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INFLUENCE OF MEMBRANE LIPIDS ON RETINAL-AMINE INTERACTONS

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Abstract — The effect of vesicular L-α-phosphatidylcholine on the rate of formation of all-*trans*-N-retinylidene-*n*-butylamine (**3**) and on the regeneration kinetics of bacteriorhodopsin pigment from retinal and bacterio-opsin have been studied. An estimate of the relative positions of retinal and *n*-butylamine in the vesicles has been made by fluoresence quenching experiments. Partition coefficient of retinal and microviscosity of the retinal-binding region have also been determined. The results are discussed in terms of the nature of chemical interaction between retinal and amine in a lipid environment.

INTRODUCTION

Signal and light-energy transducing photoreceptors such as rhodopsin and bacteriorhodopsin consist of seven membrane-spanning α-helices[1,2]. The chromophoric retinal is covalently linked to a lysine residue through a protonated Schiff base linkage in the seventh trans-membrane helix (Fig.1) of these proteins. Upon absorption of photon, bacteriorhodopsin undergoes a photocyclic reaction involving *trans-cis* isomerisation of the 13/14 C=C of retinylidene moiety which ultimately results in proton translocation across the cell membrane[3]. During this photoprocess deprotonation and protonation of the Schiff base chromophore also occurs. However the Schiff base linkage between retinal and lysine remains intact. In visual rhodopsin, on the other hand, absorption of photon causes *cis-trans* isomerisation of the double bond of the retinylidene chromophore followed by hydrolysis of the Schiff base giving retinal and opsin. This process is commonly known as the bleaching process[4]. Retinal reacts with the apoprotein opsin in vivo in subsequent dark process to regenerate rhodopsin.

The formation, stability, bleaching and regeneration of the retinal Schiff base chromophore play crucial role in the structure and function of these proteins. A complete chemical knowledge of these processes is central to our understanding of light-induced energy and sensory transduction. It is important to elucidate the role of membrane lipid on these processes as all these events take place in lipid bilayer.

It has been shown earlier that phospholipids play crucial role in the photoisomerization processes of retinal and its Schif bases[5,6]. Lipid-protein interactions are also implicated in the absorption behaviour of bacteriorhodopsin[7,8]. It has been reported that when

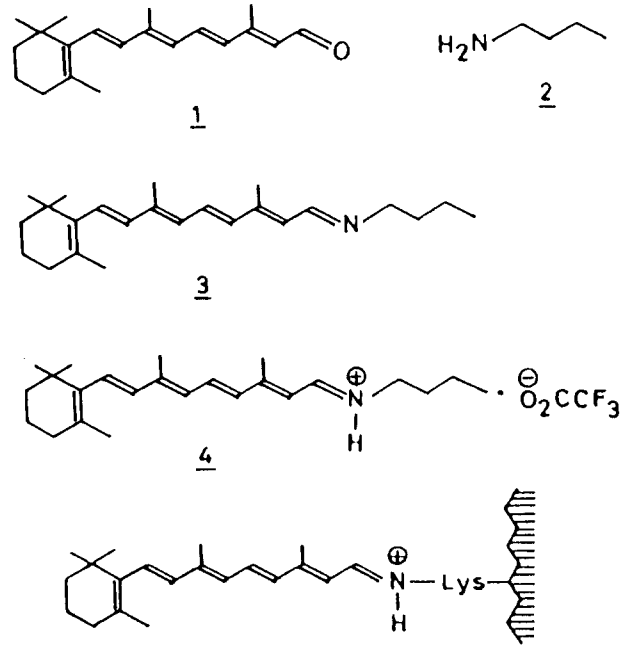

Figure 1. Schiff base chromophore in retinal proteins. Rhodopsin contains 11-*cis*; bacteriorhodopsin contains all-*trans* and 13-*cis* chromophore.

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trimer structure of bacteriorhodopsin is broken down to monomers by detergent Triton, the photocooperativity is lost[9,10]. The nature and the percentage of exogenous lipids also influences the photocycle of monomeric bacteriorhodopsin[11]. In retinal pigment epithelium membrane phospholipid is involved in acyl group transfer reaction to provide energy for the conversion of all-*trans*-retinoids to 11-*cis*-retinoids which is thermodynamically high-energy demanding[12]. These suggest an important role for the membrane, presumably the lipid structure, in the ability of light to influence and control bacteriorhodopsin photocycle and for its effective functioning. We report here a study of the effect of lipid concentration on the rate of formation of the Schiff base of retinal with *n*-butylamine and the ε-amino group of the lysine in bacterioopsin (*i.e.*, the photobleached bacteriorhodopsin, devoid of retinal chromophore). The microenvironment of retinal in phosphatidylcholine vesicles has also been characterised by fluorescence spectroscopy and the nature of retinal-amine interactions in presence of phospholipid is discussed.

MATERIALS AND METHODS

All-*trans*-Retinal **1** (Sigma, USA), trifluoroacetic acid and pyrene (Aldrich, USA), L-α-phosphatidylcholine (from Centre for Biochemical Technology, New Delhi) and all other chemicals (from Sisco Research Laboratories, Bombay) were used as received. *n*-Butylamine(Aldrich, USA) was distilled under nitrogen and stored over molecular sieves (4A) prior to its use. Double distilled water was used for preparation of buffers. The buffer solutions of pH 7.0, 7.1 and 9.4 were tris. HCl, phosphate and borate buffer, respectively. All procedures involving retinal and related Schiff bases were performed under dim red light and under nitrogen atmosphere. Bacteriorhodopsin and bacterioopsin were obtained from *Halobacterium halobium* (R M) by following the procedures described elsewhere[13,14]. The apoprotein was pelletised and washed for three times with water to remove retinal oxime and excess hydroxylamine. Finally the apoprotein was suspended in tris buffer (0.01 *M*, pH 7.0). UV-vis measurements were taken on Hitachi-U-2000 spectrophotometer. All pH measurements were made on Radiometer PHM 84 research pH meter. Sonications were done by using Branson Sonifier 450 (output power 400 W, frequency 20 kHz). Fluorescence spectra of deoxygenated samples were recorded on Spex Spectrofluorolog Spectrofluorimeter equipped with a 450 W xenon lamp as light source. Data were analysed on a DM-IB data processor interfaced with the spectrofluorimeter. Fluorescence lifetime was measured on an Applied Photophysics SP-70 nanosecond spectrofluorimeter equipped with a 250 W xenon lamp and 200 kHz nanosecond flash lamp as light source.

All-trans-N-retinylidene-n-butylamine **3** *in L-α-phosphatidylcholine vesicles.* Solution of all-*trans*-retinal (27.7 μL, 3.60 x 10^{-3} *M*) in chloroform L-α-phosphatidylcholine (0-500 μL, 2.50 x 10^{-3} *M*) in *n*-hexane were mixed in an amber coloured flask. Most of the organic solvents were removed from the mixture on a rotary evaporator under diminished pressure. The mixture was further kept in the dark under vacuum (10^{-4} Torr) for three hours to ensure complete removal of organic solvent and formation of a thin shiny film. Appropriate buffer solution (2 mL) was added in the flask containing the thin film and the contents were sonicated for ten minutes and centrifuged (5400 g, 4°C) for ten minutes. *n*-Butylamine (3 μL) was added to the solution and the formation of the Schiff base was followed by monitoring the growth of the uv-vis band 363 nm. The pseudo first order rate of formation of the Schiff base was calculated by plotting log (A_∞-A_t) versus t; where A_∞ (is the absorbance at the end of reaction and A_t is the absorbance at time t.

Regeneration of bacteriorhodopsin from vesicular bacterioopsin and retinal (**1**). Bacterioopsin and phosphatidylcholine were suspended in tris buffer(pH 7.0, 0.01 *M*) and the mixture was sonicated at 0°C for three minutes. Sonications were repeated three times with two minutes intervals with cooling. The final protein and lipid concentrations were 2 x 10^{-5} *M* and 3.4 x 10^{-4} *M*, respectively. A concentrated solution of retinal in ethanol (3 μL, 1.13 x 10^{-2} *M*) was added to the vesicle constituted bacterioopsin solution (1 mL) and the change in absorbance at 555 nm was monitored by uv-vis spectrophotometry. A control experiment was performed by adding retinal to the bacterioopsin solution in tris buffer.

Determination of partition coefficient of retinal (**1**) *in L-α-phosphatidylcholine vesicles.* Pyrene was incorporated into vesicles of different lipid concentrations following the above mentioned procedure for retinal. Pyrene concentration was maintained at 1 x 10^{-6} *M* in all the cases. Vesicular retinal (5 μL,4 x 10^{-4} *M*) was added each time to the vesicular pyrene solution (2 mL) and fluorescence spectra were recorded. Aliquots of the quencher were added at a time interval of ten minutes so that fusion of the vesicles containing probe and the quencher could occur. The final concentration of retinal in each solution was a multiple of 1 x 10^{-6} *M*. The solutions were excited at 335 nm and emission was monitored at 394 nm. Fluorescence lifetime of vesicle (lipid concentration = 1 x 10^{-3} *M*) constituted pyrene (1 x 10^{-6} *M*) were recorded using single photon counting technique and data were analysed by decovolution procedure.

Quenching of pyrene fluorescence by n-butylamine in vesicles. To the vesicle solution of pyrene measured amount (in microlitres) of amine was added. The cuvette was sealed and the spectra were recorded after an incubation time of ten minutes.

RESULTS AND DISCUSSION

Retinal Schiff base (**3**) *formation in vesicles.* Vesicles containing all-*trans*-retinal (**1**) used in this

study have been characterised by gel permeation chromatography and electron microscopy[15], and it has been shown that the reaction between retinal (**1**) and *n*-butylamine can be conveniently be monitored by uv-vis spectrophotometry. Retinal (**1**) incorporated in phosphatidylcholine vesicles exhibits uv-vis band at 380 nm. *n*-Butylamine has been shown to react with vesicular retinal and give Schiff base **3** having uv-vis absorption maximum at 363 nm. Addition of trifluoroacetic acid to vesicular **3** causes a bathochromic shift of 72 nm in its uv-vis band due to the formation of Schiff base salt **4**. It is known that retinal Schiff bases show bathochromic shift when treated with anhydrous acids, the extent of which depends on the solvents and the type of acids used[16,17]. Such bathochromic shifts in the absorption band of **3** in different media is shown in Table 1. The spectral shifts of the protonated Schiff bases derived from long conjugated polyenes are the consequence of a comperatively small delocalisation of their positive charge in the ground state compared to the excited state, and the absorption shift is controlled by the distance between the centres of cationic and anionic charges. In the present study different extent of spectral shift is the manifestation of extent of tightness in the ion pair which is influenced by solvent polarity.

As shown in Table 2 and Figure 2, the rate of formation of Schiff base **3** is influenced by lipid concentration and the pH. As the concentration of phosphatidylcholine is increased, the rate of formation of **3** decreased. Schiff base **3** is found to be stable in vesicles without any significant loss of the chromophore. However the protonated Schiff base (**4**, λ_{max}, 435 nm), with trifluoroacetate as the counterion, in vesicles was found to be less stable than **3** in the vesicles. Retinal Schiff base **3** was also formed in aqueous buffer and the pseudo first order rate of formation of **3** depended upon the pH of the medium. However, in aqueous buffers, the yield of **3** was much smaller and its hydrolysis to retinal and amine occured very fast. On the other hand, Schiff base **3** does not undergo hydrolysis and remains stable in the crafted environment of the lipid bilayer. It is clear that rate of formation of **3** is governed by the attack of the unprotonated amine on the carbonyl carbon of retinal. The increase in the rate of formation of **3** in vesicles prepared from buffer of higher pH value is compatible with the general mechanism of Schiff base formation[18,19].

Table 1. UV-vis absorption maxima of the retinal Schiff base **3** and its salt **4**

Medium	λ_{max}(nm)	
	3[a]	**4**[b]
Heptane	355	450
Methanol	358	441
AOT-heptane[c]	356	425
Phosphatidyl-choline vesicle	363	435

[a] [**3**], 1 x 10^{-5} *M* . [b] protonating agent CF_3COOH, 1 x 10^{-3} *M*. [c] [AOT], 1 x 10^{-2} *M* and w = $[H_2O]/[AOT]$= 0.

Table 2. Effect of L-α-phosphatidylcholine concentration on the rate of formation of **3** in vesicles at two different pH values.[a]

L-α-Phosphatidyl-choline, 10^{-5} M	k,10^{-3} s^{-1} (pH=7.1)	k,$10^{-3}s^{-1}$ (pH=9.4)
0	17.0	24.00
2.50	7.43	10.00
5.00	4.55	6.60
7.50	4.43	5.91
10.0	3.04	3.65
14.20	2.88	3.10
18.90	2.78	2.95
25.00	2.63	2.85

[a] Retinal **1**, 5 x 10^{-5} *M* ; n-$BuNH_2$ **2**, 3 x 10^{-2} *M*; k, rate constant.

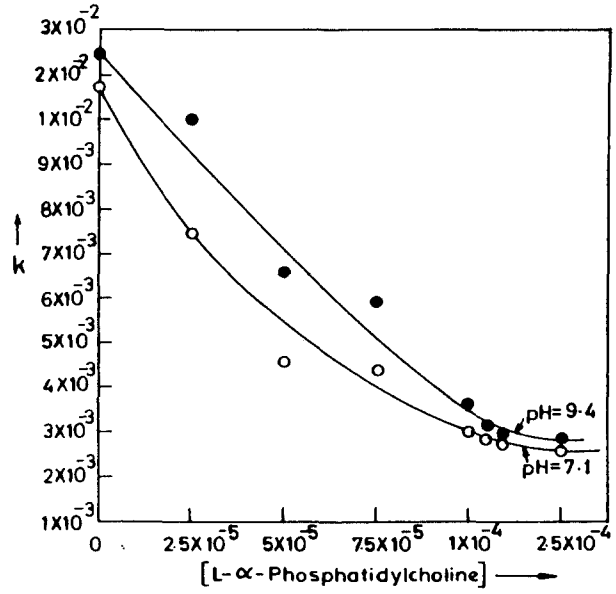

Figure 2. Plot of rate constant of formation (k, sec^{-1}) of **3** versus phosphatidylcholine concentration (*M*)

Determination of partition coefficient and location of retinal(1) in vesicles. The location of retinal in vesicles is established by determining its partition

coefficient K which is defined as follows(equation 1)

$$K = \frac{[\text{retinal}]_{\text{mem}}/[\text{lipid}]}{[\text{retinal}]_{\text{water}}} = \frac{[\text{retinal}]_{\text{mem}}}{[\text{retinal}]_{\text{water}}[\text{lipid}]} \quad (1)$$

where $[\text{retinal}]_{\text{mem}}$ is the concentration of retinal at the membrane interface and $[\text{retinal}]_{\text{water}}$ is the concentration of retinal in water. The following equation-2 has been used to calculate the partition coefficient[20-22].

$$[\text{Retinal}]_{\text{total}} = n/K + n[\text{lipid}] \quad (2)$$

where n is the mean occupation number and gives the average concentration of retinal in pseudophase. In determining the partition coefficient(K) of retinal in vesicles, pyrene was used as a fluorescent probe and retinal acted as a quencher of pyrene fluorescence (Fig. 3). Initially a plot of I_0/I versus [retinal] at different lipid concentration was obtained (Fig. 4). I_0/I is the ratio of the fluorescence of intensity of pyrene at 394 nm in absence and in presence of retinal (1) as

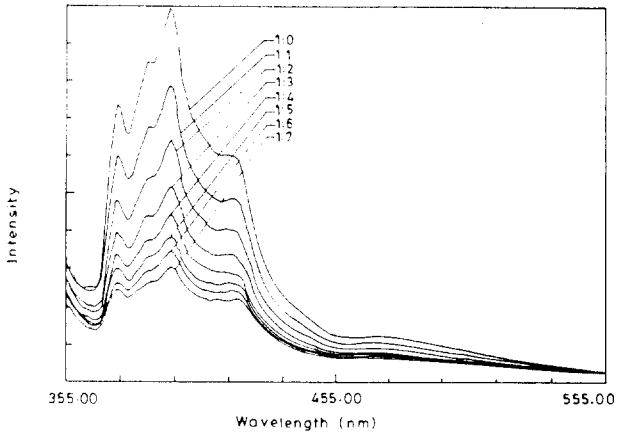

Figure 3. Quenching of pyrene (1 x 10^{-6} M) fluorescence by retinal in vesicles. Excitation is at 335nm. Molar ratio of pyrene to retinal varied from 1:0 to 1:7

quencher. The linear Stern-Volmer plots indicate a single quenching mechanism in operation. From Figure 4 it is evident that the quenching effiency decreases with increasing lipid concentration. This is because of dilution of the quencher in the lipid phase with the increase in lipid concentration. For a given I_0/I value, plot of different sets of [lipid] versus $[\text{retinal}]_{\text{total}}$ could be obtained (Fig. 5). This plot has n as the slope and n/K as the intercept (equation 2) where from K can be calculated for different I_0/I values (Table 3). Pyrene is a fluorescent probe which

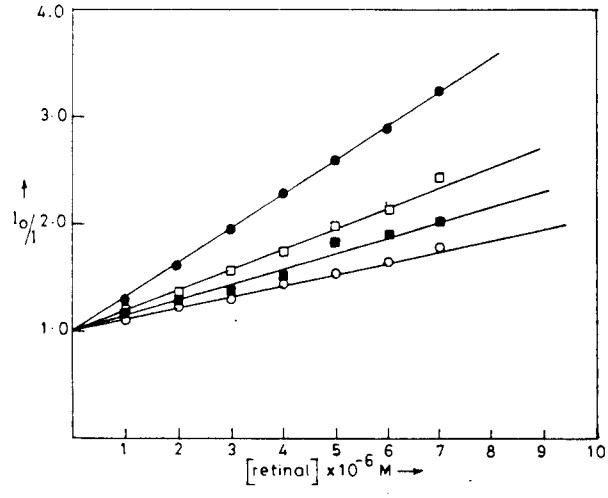

Figure 4. Stern-Volmer plots for quenching of pyrene fluorescence by retinal in vesicles. Lipid conc :(●)5 x 10^{-4} M; (□)1 x 10^{-3} M; (■)1.5 x 10^{-3} M; (○)2 x 10^{-3} M.

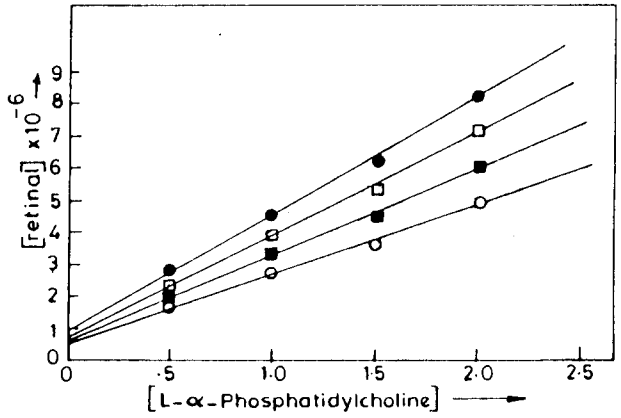

Figure 5. Plot of retinal concentration(moles/litre) against lipid concentration(moles/litre) at different I_0/I values. I_0/I values : (●)1.5; (□)1.625; (■)1.750; (○)1.875.

localises itself mainly in the hydrophobic domain and is used to study the properties of the bilayer membrane[23,24]. The average value of the partition coefficient (K_{av}) for retinal in vesicle is 4.24 x 10^3 M^{-1}. The high value of K indicates that retinal is strongly solubilised in lipid bilayer rather than in aqueous phase. From the lifetime data of pyrene in vesicles it is possible to calculate the quenching constant of the process from the following relationship(equation 3)[25].

Table 3. Values of n and K at different I_0/I values for the quenching of pyrene fluorescence by retinal at 25°C.

I_0/I	10^3 n	10^6 n/K	10^{-3} $K(M^{-1})$	10^{-3} K_{av} (M^{-1})
1.50	36.36	9.1	4.00	
1.625	32.50	7.5	4.33	4.24
1.750	26.66	6.0	4.44	
1.87	21.42	5.1	4.20	

$$I_o/I-1 = K_{sv}[Q]$$
$$= k_q \tau_o [Q] \qquad (3)$$

Again for a diffusion controlled process

$$k = k_{diff}$$
$$k = 8RT/2000\eta \qquad (4)$$

where η is the microviscosity. This equation is employed to find the microviscosity of the medium. The lifetime for vesicle incorporated pyrene is found to be 111 nanosecond. From the slope of Stern-Volmer plot K_{sv}, k_q and η for retinal (**1**) in vesicles were calculated to be 1.85 x 10^5 M^{-1}, 1.665 x 10^{12} M^{-1} s^{-1} and 5.94 x 10^{-12} N·s·m^{-2}, respectively, for the phospholipid concentration of 1 x 10^{-3} M. For *n*-butylamine, however, the K_{sv} is found to be 2.536 M^{-1} upon quenching the pyrene fluorescence (Fig. 6) which is very less compared to retinal.

These results indicate that retinal quenches pyrene emission more effectively than *n*-butylamine. A large

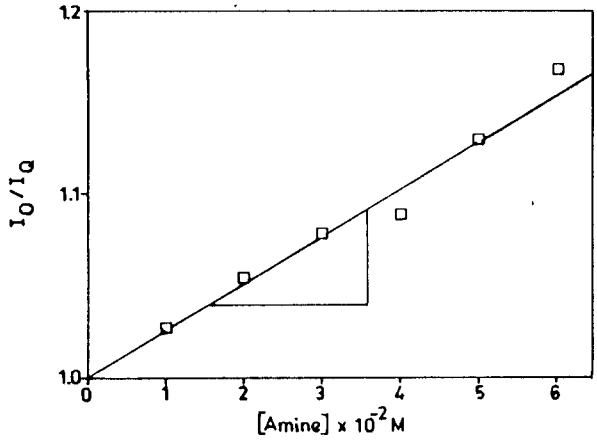

Figure 6. Stern-Volmer plot for quenching of pyrene fluorescence by *n*-butylamine in vesicles (L-α-phosphatidylcholine, 1 x 10^{-3} M)

difference in K_{sv} values suggests that retinal resides closer to pyrene in a hydrophobic region as compared to *n*-butylamine. Thus, from the fluorescence studies it is found that retinal (**1**) resides in the hydrophobic region of the vesicles. *n*-Butylamine is required to reach retinal by crossing the lipid barrier in order to react with retinal and form Schiff base **3**. On the other hand absence of such lipid barrier in non-organised aqueous buffers allows retinal and amine to interact yielding Schiff base **3** with rate greater than that found in the vesicular systems.

Regeneration of bacteriorhodopsin by allowing retinal (1) to react with vesicle constituted bacterioopsin. The regeneration of bacteriorhodopsin was carried out both in presence and in absence of external phospholipid. Percentage of regeneration was calculated from the absorbance at 555 nm at different time intervals and final constant absorbance. It is evident from Figure 7 that lipid retards the regeneration kinetics. After fifteen minutes 78% of

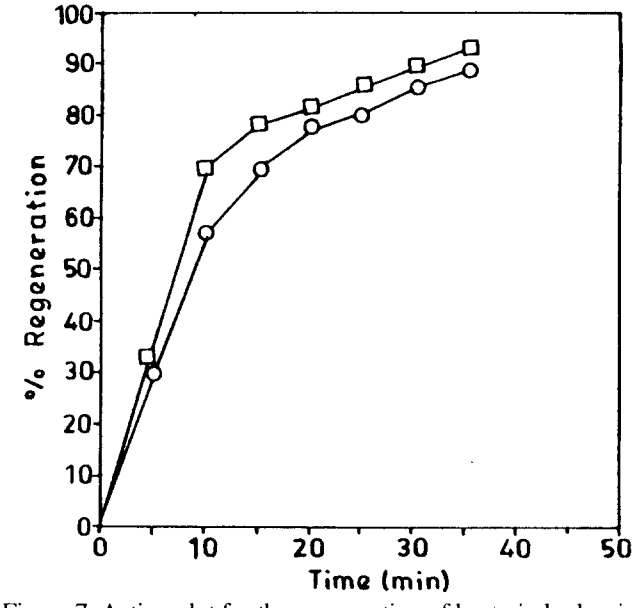

Figure 7. Action plot for the regeneration of bacteriorhodopsin. a) in absence of external lipid (L-α-phosphatidylcholine) (□), b) in presence of external lipid(3.4 x 10^{-4} M)(○).

the pigment was regenerated in aqueous buffer whereas 69% of the pigment was regenerated in vesicles. It has been shown earlier[26,27] that in the absence of exogenous lipid, delipidated bacterioopsin in deoxycholic acid and delipidated bacterioopsin in sodium dodecyl sulfate fail to regenerate bacterioopsin when incubated with retinal. This is because initially lipids are required to bring about the renaturation of the protein which gets denatured upon delipidation. Thus, in the case of delipidated bacterioopsin, regeneration process involves an additional step of renaturation. In the present case the presence of external phospholipid is found to retard the regeneration kinetics. Thus, in addition to their role in renaturation, phospholipids affect the Schiff base formation kinetics also.

The present studies show that the lipid structure plays a key role in retinal-amine interactions, *i.e.*, Schiff base formation, hydrolysis and regeneration in retinal proteins particularly, in bacteriorhodopsin.

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