ELSEVIER

Biochimica et Biophysica Acta 1297 (1996) 77–82

BBA Biochimica et Biophysica Acta

# Effect of cholesterol on rhodopsin stability in disk membranes

Arlene D. Albert [a,b,*], Kathleen Boesze-Battaglia [a,1], Zofia Paw [a], Anthony Watts [c], Richard M. Epand [d]

[a] *Department of Biochemistry, 140 Farber Hall, State University of New York at Buffalo, Buffalo, NY 14214, USA*
[b] *Department of Ophthalmology, State University of New York at Buffalo, Buffalo, NY 14214, USA*
[c] *Department of Biochemistry, Oxford University, Oxford OX1 3QU, UK*
[d] *Department of Biochemistry, McMaster University, Hamilton, Ont. L8N 3Z5, Canada*

Received 15 February 1996; revised 18 June 1996; accepted 21 June 1996

**Abstract**

The effect of cholesterol on rhodopsin stability has been investigated in intact disk membranes. Because cholesterol readily equilibrates between membranes, the disk membrane cholesterol content can be altered by incubation with cholesterol/phospholipid vesicles. The effect of membrane cholesterol on rhodopsin was investigated using three independent techniques: thermal bleaching, differential scanning calorimetry (DSC) and activation of the cGMP cascade. Rhodopsin exhibited an increased resistance to thermally induced bleaching as the membrane cholesterol level was increased. DSC also indicated that the protein is stabilized by cholesterol in that the $T_m$ increased in response to higher membrane cholesterol. A similar degree of stabilization was observed in both the unbleached and bleached states in the DSC experiments. These results suggest that cholesterol affects the disk membrane properties such that thermally induced unfolding is inhibited, thus stabilizing the rhodopsin structure. Furthermore, high membrane cholesterol inhibited the activation of the cGMP cascade. This is consistent with the stabilization of the metarhodopsin I photointermediate relative to the metarhodopsin II intermediate.

*Keywords:* Rod outer segment disk; Rhodopsin; Metarhodopsin; Cholesterol; Thermal stability; DSC

## 1. Introduction

The rod outer segment (ROS) disk membrane is the site of the primary events of visual transduction. The photopigment, rhodopsin, is the major integral membrane protein of the disk membrane, constituting greater than 90% of the membrane protein [1]. Absorption of light by 11-*cis* retinal triggers a series of conformational changes. The conformational transition which initiates the visual transduction — i.e., the cGMP cascade — is the conversion of the photointermediate metarhodopsin I to metarhodopsin II. The ability of rhodopsin to undergo these conformational changes is essential to its role as a receptor. Because rhodopsin is largely buried in the lipid bilayer, it is expected that the lipid matrix can influence rhodopsin conformational properties. This influence may be on both light induced non-denaturing conformational changes and the unfolding which occurs upon protein denaturation.

The role of cholesterol in membranes has been the subject of many investigations [2]. It is well known from model membrane studies that cholesterol broadens the phase transition of the lipid bilayer. Furthermore, cholesterol has been shown to increase membrane protein stability [3].

Cholesterol has been shown to inhibit the metarhodopsin I to metarhodopsin II conformational transitions of rhodopsin in reconstituted membrane systems. An increase in membrane cholesterol results in a decrease in the partial free volume of the bilayer. Because the Meta I to Meta II

Abbreviations: CTAB, cetyltrimethylammonium bromide; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; EDTA, ethylenediamine tetracetic acid; DTT, dithiothreitol; PDE, phosphodiesterase; DSC, differential scanning calorimetry; $T_m$, temperature maximum of the thermal transition; ROS, rod outer segment; PC, phosphatidylcholine; SUV, small unilamellar vesicles; c/p, cholesterol to phospholipid ratio; $\Delta H_{cal}$, calorimetric enthalpy; Meta I or II, metarhodopsin I or II.

* Corresponding author. Fax: +1 (716) 8292725; e-mail: aalbert@ubmedg.buffalo.edu.

[1] Present address: Department of Molecular Biology, UMDNJ-SOM, 2 Medical Ct. Dr., Stratford, NJ 08084, USA.


*PII* S0167-4838(96)00102-1

transition involves an increase in volume, the Meta I state is stabilized by membrane cholesterol [4,5]. Additional studies in reconstituted membranes have been presented which indicate that cholesterol increases the thermal stability of rhodopsin in reconstituted membranes [6]. While the previous studies investigated the effect of membrane cholesterol on rhodopsin in reconstituted membranes, high membrane cholesterol has also been shown to inhibit the cGMP cascade in ROS plasma membranes [7].

In this study, the effect of cholesterol on the thermal stabilization of rhodopsin in the intact ROS disk membrane was investigated. This stabilization to irreversible thermal unfolding is compared to the effect of cholesterol on the non-denaturing, reversible, light induced conformational changes of this receptor. The light induced conformational changes were monitored through its ability to activate the cGMP cascade. Rhodopsin thermal stability was evaluated by differential scanning calorimetry (DSC) and by thermal bleaching. By using intact disk membranes, the phospholipid composition and asymmetry of the original disk membrane are maintained. These studies demonstrate that cholesterol can enhance the thermal stability of rhodopsin to unfolding. In addition, the data are also consistent with an inhibition of the conformational change which occurs upon conversion of Meta I to Meta II. Since rhodopsin is a member of the large family of G protein receptors, it is likely that the conclusions from this study will be relevant to other G-protein receptors.

## 2. Materials and methods

### *2.1. Preparation of bovine rod outer segment (ROS) disk membranes*

Retinal rod outer segment disk membranes were prepared from frozen bovine retinas (J. Lawson Inc., Lincoln, Nebraska) as described [8]. All manipulations of the rod outer segment and disk membranes were performed under a Kodak 1A red filter. The isolated disks were washed and resuspended in 10mM Hepes, 1mM EDTA, 0.1mM DTT pH 7.0 to a final rhodopsin concentration of 4–6 mg/ml. The buffers used were made 1mM in EDTA, 0.5 mM in DTT and perfused with nitrogen or argon to reduce lipid oxidation [9]. Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL).

### *2.2. Modulation of the cholesterol content of ROS disk membranes*

The procedure used is essentially that described previously [10]. Small unilamellar vesicles (SUV) composed of PC and cholesterol or of PC only were incubated with disk membranes in a shaking water bath at 37°C for 3–5 h. The disk membranes were then separated from the SUV by centrifugation at 30000 rpm for 20 min. The pellet was washed twice with 10 mM Hepes, 100 mM NaCl (pH 7.2). The phospholipid/protein ratio was determined in the cholesterol-modified membranes as a control for sticking or fusion of the PC SUV to the disk membranes. In most cases, no sticking or fusion was observed. In the rare case of an increase in this ratio, the material was discarded. After isolation of the disk membranes, the cholesterol/phospholipid ratio was also determined. Disks were incubated with three types of SUV: PC vesicles, PC:cholesterol (9:1 mole ratio) and PC:cholesterol (1:1 mole ratio).

### *2.3. Phosphodiesterase activity measurements*

The ability of the disks to activate phosphodiesterase (PDE) was determined as previously described [7]. Briefly, transducin and PDE-rich extracts were prepared from ROS by washing with a hypotonic buffer. The soluble enzymes were added to disk membranes along with cGMP and GTP. Upon exposure to light, the hydrolysis of cGMP was monitored by the change in pH.

### *2.4. Differential scanning calorimetry measurements*

Initial measurements of $T_m$ were made on a Perkin-Elmer DSC7 scanning calorimeter using a heating scan rate of 2°C/min. The reference chamber contained only the buffer. Additional measurements were made on a Microcal MC-2 scanning calorimeter with a heating scan rate of 0.6°C/min.

### *2.5. Thermal bleaching of rhodopsin*

The disk membranes were incubated for various times at 70°C, then rapidly cooled by immersing them into liquid nitrogen. The aliquots were solubilized in 1.12% CTAB in the presence of hydroxylamine. The concentration of rhodopsin for each time point was determined by the change in absorption at 500 nm using an extinction coefficient of 40000. The initial concentration of rhodopsin was approx. 1 mg/ml.

### *2.6. Protein, phospholipid, cholesterol assays*

Phospholipid was determined by the method of Bartlett [11] as modified [12], protein by the method of Lowry [13], and cholesterol by enzymatic assay using cholesterol oxidase [14].

### *2.7. Statistical analysis*

Regression analyses were carried out using the program Stata (Stata, College Station, TX).

## 3. Results

### 3.1. Modification of disk membrane cholesterol content

Disk membrane cholesterol content can be modified by cholesterol equilibration between disk membranes and unilamellar lipid vesicles [15]. Disk membranes depleted in cholesterol (c/p < 0.1) were prepared by incubation with phosphatidylcholine vesicles containing no cholesterol. Disk membranes were also incubated with PC vesicles containing 10 mole percent cholesterol. Cholesterol is present at approximately 10 mole percent in native disk membranes. Therefore, these incubated disk membranes exhibited the average native membrane cholesterol level (c/p = 0.1). Finally, cholesterol enriched disk membranes were prepared by incubation with phospholipid vesicles containing 50 percent cholesterol. The phospholipid composition of the disk membrane is unfavorable for a high membrane cholesterol level [15]. Therefore c/p ratios above 0.4 were never achieved. However, disks with c/p ratios greater than 0.3 were produced. Cholesterol rapidly equilibrates among disk membranes to produce a uniform cholesterol distribution [16]. Therefore each of these preparations was uniform with respect to membrane cholesterol levels. This process does not alter the disk membrane protein content. Therefore in each preparation the phospholipid: protein ratio was that found in the native membrane (70:1).

### 3.2. Activation of the cGMP cascade by disks with modified cholesterol content

In response to light, rhodopsin undergoes a series of conformational changes. The conversion of the photo intermediate Meta I to Meta II activates an enzymatic cascade which culminates in the activation of a phosphodiesterase (PDE) and the hydrolysis of cGMP. Thus a decrease in the ability of rhodopsin to form Meta II in response to light will be reflected by a decrease in PDE activity. The ability of rhodopsin to activate this enzymatic cascade was determined in ROS disks whose cholesterol content had been modified as described above. The PDE activity was investigated by monitoring the change in pH as protons were released by cGMP hydrolysis as described in the methods. The data shown in Fig. 1 indicate that for each of the disk samples increasing light intensity resulted in an increased activity until the system is fully activated. Prior to the light flash no activity was detected. The ability of rhodopsin in these disk membranes to initiate the activation of PDE decreased by approx. 50% in disks with c/p ratios above 0.3 as compared to those with c/p ratios of 0.1.

Based on previously published studies [5,17] the most likely interpretation of these results is an inhibition of the Meta I to Meta II transition due to the presence of cholesterol in the membrane. However, membrane cholesterol may have also alter the ability of the cascade G protein,

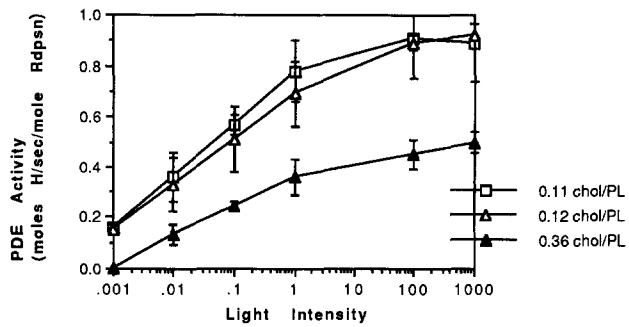


Fig. 1. PDE activity initiated by bleaching disk membranes with cholesterol to phospholipid ratios of 0.11 (□), 0.12 (△) and 0.36 (▲). PDE and transducin were added to disk membranes which exhibited no PDE activity in the presence or absence of light. PDE activity is expressed as μmoles $H^+$ released/second/mole rhodopsin × $10^6$. Light-stimulated PDE activity was assayed by continuous pH monitoring. A one to one relationship between $H^+$ produced and cGMP hydrolyzed is assumed. The rates were corrected for dark activity. All manipulations of rhodopsin containing membranes were done in complete darkness. Light intensity was calibrated using neutral density filters.

transducin to bind to the disk membrane. This would also result in a decrease in PDE activity. No difference in binding was detected (data not shown).

### 3.3. Thermal bleaching of rhodopsin in modified disks

Prolonged heating of rhodopsin results in its denaturation. This thermal bleaching can be followed as a decrease in absorption at 500nm. The stability of rhodopsin to thermally induced bleaching is affected by its lipid environment. Therefore the thermal stability of rhodopsin in disks with modified cholesterol content was examined. Disk membranes with c/p ratios between 0.05 and 0.35 were incubated at 70°C and the amount of rhodopsin which remained after various lengths of time was determined. In Fig. 2 data from five independent experiments are shown. The percent of rhodopsin bleached in these disks is presented as a function of c/p ratio after incubation at 70°C for 15 and 30 min. Linear regression analysis of the 30 minute data produced a slope of value −54 ($P < 10^{-4}$; $R^2 = 0.85$). The same analysis of the 15 min data produced a slope of −100 ($P < 0.03$; $R^2 = 0.40$). These data therefore reveal a statistically significant increase in the stability of rhodopsin to thermal bleaching when the cholesterol content of the ROS disk membrane is increased. The percent of rhodopsin bleached before incubation ($t = 0$) is also shown in Fig. 2. For each of the c/p ratios, there was no detectable bleaching (at $t = 0$). Thus changing the membrane cholesterol level did not by itself induce bleaching.

### 3.4. Differential scanning calorimetry

ROS disks with modified cholesterol content were examined by differential scanning calorimetry (DSC) be-

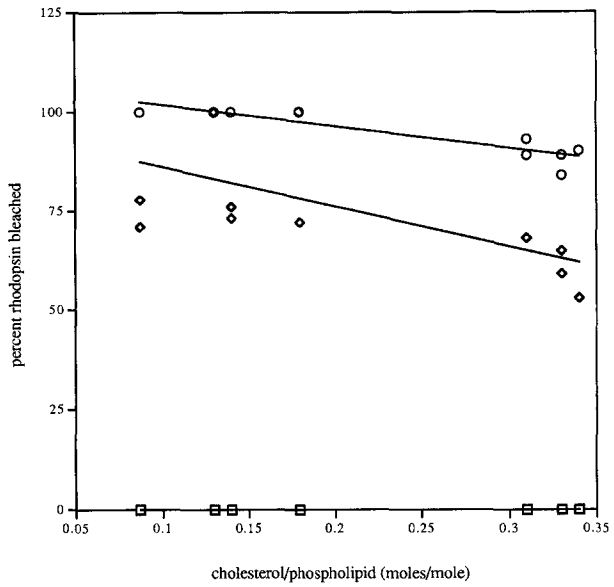


Fig. 2. The percent of rhodopsin thermal bleached as a function of the cholesterol to phospholipid ratio in disk membranes. Samples were incubated at 70°C for 0 min (□), 15 min (◇) or 30 min (○) then rapidly cooled. Rhodopsin concentration was determined by the change in absorbance at 500 nm upon exposure to light. Data represent five independent experiments. Linear regression analysis produced a linear correlation at greater than the 95% confidence level.

tween 20°C and 90°C. It was previously reported that native disk membranes exhibited a single thermal transition due to the denaturation of rhodopsin, the major disk membrane protein [18]. In the experiments reported here, only a single thermal transition was observed in this temperature range, in agreement with the previous report. Typical scans are shown in Fig. 3. The thermal transition was not observed on a second heating scan and therefore can not be proven to be an equilibrium property. The maximum of this transition, $T_m$, was measured and plotted

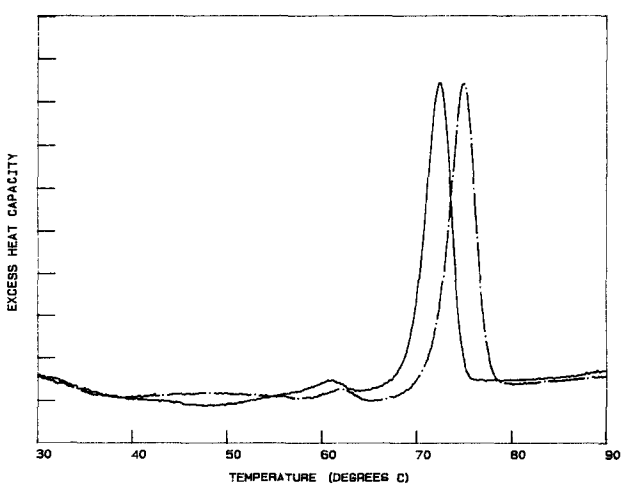


Fig. 3. Representative DSC heating scans of disk membranes with c/p ratios of 0.05 (————) and 0.23 (-·-·-). These scans were preformed on a MicroCal scanning calorimeter. Each tic mark on the ordinate represents 5 kcal $mol^{-1}$ $degree^{-1}$.

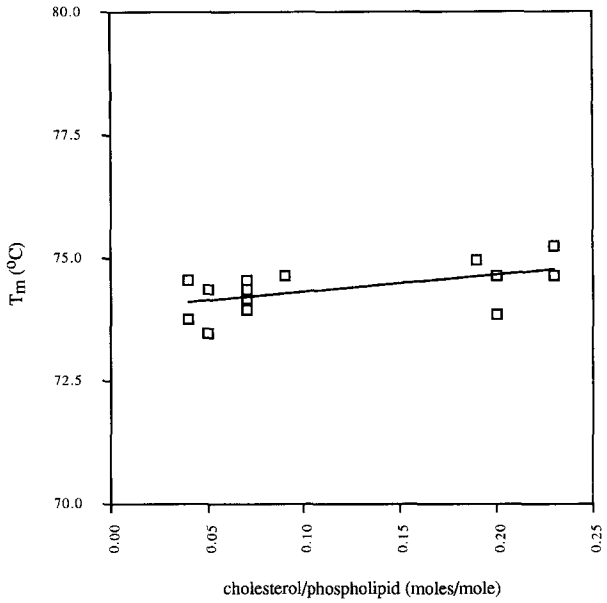


Fig. 4. $T_m$ (°C) of rhodopsin thermal denaturation as a function of the cholesterol to phospholipid ratio in disk membranes as determined by DSC. These measurements were preformed on a MicroCal as above.

in Fig. 4 as a function of c/p. Linear regression of these data produced a positive slope of 3.5 ($P = 0.03$; $R^2 = 0.30$). Thus, a statistically significant (greater than 95% confidence level) increase of the transition temperature was observed as the cholesterol content of the disks was increased.

Upon exposure to bright room light for several minutes, rhodopsin bleaches to form the apo protein, opsin. This bleaching is to be distinguished from the thermally induced bleaching in that the protein is not denatured and in the presence of the chromophore, 11-*cis* retinal will re-

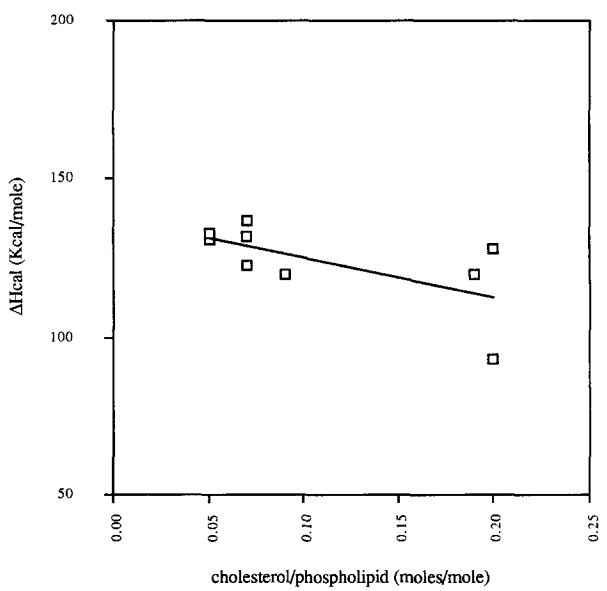


Fig. 5. The enthalpy ($\Delta H_{cal}$ in kcal/mol of protein) of rhodopsin thermal denaturation as a function of the cholesterol to phospholipid ratio.

combine to form rhodopsin. In agreement with earlier studies [18], the $T_m$ of the thermal transition of bleached disks shifts to approx. 15°C less than that observed in the unbleached disks. This transition also does not exhibit reversibility. A similar dependence of $T_m$ on the c/p was observed in bleached membranes as in unbleached disks (data not shown). Collectively, these findings indicate a stabilization of the protein by cholesterol in the membrane.

The calorimetric enthalpy ($\Delta H_{cal}$) is plotted as a function of c/p in Fig. 5. A linear regression of the dependence of $\Delta H_{cal}$ produced a $P$-value of 0.06. Therefore, the $\Delta H_{cal}$ may be independent of the c/p in the disk membranes. As this system can not be demonstrated to be reversible, this parameter can be regarded phenomenological, as the difference in enthalpy between the native and thermally unfolded states [18].

## 4. Discussion

These studies investigate the influence of membrane cholesterol on rhodopsin in the intact disk membrane. The effect of membrane cholesterol on the Meta I to Meta II transition has been studied in reconstituted systems [5,17]. It was also reported that cholesterol can shift the temperature of rhodopsin unfolding in a reconstituted membrane [6]. While reconstituted membranes are chemically well defined, they lack the transbilayer asymmetry of the native disk. In the modified disk membranes used for this study, the native rhodopsin to phospholipid ratio, the native phospholipid membrane composition and both the phospholipid and protein asymmetry are maintained. These results show that the conclusions drawn from reconstituted systems can now be extended to the biological membranes of the ROS. Furthermore, they can be used to explain the effect of cholesterol on rhodopsin activation of the cGMP cascade.

The data presented here show that the activity of the cGMP cascade is inhibited in disks with high membrane cholesterol as compared to those with the normal cholesterol content when transducin and PDEase were exogenously added. It was demonstrated in reconstituted systems that the Meta I to Meta II equilibrium is shifted to favor Meta I as the level of membrane cholesterol is increased. The cholesterol exerts this effect by decreasing the partial free volume of the bilayer. This inhibits Meta II formation because the conformational change of Meta I to Meta II requires an increase in the protein volume. [5,17]. Although the Meta I to Meta II equilibrium reflects a millisecond time scale and the experiments presented here were carried out on a minutes scale, the results are consistent.

These studies further demonstrate that membrane cholesterol can enhance the stability of the major ROS disk membrane protein, rhodopsin, to thermal denaturation. Differential scanning calorimetry provides information on the stability of proteins by determining the temperature at which unfolding occurs, the heat absorbed upon denaturation, the cooperativity of the transition and the $\Delta C_p$ of the process [19]. The DSC measurements revealed a stabilization of the protein to thermal denaturation by the presence of cholesterol. Rhodopsin constitutes approx. 90% of the ROS disk integral membrane proteins. Consistent with previous work [18] unbleached ROS disk membranes exhibited a single major endothermic transition. This transition occurred at approx. 74°C in this study. Upon bleaching rhodopsin to opsin, the transition is shifted to approx. 59°C. As the level of cholesterol in the disk membrane was increased, the temperature of unfolding also increased. This effect of cholesterol was similar for both rhodopsin and opsin.

The thermal stability of rhodopsin was also examined by rates of thermal bleaching. These bleaching experiments indicated a stabilization of rhodopsin by the cholesterol in the disk membrane. The data are consistent with the DSC measurements and indicate that cholesterol has a stabilizing effect on rhodopsin.

Together these studies examined the effect of cholesterol on different protein transitions which may share certain aspects of their transition pathway. The mechanism by which cholesterol inhibits protein denaturation may involve its ability to order phospholipid acyl chains and reduce packing defects. These packing defects may be recruited to provide the volume necessary for conformational changes leading to denaturation of rhodopsin just as they are necessary for the conformational changes of rhodopsin in transduction processes which require the Meta I to Meta II transition [5]. It has been reported that when rhodopsin is incorporated into Langmuir–Blodgett films, a dry state, its thermal stability exceeded 150°C [20]. Additionally, it has been observed that weakly hydrated bacteriorhodopsin (dry state) is stable to 140°C [21]. Since cholesterol is weakly hydrated [22] its affect in raising the temperature of denaturation may also be a consequence of its effect on the hydration of the membrane surface, although hydration and free volume are likely to be interdependent properties.

In these studies membrane cholesterol had a more dramatic effect on the activation of the cGMP cascade than on the thermal stability (either by DSC or thermal bleaching). The protein conformational change necessary for the Meta I to Meta II transition may be more sensitive to cholesterol than the denaturation of either rhodopsin or opsin. This may be due to a larger change of intramembraneous protein volume than that which occurs during thermal denaturation.

## Acknowledgements

Supported by RO1-EY03366 (ADA), Fight for Sight, NSRPB post-doctoral fellowship (KBB), Medical Research Council of Canada, Grant MT-7654 (RME), Medical Research Council of the United Kingdom fellowship (ADA).

## References

[1] Papermaster, D. and Dreyer, W. (1974) Biochemistry 13, 2438–2444.
[2] Yeagle, P.L. (1988) The Biology of Cholesterol, CRC Press, Boca Raton, FL.
[3] Maneri, L.R. and Low, P.S. (1988) J. Biol. Chem 263, 16170–16178.
[4] Straume, M. and Litman, B.J. (1988) Biochemistry 27, 7723–7733.
[5] Mitchell, D., Straume, M., Miller, J. and Litman, B.J. (1990) Biochemistry 29, 9143–9149.
[6] Mone, A.P. and Litman, B.J. (1990) Biophys. J. 57, 74a.
[7] Boesze-Battaglia, K. and Albert, A. (1990) J. Biol. Chem. 265, 20727–20730.
[8] Smith, H.G., Stubbs, G.W. and Litman, B.J. (1975) Exp. Eye Res. 20, 211–217.
[9] Stone, W.L., Farnsworth, C.C. and Dratz, E.A. (1979) Exp. Eye Res. 28, 387–397.
[10] Yeagle, P.L. (1983) Biochim. Biophys. Acta 727, 39–44.
[11] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–473.
[12] Litman, B.J. (1973) Biochemistry 13, 2545–2554.
[13] Lowry, O.H., Rosenborough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–272.
[14] Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Clin. Chem. 20, 470–475.
[15] House, K., Badgett, D. and Albert, A.D. (1989) Exp. Eye Res. 49, 561–572.
[16] Boesze-Battaglia, K., Hennessey, T. and Albert, A.D. (1989) J. Biol. Chem. 264, 8151–8155.
[17] Straume, M., Mitchell, D., Miller, J. and Litman, B.J. (1990) Biochemistry 29, 9135–9142.
[18] Khan, S.M.A., Bolen, W., Hargrave, P.A., Santoro, M.M. and McDowell, J.H. (1991) Eur. J. Biochem. 200, 53–59.
[19] Sturtevant, J.M. (1987) Annu. Rev. Phys. Chem. 38, 463–488.
[20] Maxia, L., Radicchi, G., Pepe, I.M. and Nicolini, C. (1995) Biophys. J. 69, 1440–1446.
[21] Shen, Y. Safinya, C.R., Liang, K.S., Ruppert, A.F. and Rothschild, K.J. (1993) Nature 366, 48–50.
[22] Yeagle, P.L. (1985) Biochim. Biophys. Acta 822, 267–287.