this ongoing research are summarized below.

(i) *Mixtures of lipids* are necessary for native-like photochemical behavior of rhodopsin in recombinant membranes; no single 'magic lipid' has been discovered which is sufficient to yield native-like rhodopsin function.

(ii) *Phosphatidylcholines* alone, either as single components, e.g., di(*n*:0)PC or di(22:6)PC, or in mixtures, e.g., egg PC or di(22:6)PC/egg PC, are not sufficient to support native-like photochemical function of rhodopsin, regardless of the degree of polyunsaturation of the chains.

(iii) An influence of the acyl *chain length* is found, e.g., as predicted by models involving matching of the bilayer thickness to the hydrophobic protein surface [103,104]. Although a minimum bilayer thickness may be necessary for the formation of MII, the acyl chain length is not the key bilayer property; no optimal thickness exists in single lipid component systems.

(iv) The presence of phospholipids besides phosphatidylcholine is important; *phosphatidylethanolamine* and *phosphatidylserine* both promote formation of MII in mixtures with PC, but probably by different mechanisms. PE is a neutral phospholipid having a relatively small head group, leading to a more condensed bilayer surface. By contrast, PS is an acidic phospholipid bearing a net negative charge at neutral pH. The resulting electrical double layer means that the concentration of hydronium ions, $H_3O^+$, is higher adjacent to the membrane lipid/water interface, driving the MI-MII equilibrium to the right. But neither PE nor PS together with PC in unsaturated membranes is sufficient for native-like photochemical behavior.

(v) *Polyunsaturation* of the chains promotes the formation of MII but is not sufficient for native-like behavior, either in single component phosphatidylcholines or their mixtures.

(vi) The influences of membrane lipids on the MI-MII transition are not chemically specific; but rather *material properties* of the bilayer are involved [41] as indicated by lipid substitution experiments.

(vii) Lipids that favor native-like photochemical function tend to form *reverse hexagonal* ($H_{II}$) phases at slightly higher temperatures [41,79]. The MI-MII transition of rhodopsin is promoted by neutral membrane lipids in which the polar head group favors a relatively small interfacial area; whereas the nonpolar acyl chains favor a larger cross sectional area.

(viii) The membrane bilayer can provide a source of work for conformational changes of integral membrane proteins such as rhodopsin; the protein can go uphill in free energy if coupled to a downhill movement of the bilayer. The resulting force imbalance due to the presence of $H_{II}$ forming lipids in the planar state can lead to a *curvature elastic stress*. This *frustration* can be relieved by a protein conformational transition coupled to changes in the membrane curvature free energy. The latter may involve but is not necessarily restricted to a change in the physical curvature of the membrane lipid/water interface in the vicinity of an integral membrane protein.

(ix) The influence of lipids on the conformational energetics of the MI-MII transition of rhodopsin may be related to the biological effects of alterations of the retinal mem-

brane lipid composition, e.g., through dietary means [26–29] or possibly lipid diseases such as retinitis pigmentosa [34,124]. However, the influences of lipid-rhodopsin interactions on later steps of the phototransduction cascade remain to be shown.

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