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Rhodopsin-cholesterol interactions in bovine rod outer segment disk membranes

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Abstract

Cholesterol modulates the function of rhodopsin in the retinal rod outer segment (ROS) disk membranes. One mechanism for such modulation is cholesterol modulation of the properties of the membrane bilayer. This has been explored previously. Another possible mechanism is an interaction between the sterol and the protein, which has not been previously explored. In this study, the fluorescent sterol, cholestatrienol, was used to probe interactions between cholesterol and rhodopsin in bovine ROS disk membranes. Cholestatrienol was incorporated into the disk membranes by exchange from donor phospholipid vesicles. Fluorescence energy transfer from protein tryptophans to cholestatrienol was observed indicating close approach of this fluorescent sterol to the tryptophan. The effectiveness of the energy transfer was measured by the quenching of tryptophan fluorescence by cholestatrienol. The quenching of tryptophan fluorescence was directly related to the cholestatrienol content of the membranes. Cholesterol was incorporated into the disk membranes by exchange from donor phospholipid vesicles. The effect of increasing membrane cholesterol on the ability of cholestatrienol to quench rhodopsin tryptophan fluorescence was determined. This quenching was inversely proportional to the membrane cholesterol content. Furthermore the observed quenching was greater than could be explained by a simple dilution of the cholestatrienol by the addition of cholesterol to the membrane. These data suggest an interaction between the sterol and the protein. The specificity of this interaction was explored by the addition of ergosterol, instead of cholesterol, to the disk membranes. Ergosterol was not able to inhibit the quenching of protein trytophans beyond that due to dilution of the cholestatrienol by addition of ergosterol to the membrane. The ability of cholesterol to compete with cholestatrienol for that interaction suggests a 'site' at which cholesterol contacts rhodopsin. The inability of ergosterol to compete with cholestatrienol for this 'site' suggested that the site was specific for the structure of cholesterol.

Keywords: Rhodopsin–cholesterol interaction; Cholesterol; Cholestatrienol; Ergosterol; Tryptophan fluorescence; Rod outer segment disk membrane; (Bovine)

1. Introduction

Retinal rod outer segment disk membranes are arranged as a stack of flattened sacks along the length of the outer segment of the rod cell. Greater than 90 percent of the protein found in the disks is the photoreceptor protein, rhodopsin [1]. Therefore this integral membrane protein is well suited to investigations of the effects of bilayer components on the protein. Rhodopsin, is responsible for initiating visual

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transduction at low light levels. When a photon of visible light is absorbed by rhodopsin a series of spectrally defined intermediates is observed. This is followed by the dissociation of the retinal chromophore leaving the apoprotein, opsin. During this process a cascade of signal transduction events is mobilized. This cascade is analogous to signal transduction by other G-protein receptors.

Cholesterol has been shown to affect rhodopsin function and stability. Upon absorption of light, rhodopsin undergoes a series of conformational changes. The conversion of the photointermediate, Metarhodopsin I (Meta I), to Metarhodopsin II (Meta II) produces the activated form of the receptor. The equilibrium between Meta I and Meta II for rhodopsin in lipid bilayers is sensitive to the membrane cholesterol concentration [2,3]. In isolated ROS plasma membrane, the high cholesterol level inhibits activation of the transduction cascade [4]. Rhodopsin thermal denaturation is also inhibited by cholesterol (Albert, A.D. et al., unpublished observations).

The mechanism by which cholesterol influences rhodopsin may be through alteration of the membrane bilayer properties, or by direct interaction with the rhodopsin. In reconstituted rhodopsin-phospholipid membranes, modulation of the Meta I ⇄ Meta II equilibrium by cholesterol was explained by a reduction in the free volume characterizing the lipid bilayer [2]. Whether a direct interaction of rhodopsin and cholesterol may also play a role has not yet been examined. Previous studies suggested that the protein of the disk membrane influenced the dynamics of cholesterol. The correlation time for wobble (time dependence of the orientation of the director for axial rotation for the sterol in the membrane) of cholestatrienol in disk membranes was a factor of 3 shorter in the biological membrane than in bilayers made from lipid extracts of that membrane [5]. This observation may have been the result of an interaction between the sterol and rhodopsin.

To better understand the structural basis for the role of cholesterol in ROS disk membranes, the interaction between sterol and rhodopsin was probed with cholestatrienol, a fluorescent derivative of cholesterol. This probe has been used to study cholesterol dynamics in membranes [5] and to study cholesterol distribution across membranes [6]. In the present study, a novel use of cholestatrienol is introduced to study interactions between sterol and a membrane protein in the biological membrane. The fluorescence emission from protein tryptophans overlaps the absorption bands of cholestatrienol. R_0 can be estimated for this donor-acceptor pair to be about 15–20 Å. Therefore it could be expected that if the sterol closely approached the membrane protein, fluorescence energy transfer would occur from the protein tryptophans to the cholestatrienol.

In this study, fluorescence energy transfer from rhodopsin tryptophans to cholestatrienol was observed. The energy transfer was manifest as a quenching of the rhodopsin tryptophan fluorescence. The effectiveness of the fluorescence quenching was inversely related to the cholesterol content of the membranes. Ergosterol did not inhibit the quenching to the same degree as cholesterol. These data suggested a previously unreported property of cholesterol in ROS disk membranes: an ability of cholesterol to interact at a structurally specific site on rhodopsin of the ROS disk membrane.

2. Materials and methods

Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL). Cholestatrienol was synthesized and purified by HPLC as described previously [7]. Retinas were obtained from Lawson (Lincoln, NE). Cholesterol and ergosterol were obtained from Sigma.

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

Retinal rod outer segment disk membranes were prepared from frozen bovine retinas as described [8]. Rhodopsin levels were determined from the absorbance at 500 nm, using the extinction coefficient of 40000. Disks typically exhibited a ratio of the absorbance at 280 nm to that at 500 nm of 2.2. Unless otherwise stated, all manipulations of the rod outer segment disk membranes were performed under a Kodak 1A red filter. The isolated disks were washed and resuspended in 100 mM NaCl, 20 mM Hepes, 0.1 mM EDTA, pH 7.5 (buffer A) to a final rhodopsin concentration of 4–6 mg/ml. The buffers used were

perfused with nitrogen or argon to reduce lipid oxidation [9].

2.2. Modulation of the cholesterol content of ROS disk membranes

The procedure used is essentially that described previously [10]. Small unilamellar phosphatidylcholine (PC-SUV) and PC-cholesterol vesicles were formed by sonication in a Branson 350 sonifier. SUV (PC/cholesterol or PC) and membranes were incubated together in equimolar (with respect to phospholipid) or 2 × molar (SUV/disks) in a shaking water bath at 4, 24, or 37°C for varying lengths of time. The biological membranes were then separated from the SUV by centrifugation at 20000 rpm for 20 min. The pellet was washed twice with buffer A. The phospholipid/protein ratio was determined in the cholesterol-modified membranes as a control for sticking or fusion of the PC SUV to the biological membranes. In most cases, no sticking or fusion was observed. After isolation of the biological membranes, the cholesterol/phospholipid ratio was also determined.

2.3. Introduction of cholestatrienol into ROS disk membranes

Cholestatrienol was introduced into the disk membranes by transfer from donor SUV containing the fluorescent probe. Cholestatrienol was dried from acetonitrile/methanol (95:5, v/v) using a flash evaporator. The PC was then cosolubilized with the probe in chloroform/methanol (2:1, v/v), dried under a stream of nitrogen and then under partial vacuum overnight in the dark. The lipids were then hydrated with buffer A overnight. The suspension was sonicated 4 times (5 min each) using a Branson 350 Sonifier. The large phospholipid vesicles and the multilamellar species were removed by centrifugation at 45000 rpm for 60 min in a Beckman Ti-50 rotor as described [11]. The ROS disk membranes were then incubated with these cholestatrienol-containing vesicles for varying length of time, up to 24 h, under nitrogen at 4, 24, and 37°C and the fluorescent sterol was incorporated by exchange into the disk membranes.

2.4. Introduction of ergosterol into ROS disk membranes

Ergosterol was introduced into ROS disk membranes by incubation with PC vesicles containing ergosterol, following protocols described above. The disks were first depleted of cholesterol, to a level of 0.05 mole percent (about as low a cholesterol level as can be achieved). Then ergosterol was added to the disks by incubation of the disks with vesicles containing ergosterol. The ergosterol concentration in the modified disks was determined by absorbance at 290 nm (absorbance in excess of the absorbance observed from the protein), using a standard curve as reference. Ergosterol contents from 0 to 4 mole percent were achieved in the disks with this method (above the 5 mole percent of cholesterol). Ergosterol was much less able to enter the disk membrane than was cholesterol, under the same experimental conditions. The relative insolubility of ergosterol (compared to cholesterol) in lipid bilayers was documented previously [12].

2.5. Assays

The level of cholestatrienol in the membrane was determined by fluorescence emission at 370 nm, using a standard calibration curve constructed from the fluorescence emission from samples containing known amounts of cholestatrienol in sonicated lipid vesicles. The phospholipid content (±0.2%) was determined by the method of Bartlett [13] as modified by Litman [14], the protein content by the method of Lowry et al. [15], and the cholesterol content (±1%) by the method of Allain et al. [16].

2.6. Fluorescence measurements

Fluorescence measurements were made on a Perkin–Elmer LS50B spectrofluorometer in a thermostated cuvette holder. These measurements produced no detectable bleaching of the disk membranes. Bleaching was achieved by irradiation under a bright light for at least five minutes.

2.7. Statistical analysis

Linear regression analysis was performed using Stata (Stata Corporation, College Station, TX) on a Power Macintosh.

3. Results

Cholestatrienol is a fluorescent analog of cholesterol that has been used to probe sterol behavior in membranes [17,18]. The excitation spectrum of cholestatrienol ($\lambda_{max} = 327$ nm) overlaps the emission spectrum of protein tryptophans ($\lambda_{max} = 340$ nm). Fluorescence energy transfer from tryptophan to cholestatrienol can therefore occur in membranes if there is a close approach of the two fluorophores. Consequently fluorescence energy transfer from rhodopsin tryptophans to cholestatrienol, manifest as quenching of tryptophan fluorescence, could be used to probe cholesterol–protein interactions in rod outer segment disk membranes. Since rhodopsin represents greater than 90% of the protein in the disk membrane [1], tryptophan fluorescence from the disk membrane would be dominated by rhodopsin tryptophans.

The influence of cholestatrienol on the fluorescence of rhodopsin tryptophans was examined as a function of cholestatrienol concentration in the biological membrane. Cholestatrienol was introduced into the unbleached bovine ROS disk membranes as described in Section 2. Fig. 1 shows a representative experiment. Tryptophans were excited at 280 nm. Emission was measured from 300 nm to 450 nm. In the absence of cholestatrienol, the expected emission band from protein tryptophan fluorescence was observed, with a maximum at 340 nm. In the presence of cholestatrienol and at the same protein concentration, the intensity of the tryptophan fluorescence was diminished. Concurrently, emission from the cholestatrienol was observed in the region of 370 nm, arising indirectly from excitation of the protein tryptophans. These observations suggested energy trans-

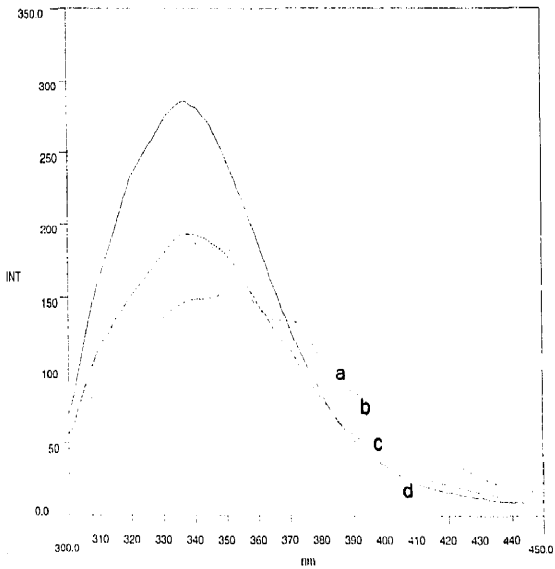

Fig. 1. Fluorescence emission spectra of ROS disk membranes containing varying amounts of cholestatrienol. Excitation was at 280 nm with emission range as shown. Tryptophan emission maximum is at 340 nm. Cholestatrienol emission (through energy transfer) is at 360 nm, 375 nm, and 395 nm. Curves: (a) 2.4 mole percent cholestatrienol; (b) 1.6 mole percent cholestatrienol; (c) 0.7 mole percent cholestatrienol; (d) 0 mole percent cholestatrienol.

fer from the protein tryptophans to the cholestatrienol. Such energy transfer was consistent with a close approach of the cholestatrienol to some of the protein tryptophans of rhodopsin.

The extent of this interaction could be measured as the quenching of rhodopsin tryptophan fluorescence by cholestatrienol. The quenching of tryptophan fluorescence was therefore measured as a function of cholestatrienol content of the disk membrane. As shown in Fig. 2, increased cholestatrienol content in the disk membrane led to increased quenching of the rhodopsin tryptophan fluorescence.

The previous data indicated that cholestatrienol could closely approach rhodopsin in the disk membrane. In the following experiments, the ability of cholestatrienol to quench tryptophan fluorescence was measured as a function of cholesterol content of the disk membranes. Cholesterol content of the disk membranes was modified as described in methods to produce disk membranes in which the cholesterol/phospholipid mole ratio ranged from 0.05 to 0.4. Cholestatrienol was then introduced into the membranes and the quenching of the tryptophan fluorescence was determined. In these experiments the concentration of membrane cholesterol was the variable of interest. However, it was not possible to reproducibly introduce a precise amount of cholestatrienol into each disk membrane preparation. Therefore, the concentration of cholestatrienol also became a variable. Fig. 3 shows a subset of the quenching measurements on disk membranes in which the cholestatrienol concentration in the membrane was between 0.5 mole percent and 1.0 mole percent (with respect to total lipid content). As shown in Fig. 2, in this range a change in cholestatrienol produces only a small variation in the quenching. Furthermore, that variation is small relative to the observed scatter in the quenching measurements as a function of cholesterol concentration. Many individual measurements were acquired because of the difficulty in controlling two independent sterol concentrations in a biological membrane.

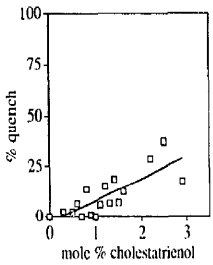

Fig. 2. Plot of the percent quench of ROS disk membrane tryptophan fluorescence as a function of the cholestatrienol content of the membranes.

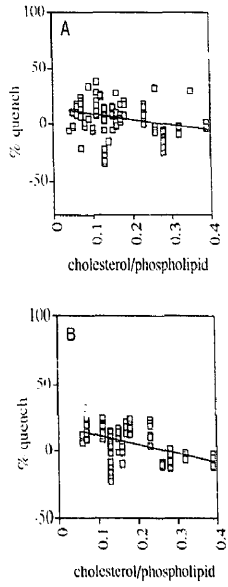

Fig. 3. Plot of the percent quench of ROS disk membrane tryptophan fluorescence by cholestatrienol as a function of the cholesterol content. Cholestatrienol was present in these membranes at a level of at least 0.5 but less than 1.0 mole percent, with respect to the membrane phospholipid. The solid lines are linear regressions as described in the text. (A) Unbleached disk membranes. 106 separate experiments are plotted. (B) Bleached disk membranes. 82 separate experiments are plotted.

Upon exposure to light (bleaching) rhodopsin undergoes spectrally defined conformational changes which eventually produce the apoprotein, opsin, and all trans retinal. Therefore, quenching experiments were performed on both unbleached and bleached disk membranes.

Fig. 3A shows the quenching of tryptophan fluorescence as a function of cholesterol content of un-

bleached disk membranes. 106 determinations from separate preparations are represented. For experiments on bleached disks, the membranes were prepared under dim red light in an identical manner as the unbleached samples were prepared. They were exposed to bright white light immediately prior to the fluorescence measurements for at least 5 minutes. Fig. 3B shows the quenching of tryptophan fluorescence by cholestatrienol as a function of cholesterol content of bleached disk membranes. 82 determinations from separate preparations are represented.

Table 1 shows the results of the statistical analysis of the data in Fig. 3. The data were fit both with a logarithmic function and with a linear function. From regression of both the logarithmic and the linear model, a statistically significant inverse relationship was found between the quenching and the cholesterol content of both the unbleached and bleached membranes. For example, the negative slope of the linear fit for the data from the quenching of tryptophan fluorescence as a function of cholesterol/phospholipid ratio was significantly different from zero at substantially greater than the 95% confidence level ($P = 0.00001$) for the bleached disk membranes. The negative slope of the linear fit for the data from the quenching of tryptophan fluorescence as a function of cholesterol/phospholipid ratio was significantly different from zero at greater than the 95% confidence level ($P = 0.0024$) for the unbleached disk membranes. The solid lines in Fig. 3 represent the linear fits to the data. Therefore the quenching of rhodopsin tryptophan fluorescence decreased with increasing cholesterol content of the disk membrane.

The specificity of the interactions observed with cholesterol (above) was examined using another sterol, ergosterol, which differs from cholesterol in the chemical structure of the hydrophobic tail and the fused ring system. Quenching of rhodopsin tryptophan fluorescence was measured in cholestatrienol-labeled, cholesterol depleted ROS disks as a function of ergosterol concentration in the membrane. Ergosterol levels in the membrane varied from 0 to 4 mole percent (see Section 2). Fig. 4 shows these data. 63 observations were recorded for unbleached membranes containing ergosterol (Fig. 4A), and 63 observations were recorded for bleached membranes containing ergosterol (Fig. 4B). The negative slope of the linear fit for the data (−2.1) from the quenching of tryptophan fluorescence as a function of ergosterol/phospholipid ratio was significantly different from zero at greater than the 95% confidence level ($P = 0.038$) for the unbleached disk membranes. The negative slope of the linear fit for the data (−2.1) from the quenching of tryptophan fluorescence as a function of ergosterol/phospholipid ratio was significantly different from zero at about

Table 1
Regression of quenching data as a function of cholesterol/phospholipid ratio

	Logarithmic regression		Linear regression	
	P	R^2	P	R^2
Bleached	0.0001	0.18	0.00001	0.22
Unbleached	0.036	0.042	0.0024	0.085

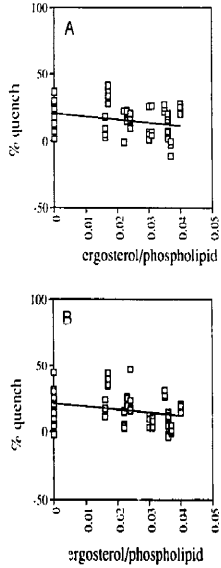

Fig. 4. Plot of the percent quench of ROS disk membrane tryptophan fluorescence by cholestatrienol as a function of the membrane ergosterol content. Cholestatrienol was present in these membranes at a level of at least 0.5 but less than 1.0 mole percent, with respect to the membrane phospholipid. The solid lines are linear regressions as described in the text. (A) Unbleached disk membranes. 63 separate experiments are plotted. (B) Bleached disk membranes. 63 separate experiments are plotted.

the 95% confidence level ($P = 0.047$) for the bleached disk membranes. This slope is different from the slopes obtained from the cholesterol experiments described above.

4. Discussion

ROS membranes are heterogeneous with respect to cholesterol content. Newly formed basal disk membranes are relatively high in cholesterol (about 25 mole percent), while the oldest disk membranes are much lower (approximately 5 mole percent cholesterol) [19]. The cholesterol content of the ROS plasma membrane from which disks are formed is approximately 25 mole percent [20]. Rhodopsin function in the ROS plasma membrane is impaired by the high cholesterol content [4]. The variation in cholesterol content may cause rhodopsin function to change in the disks as a function of disk age. Cholesterol stabilizes rhodopsin in ROS disk membranes to denaturation and to bleaching (Albert, A.D. et al., manuscript in preparation).

In reconstituted systems, it was shown that the Meta I ⇄ Meta II equilibrium was modulated by the cholesterol content of the membrane bilayer [21]. Physical measurements revealed that cholesterol reduction of packing defects, or free volume, in the lipid bilayer through cholesterol ordering of the phospholipid hydrocarbon chains explained the influence of cholesterol on the Meta I ⇄ Meta II equilibrium [2]. Thus one mechanism for cholesterol modulation of rhodopsin is cholesterol interaction with membrane phospholipids that compose the bilayer in which rhodopsin is identified.

An additional mechanism for cholesterol modulation of rhodopsin function and stability that has not previously been explored is cholesterol interaction with rhodopsin. The experiments in this work were designed to probe such interactions between cholesterol and rhodopsin in the biological membrane. Because these measurements were made on isolated disk membranes and the cholesterol levels investigated were those found in native disks from the ROS, these measurements suggested that such an interaction may occur in the ROS.

Cholesterol is inert to the biophysical measurements used to study biological membranes. Therefore, cholestatrienol was used as a fluorescent analog to sterol behavior in the disk membrane. Cholestatrienol is a cholesterol analog in that it contains the same side chain and 3β-hydroxyl as cholesterol. Previous reports had indicated that cholestatrienol functioned as a probe of cholesterol in membranes [17,22]. Therefore, the behavior of cholestatrienol can be taken as representative of cholesterol behavior in the membrane. However, the experiments reported here were not dependent upon the fidelity with which cholestatrienol reported on cholesterol behavior, since it was the displacement of the probe by cholesterol that was measured.

Whether the observed reduction in quenching, arising from the increase in membrane cholesterol content, resulted from dilution of the cholestatrienol in the lipid bilayer of the ROS membrane (the lipid-protein ratio increases due to the addition of cholesterol) had to be addressed. From Fig. 2, it can be deduced that a reduction of 50 percent in cholestatrienol content (or a dilution of cholestatrienol concentration by a factor of 2) will reduce the observed quenching by 50 percent. In Fig. 3A (unbleached disks) where dilution of cholestatrienol concentration is caused by addition of cholesterol to the membrane, a line with a slope of -7.5 would represent the dilution effect (such a line would begin at the same intercept at zero cholesterol, and would connect with a point with coordinates of one half the quenching of the intercept and a cholesterol/phospholipid ratio of 1, corresponding to a dilution by one half of the cholestatrienol concentration). The linear regression in Fig. 3A gives a slope of -47 ± 15. The corresponding slope in Fig. 3B (bleached disks) is -70 ± 15. The range in values of these slopes at the 95% confidence level did not include -7.5. Therefore dilution of cholestatrienol by addition of cholesterol to the membrane cannot explain the observed quenching of rhodopsin tryptophan fluorescence. The slopes in Fig. 3A and B are not statistically distinguishable from each other. Therefore these data cannot distinguish conformational changes of rhodopsin upon bleaching.

The magnitude of the quenching of rhodopsin tryptophan fluorescence was inversely related to the level of cholesterol in the disk membrane. This observation, which could not be explained by probe dilution, could be explained by a competition of cholesterol with cholestatrienol for a 'site' of interaction

between sterol and one or more rhodopsin tryptophans. Such a competition is reasonable, based on the similarity of the structure of cholestatrienol and cholesterol. That is, as membrane cholesterol content increased, the cholesterol would be expected to replace cholestatrienol in any sites on the protein surface suited for such sterols. This replacement would decrease the occupancy of such a site by cholestatrienol, thereby reducing the possible energy transfer which is dependent upon the number of fluorophores in a position to accept fluorescence energy transfer from the protein tryptophans. The mechanism for the observed fluorescence quenching in this case was fluorescence energy transfer, and the quenching of the protein tryptophan fluorescence should be decreased, as observed. The observation of quenching of rhodopsin fluorescence by cholestatrienol suggested that the cholestatrienol could approach closely the tryptophans of rhodopsin.

This hypothesis was further tested by the ergosterol experiments. If cholesterol was indeed interacting with rhodopsin at a site on the protein, that site may exhibit specificity with respect to the chemical structure of the sterol. The ability of ergosterol to inhibit the quenching of the protein tryptophan fluorescence was then determined and compared to the results from the cholesterol experiments. A statistically significant decrease in quenching was observed upon increase in membrane ergosterol concentration. However, the slope was small and in the range of the value expected from simple dilution of the concentration of cholestatrienol by the addition of another lipid to the membrane (see above). This result contrasts with the results from the cholesterol experiments. The most simple interpretation of these results is that ergosterol cannot inhibit quenching of tryptophan fluorescence by cholestatrienol because it has a relatively low affinity for the 'site' on rhodopsin at which the most effective quenching takes place. Cholesterol can inhibit the quenching because it has a reasonable affinity for the 'site' relative to the cholestatrienol that is doing the quenching. The conclusion is that the 'site' on rhodopsin must be structurally specific, with higher affinity for cholesterol than for ergosterol.

An alternative explanation for the change in quenching due to an increase in membrane cholesterol might be a change in the orientation of the fluorophores relative to each other. An increase in cholesterol concentration might lead to an increase in ordering of the cholesterol, thereby altering the geometry between the donor and acceptor. For cholestatrienol, the emission dipole and the absorption dipole are nearly co-linear, parallel to the long axis of the molecule [17]. The orientation of the cholestatrienol is perpendicular to the membrane surface [5]. The ordering of the axis for rotational diffusion of the cholestatrienol has been determined as a function of cholesterol concentration in the membrane. No significant change in the order parameter was observed [5]. Therefore, this alternative is not likely.

The quenching of the tryptophan fluorescence at 340 nm, and the increase in fluorescence in the emission of cholestatrienol (Fig. 1), were indicative of energy transfer from protein tryptophans to the cholestatrienol. In principle, such energy transfer could be used to determine the through-space distance between the emission dipole of the tryptophan and the absorption dipole of the cholestatrienol, located in the putative 'site' suggested above. The lack of knowledge of κ and the uncertainty of the occupancy of any site on the protein prohibit a precise calculation of that distance.

However, taking into account the overlap of the tryptophan emission and the absorbance of cholestatrienol, the quantum yield of the tryptophan, and the extinction coefficient of the cholestatrienol [17], and assuming a value for κ^2 (0.476) [23], R_0 can be estimated for this donor-acceptor pair to be about 15–20 Å. Thus the observation of quenching does imply a relatively close approach of the donor and acceptor. This observation is most simply explained by a close approach of the cholestatrienol to at least one of the tryptophans in the transmembrane domain of rhodopsin. If such a close approach involved direct contact with the hydrophobic transmembrane surface of rhodopsin, the geometry of that 'site' could induce a preference for cholesterol over ergosterol, as was observed in these experiments.

This model could provide an explanation for other data on cholesterol in the disk membrane. Recent data from cholesterol oxidase experiments on the accessibility of disk membrane cholesterol to oxidation suggests that about 20% of the membrane sterol is relatively inaccessible to oxidation (Albert and Paw, manuscript in preparation). That percent corre-

sponds to approximately one cholesterol per rhodopsin. One possible explanation is that one cholesterol per rhodopsin was inaccessible to cholesterol oxidase because of interaction with the above mentioned putative 'site' in the surface of rhodopsin. Furthermore, spin label experiments defining the phospholipid annulus of rhodopsin in ROS disk membranes led to the conclusion that one sterol was found at the lipid/protein interface [24].

Acknowledgements

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