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FURTHER CHARACTERIZATION OF THE LIPID-DEPLETED BOVINE RHODOPSIN OBTAINED BY CHOLATE-AMMONIUM SULFATE FRACTIONATION

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Summary

The rhodopsin preparation obtained by the method of ammonium sulfate fractionation contained 3—6 mol phospholipid and about 18 mol cholate per mol rhodopsin. The purified rhodopsin had 74% helical structure and showed a visible CD spectrum different from that of rhodopsin in the membrane. The rhodopsin was stable below but denatured gradually above 20°C. The lifetime of metarhodopsin I was long in this preparation. Regeneration capacity was low and only 30% of the original rhodopsin was regenerable by addition of 11-*cis*-retinal after bleaching.

50 mol of phosphatidylcholine were maximally bound to 1 mol rhodopsin when the purified rhodopsin was mixed with phosphatidylcholine in 0.5% cholate. The rhodopsin recombined with lipid had properties similar to those of the original rhodopsin in the membrane. Exchange of cholate for other detergents was easily performed by dialysis. The rhodopsin preparation in which cholate was exchanged for digitonin gave almost the same CD, thermal stability and regenerability as those of a native rhodopsin in the membrane but metarhodopsin I still retained its long lifetime.

Introduction

Rhodopsin, the major protein in the membrane of rod outer segments, has been solubilized with detergents such as bile salts, digitonin, cetyltrimethylammonium bromide, Triton X-100, Emulphogen BC-720, Ammonyx LO and

Abbreviation: SDS, sodium dodecyl sulfate.

alkyl glucosides [1—7]. The solubilized rhodopsin was purified by various types of column chromatography, but the preparations were limited in quantity. Recently, the large scale purification of rhodopsin was performed by the method of ammonium sulfate fractionation from rod outer segment membranes solubilized with cholate [8]. The excellent features of this method are listed as follows. (1) Rhodopsin is separated from opsin and lipid at the same time; (2) rhodopsin is easily concentrated by salting-out and centrifugation; (3) cholate is easily removed from rhodopsin and exchanged for other detergents by dialysis.

Generally, membrane protein is modified in its properties by solubilization with detergent [9]. Rhodopsin is also affected by the solubilization. We found that the circular dichroism (CD) of rhodopsin purified by the ammonium sulfate fractionation was reversibly changed by removal and recombination of lipid [10]. In this paper, we further study the molecular and spectral characterization of this purified rhodopsin. Effects of lipid recombination and detergents exchange are also studied.

Materials and Methods

Preparation of the rod outer segment membranes and purification of rhodopsin. The method of preparation was the same as that described in the previous paper [8]. The rod outer segment membranes were isolated and purified from bovine retinas by the method of sucrose density gradient centrifugation. Rhodopsin was purified from the rod outer segment membranes solubilized with cholate by the method of ammonium sulfate fractionation. Rhodopsin was further purified by repeating ammonium sulfate salting-out and by column chromatography on Sephadex G-100. Rhodopsin was eluted from the column with 1% cholate containing 0.05 M Tris-HCl buffer (pH 7.5).

Absorption spectra were determined with a Hitachi 323 spectrophotometer and a Shimadzu UV-200 spectrometer. The molar extinction coefficient (ϵ_{max}) of rhodopsin in 1% cholate was determined by using the method of Wald and Brown [11]. It was determined from the difference absorbance at 499 nm in the presence of 0.1 M NH_2OH before and after irradiation (using a value of ϵ_{max} = 51 600 for all-*trans*-retinal oxime).

Lipid analysis. The analysis of phospholipids in the purified rhodopsin was carried out as follows. Solid $NaBH_4$ was added to the rhodopsin and the solution was irradiated with white light in order to produce *N*-retinyl opsin. This procedure was necessary because retinal disturbs the lipid analysis by thin-layer chromatography. After dialysis against 0.01 M Tris-HCl buffer (pH 7.5) to eliminate cholate, the retinyl opsin was collected by centrifugation and then the precipitate was lyophilized. Total lipid was extracted from the lyophilized opsin with chloroform/methanol by using the method of Bligh and Dyer [12]. Lipid was separated by thin-layer chromatography (HP-TLC Silicagel 60, Merck) with chloroform/methanol/acetic acid (25 : 16 : 4, v/v). After treating the TLC plate with 0.1% ninhydrin and I_2 vapor, spots of phospholipid were collected and the amount of phosphate was determined by using the methods of Bartlett [13] and of Martin and Doty [14].

Analysis of cholate binding to rhodopsin. The binding curves of cholate

to the purified rhodopsin and the lipid-recombined rhodopsin were determined by the method of equilibrium dialysis. 0.5 ml of rhodopsin solution containing differerent percentage of cholate was dialyzed against 20 ml of the same percent of cholate solution (0.05 M Tris-HCl, pH 7.5) at 4°C for 4 days. We confirmed that 4 days dialysis was enough to reach equilibrium. After dialysis, the concentrations of cholate of outer and inner solutions were determined by using the method of Kier [15]. The amount of cholate binding to rhodopsin was calculated from the difference of the concentration between outer and inner solutions.

Measurement of the particle weight. The ultracentrifugal analysis of the rhodopsin solution was performed with a Hitachi 282 analytical ultracentrifuge at a speed of 50 000 rev./min. The values of the sedimentation coefficient (S) were determined at 20°C for different concentrations of the purified rhodopsin in the presence of 1% cholate, 0.2 M KCl and 0.02 M Tris-HCl buffer (pH 7.5). The absorbance at 280 nm was recorded every 3 min. The Stokes' radius (a) of rhodopsin was determined by Sephadex G-200 gel filtration in the same solvent as that used in the analytical ultracentrifugation. A Whatman column (1 cm diameter, 80 cm height) used in this experiment had been calibrated with 1–2 mg ferritin (a = 60 Å), catalase (52 Å), aldolase (46 Å), bovine serum albumin (35 Å, Boehringer-Mannheim GmbH) and blue dextran (Pharmacia).

The particle weight (M) was calculated from the equation:

$$M = \frac{6\pi\eta_{20,w} \cdot N \cdot a \cdot S_{20,w}}{1 - \bar{v}\rho_{20,w}}, \qquad S_{20,w} = \frac{\eta_{20,solv}(1 - \bar{v}\rho)_{20,w}}{\eta_{20,w}(1 - \bar{v}\rho)_{20,solv}} \times S_{obs} \tag{1}$$

where $\eta_{20,w}$ and $\eta_{20,solv}$ are the viscosities and $\rho_{20,w}$ and $\rho_{20,solv}$ are the densities of water and solvent at 20°C, respectively, and N is Avogadro's number. The partial specific volume of the rhodopsin-lipid-cholate complex ($\bar{v}$) was calculated assuming the additivity of the partial specific volumes of its components; $\bar{v}_{rhodopsin}$ = 0.71 ml/g (calculated from the amino acid and carbohydrate composition [16]), $\bar{v}_{cholate}$ = 0.83 ml/g (determined by pycnometry in this experiment) and $\bar{v}_{phospholipid}$ = 0.98 ml/g (using the value of egg phosphatidylcholine [17]).

Determination of the CD spectrum and helix content. The CD spectrum was measured with a JASCO J-20 recording spectropolarimeter below 20°C. The helix content of rhodopsin was estimated by the formula, percent helix = 100 ($[\theta]_{222}$ + 2340/–30 300) [18]. The value of $[\theta]$ was calculated from the equation, $[\theta] = \theta/(c \times l) \times 100$ degree · cm^2 · dmol^{-1}, where c is the molar concentration of rhodopsin determined as A_{499}/ϵ_{max} and l is cell length. ϕ was the ellipticity read directly from the record. The value of 115 was used for a mean residue molecular weight and the value of 37 000 for the molecular weight of rhodopsin was determined by SDS-polyacrylamide gel electrophoresis [8].

Recombination of rhodopsin with lipid. Egg phosphatidylcholine dissolved in a small volume of 10% cholate was added to purified rhodopsin in the molar ratio of 50 : 1 or 20 : 1. The mixture was dialyzed against different percentages of cholate solutions (0.05 M Tris-HCl, pH 7.5) at 4°C for 3 days.

The analysis of lipid binding was carried out by the method of sucrose gradient centrifugation. 0.5 ml of rhodopsin was put on 4 ml sucrose solution

with a linear gradient (10—40%, w/v) containing the different percentages of cholate and 0.05 M Tris-HCl buffer (pH 7.5). The solution was centrifuged for 20 h at 4°C with a Beckman SW 50 rotor at 200 000 × *g*. After centrifugation, 16 fractions of 6 drops each were collected from the bottom of the tube with a syringe needle. The absorbance at 500 nm and the amount of phospholipid in each fraction were determined.

Exchange of detergent. The exchange of cholate for digitonin was carried out as follows. Digitonin was added to the rhodopsin in cholate to a final concentration of 0.2%. Then the mixture was dialyzed against 0.2% digitonin (0.05 M Tris-HCl, pH 7.5) at 4°C for 3 days with two changes of the outer solution. After dialysis, the cholate content was reduced to less than 5 mol per mol rhodopsin.

Regeneration of rhodopsin. Rhodopsin (a) was bleached by irradiation with orange light ($\lambda > 560$ nm) for 10 min at 15°C. The solution was divided into two parts (b,c) and 11-*cis*-retinal was added to one part (b). Both parts were kept in the dark at 15°C for 3 h. Finally, sufficient NH_2OH was added to the two parts (b′, c′) to bring the final concentration of NH_2OH to 0.05 M and the absorbance at 500 nm was then determined. Regenerability was defined as follows:

$$\text{Regenerability (\%)} = \frac{\text{Amount of regeneration}}{\text{Amount of bleaching}} \times 100$$

$$= \frac{A_{500}(\text{b}') - A_{500}(\text{c}')}{A_{500}(\text{a}) - A_{500}(\text{c}')} \times 100 \qquad (2)$$

The 11-*cis*-retinal used here was prepared by high-performance liquid chromatography (Shimadzu-Du Pont, Sorbax SIL) from a mixture of retinal isomers obtained by irradiating all-*trans*-retinal.

Thermal stability. Thermal stability was studied by incubating rhodopsin samples at constant temperature. The rate of thermal denaturation of rhodopsin was determined by measuring a decrease in absorbance at 500 nm.

Photolysis of rhodopsin. Spectral changes were recorded at 15°C after irradiation with yellow light ($\lambda > 500$ nm) for 3 s. The flash photolysis was performed with the apparatus described previously [19]. The flash lamp (Xenon, 300 J, with half duration of 300 μs) was used as an exciting light. The bleaching rate was calculated from the transmittance change at 480 nm at 20°C in the presence of 0.1 M NH_2OH.

Results

Molecular characterization of the lipid-depleted rhodopsin

(a) Optical purity and phospholipid composition. We obtained purified rhodopsin ($A_{280}/A_{500} = 1.6$—1.9) by the method of ammonium sulfate fractionation. The molar ratio of phospholipid : rhodopsin was reduced to 3—6 from the value of about 100 which is characteristic of the rod outer segment membrane. The phospholipid composition of this purified rhodopsin was phosphatidylcholine : phosphatidylserine : phosphatidylethanolamine = 0.8 : 0.15 : 1.0, which was similar to that of the rod outer segment membrane

[20]. Hereinafter, we call this sample 'lipid-depleted rhodopsin'.

(b) Cholate-binding. Fig. 1 shows the binding curve of cholate to rhodopsin. About 18 mol of cholate molecules were bound to the lipid-depleted rhodopsin and the number of cholate molecules bound to the lipid-depleted rhodopsin was independent of the cholate concentration in the solution (curve 1).

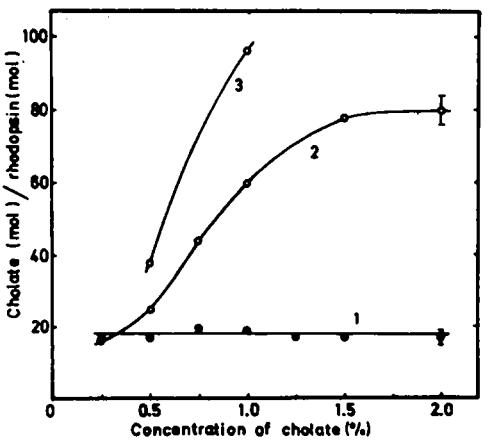
(c) The particle weight of the lipid-depleted rhodopsin. The rhodopsin preparation used for this experiment contained 6 mol phospholipid and 18 mol cholate per mol rhodopsin. The value of s_{obs} was 6.7 (Fig. 2) and the Stokes' radius was 55 Å. The particle weight of rhodopsin was 171 000 calculated from Eqn. 1. The weight of one unit of rhodopsin is 49 000 (1 mol rhodopsin = 37 000, 18 mol cholate = 7200 and 6 mol phospholipid = 4800). The value of 171 000 corresponds to 3.5 units of rhodopsin.

(d) Thermal stability and regenerability. The lipid-depleted rhodopsin was stable at 20° C in 1% cholate at pH 7.5 for at least 3 h. In 1 h at 30° C, 10 and 5% of the lipid-depleted rhodopsin bleached in 1 and 0.5% cholate solution (pH 7.5), respectively. Larger amounts of rhodopsin denatured at the higher temperature, pH and cholate concentration (Table I).

Fig. 3 shows the regenerability of rhodopsin after bleaching. The lipid-rich rhodopsin extracted with 0.8% cholate from the rod outer segment membranes regenerated about 100% and the optimum pH of the regeneration was about 7.5 (curve 1). The regeneration capacity in 0.6% cholate solution was reduced to about 30% after removing lipid from rhodopsin (curve 2).

Spectral characterization of the lipid-depleted rhodopsin

(a) Absorption maximum and the molar extinction coefficient. The absorption maximum of the lipid-depleted rhodopsin was located at 499 nm in



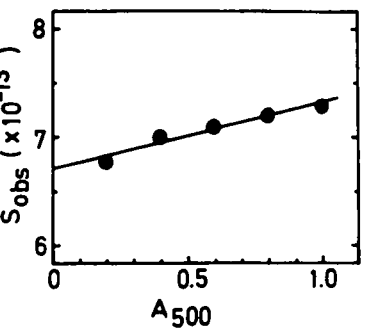


Fig. 1. The binding of cholate to purified rhodopsin. The molar ratio was determined by the method of equilibrium dialysis at 4° C for 4 days. Curve 1, purified rhodopsin (phospholipid 4); curve 2, mixture of rhodopsin : phosphatidylcholine (1 : 20); curve 3, mixture of rhodopsin : phosphatidylcholine (1 : 50).

Fig. 2. The sedimentation coefficient (s) of purified rhodopsin (phospholipid 6). Samples were centrifuged at 20° C for different concentration of protein in a solvent of 0.2 M KCl, 1% cholate and 0.05 M Tris-HCl buffer (pH 7.5).

TABLE I

EFFECTS OF LIPID-RECOMBINATION AND DETERGENT-EXCHANGE

Values of k_1 and k_2 expressed in s^{-1}.

	Lipid-depleted rhodopsin (phospholipid 3—6) in 0.5% cholate	Lipid-recombined rhodopsin (phosphatidylcholine 50) in 0.5% cholate	Detergent-exchanged (phospholipid 3—6) in 0.2% digitonin
Thermal stability (%) (A_{500} after 60 min at 40°C, pH 7.5)	76 43 *	82 83 *	90
The decay rate of metarhodopsin I (20°C in the presence of 0.1 M NH_2OH, pH 7.2—7.3)	k_1 — k_2 $1.10 \cdot 10^{-2}$	$5.73 \cdot 10^{-1}$ $4.59 \cdot 10^{-2}$	$1.56 \cdot 10^{-1}$ $1.31 \cdot 10^{-2}$

* in 1.0% cholate.

cholate solution and the molar extinction coefficient at 499 nm was 40 000.

(b) CD spectrum. Fig. 4 shows the CD spectra of rhodopsin in the visible-near ultraviolet (a) and in the far ultraviolet (b) regions. In comparison with rhodopsin prepared by sonication of the rod outer segment membranes, the lipid-depleted rhodopsin showed a small α-band (500 nm) and a large γ-band (280 nm) CD.

The helical content of the lipid-depleted rhodopsin in 0.5% cholate solution was 74% and it was reduced to 67% on irradiation with light.

(c) Photobleaching. Fig. 5 shows the spectral changes of the lipid-depleted rhodopsin and the sonicated rod outer segment membrane after irradiation with yellow light ($\lambda > 500$ nm) for 3 s at 15°C. The sonicated rod outer segment membrane immediately formed metarhodopsin II ($\lambda_{max} = 380$ nm) followed by the transition to metarhodopsin III (Fig. 5a). Only the extremely

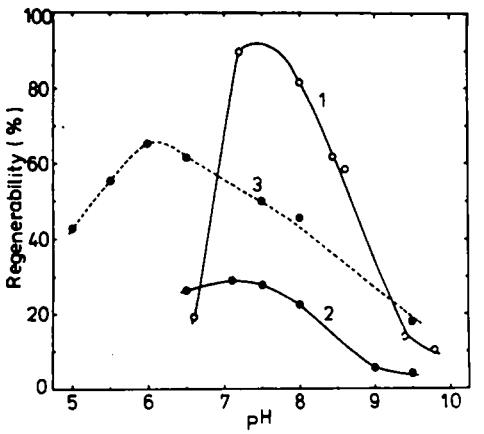


Fig. 3. The pH dependence of rhodopsin regeneration. Curve 1, lipid-rich rhodopsin (phospholipid 100) extracted with 0.8% cholate; curve 2, purified rhodopsin (phospholipid 5) in 0.6% cholate; curve 3, detergent-exchanged rhodopsin (phospholipid 5) in 0.2% digitonin.

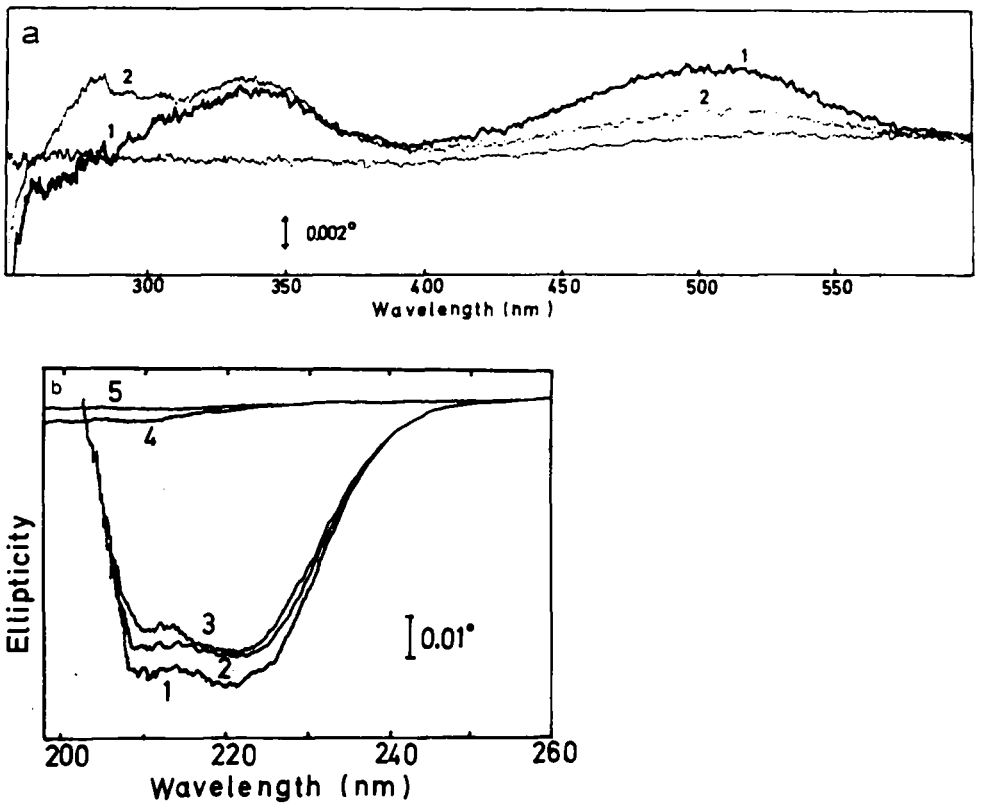


Fig. 4. Circular dichroism of rhodopsin. (a) Visible-near ultraviolet region (A_{500} = 0.36, 1 cm cell length). Curve 1, sonicated rod outer segment membrane; curve 2, purified rhodopsin (phospholipid 6) in 0.5% cholate and the base-line was determined by 0.5% cholate solution (0.05 M Tris-HCl, pH 7.5). (b) Far ultraviolet region (A_{500} = 0.35; phospholipid : rhodopsin, 3 : 1; 1 mm cell length; 0.05 M Tris-HCl, pH 7.5). Curve 1, purified rhodopsin in 0.6% cholate; curve 2, after bleaching of rhodopsin of curve 1 in 0.6% cholate, irradiated with yellow light ($\lambda > 500$ nm, 10 min, at 15°C) in the presence of 0.1 M NH_2OH; curve 3, detergent-exchanged rhodopsin in 0.2% digitonin; curve 4, 0.6% cholate solution; curve 5, 0.2% digitonin solution.

slow transition of metarhodopsin I to metarhodopsin II was observed in the lipid-depleted rhodopsin in cholate following the same irradiation (Fig. 5b).

(d) Spectral changes during storage. In cholate solution, the absorbance below 400 nm increased during storage of rhodopsin at 4°C in the dark although the absorbance at 500 nm never changed. This absorbance increase was remarkable in the lipid-rich preparation. The substance having the absorbance below 400 nm was separated by column chromatography on Sephadex G-100 and it contained lipid but not protein. This absorbance increase was prevented by exchanging cholate for digitonin. These findings show that lipid is easily oxidized in the micelle of cholate but not in the micelle of digitonin.

Lipid recombination and detergent exchange

(a) Lipid recombination. Fig. 6a shows the sedimentation pattern of the lipid-recombined rhodopsin (molar ratio of phosphatidylcholine : rhodopsin =

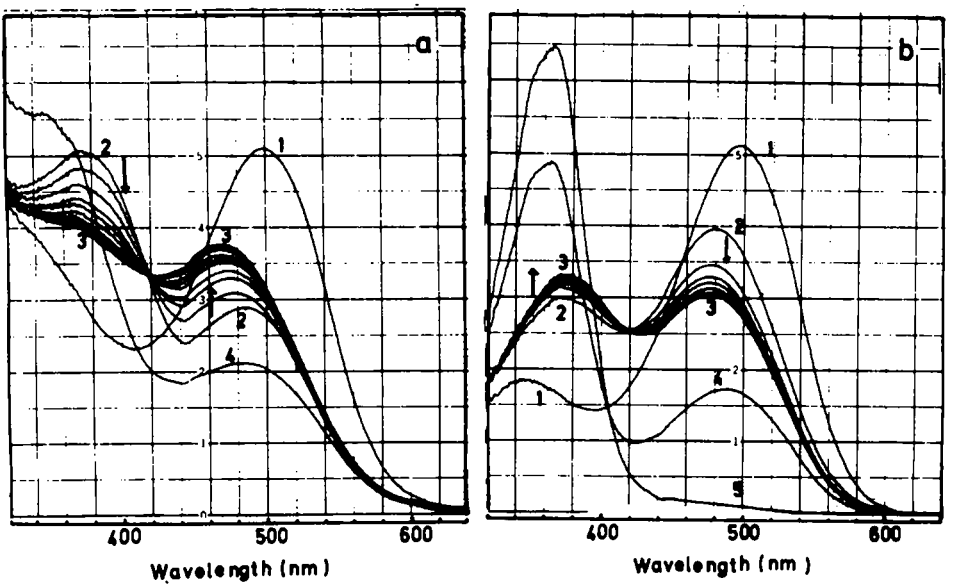


Fig. 5. The lifetime of meta-intermediate at 15°C after irradiation with yellow light ($\lambda > 500$ nm) for 3 s (0.05 M Tris-HCl, pH 7.5). (a) Sonicated rod outer segment membranes. (b) Purified rhodopsin (phospholipid 6) in 0.5% cholate solution. Curve 1, unirradiated rhodopsin; curves 2 and 3, spectra measured with 5 min repeat scan after irradiation; curve 4, spectra on addition of 1 M NH_2OH (final concentration of 0.1 M) after repeat scan; curve 5, reirradiation ($\lambda > 500$ nm) for 10 min at 15°C.

50 : 1) in 0.5% cholate. Free phosphatidylcholine was separated from a rhodopsin-phosphatidylcholine complex by sucrose gradient centrifugation. 45 mol phosphatidylcholine combined with 1 mol rhodopsin in 0.5% cholate

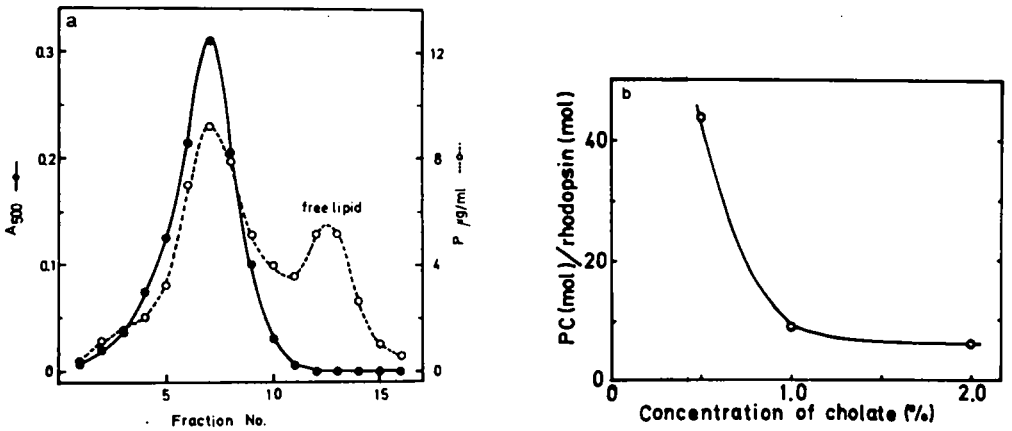


Fig. 6. The binding of phosphatidylcholine to purified rhodopsin in cholate solution. (a) Separation of lipid (phosphorus o- - - - - -o) from rhodopsin (absorbance at 500 nm, •———•) by sucrose density gradient centrifugation. 0.5 ml of the mixture (rhodopsin : phosphatidylcholine, 1 : 50) was put on 4 ml of a linear sucrose gradient (10—40%, w/v; 0.5% cholate; 0.05 M Tris-HCl buffer, pH 7.5) and centrifuged for 20 h at 200 000 × g at 15°C. Fractions of 6 drops were collected from bottom. (b) The dependence of phosphatidylcholine binding to rhodopsin on cholate concentration. The mixture (rhodopsin : phosphatidylcholine, 1 : 50) was analyzed by the same method as in a for each percent of cholate. The molar ratio of phosphatidylcholine : rhodopsin was determined for the fraction having maximum A_{500}. PC, phosphatidylcholine.

solution. We also examined the mixtures of phosphatidylcholine : rhodospin of ratios 20 and 100. All phosphatidylcholine combined with rhodopsin in the mixture of phosphatidylcholine : rhodopsin of ratio 20 : 1 and 49 mol phosphatidylcholine in the mixture of phosphatidylcholine : rhodopsin of ratio 100 : 1. Maximum binding of phosphatidylcholine was about 50 mol per mol rhodopsin in 0.5% cholate solution.

Fig. 6b shows the dependence of lipid binding on various concentrations of cholate. The molar ratio of phosphatidylcholine : rhodopsin decreased to 6 as the concentration of cholate in the solution increased to 2%.

When phosphatidylcholine recombined with rhodopsin in 0.5% cholate solution, the ratio of binding of cholate to rhodopsin increased in proportion to the binding of phosphatidylcholine, and it was 38 at phosphatidylcholine : rhodopsin = 50 : 1 (Fig. 1). The apparent ratio of cholate binding to rhodopsin increased as the cholate concentration increased.

(b) Effects of lipid-recombination and detergent-exchange on various properties. The CD spectra of lipid-depleted rhodopsin showed a small α-band (500 nm) and a large γ-band (280 nm) as shown in Fig. 4a. The native CD was recovered by the recombination of rhodopsin with lipid and partially recovered by exchanging cholate for digitonin [10]. The helical content of the detergent-exchanged rhodopsin in digitonin was 65% and it did not change after irradiation.

The effects on thermal stability and the decay rate of metarhodopsin I are summarized in Table I. Rhodopsin was stabilized by recombination with lipid or by exchanging cholate for digitonin.

Regenerability was recovered by lipid recombination and detergent exchange with digitonin. The original regenerability was partially recovered (54%) by recombination with lipid and almost totally recovered by detergent exchange with digitonin. The optimum pH for regeneration was about 6.2 in the case of rhodopsin containing digitonin (Fig. 3).

The rate of conversion from metarhodopsin I to metarhodopsin II was very slow in lipid-depleted rhodopsin and the rate was not changed by exchanging cholate for digitonin. In the lipid-recombined rhodopsin, the decay rate of metarhodopsin I was much faster than that of the lipid-depleted rhodopsin (Table I).

Discussion

The purified, lipid-depleted rhodopsin obtained by ammonium sulfate fractionation was different from rhodopsin in the membranes of rod outer segments in all properties examined here; CD, thermal stability, regenerability and the decay rate of metarhodopsin I. Rhodopsin recovered its original properties on recombination with lipid. The lipid-recombined rhodopsin was still soluble in cholate solution. Therefore, the modified properties of the lipid-depleted rhodopsin are not due to the solubilization with detergent but due to the depletion of lipid from the rhodopsin molecule.

The purified rhodopsin preparation contained 3—6 mol of phospholipid per mol rhodopsin. The composition of the lipid was similar to that of the rod outer segment membrane [20]. There is no special lipid which interacts with

rhodopsin. In fact, the recombination of the lipid-depleted rhodopsin with egg phosphatidylcholine yielded a rhodopsin preparation having properties similar to those of rhodopsin in rod outer segment membranes. Furthermore, digitonin could be substituted for lipid in recovering the native properties of rhodopsin.

The 1 mol rhodopsin combined with maximally 50 mol phosphatidylcholine in 0.5% cholate solution. The lipid was easily removed from rhodopsin at the higher concentration of cholate. However, the lipid-recombined rhodopsin retained similar properties to the rhodopsin in the membrane even in 1% cholate solution. This shows that the lipid-protein binding is not strong and also that the lipid molecules are in an equilibrium state containing free and bound forms. The equilibrium state of lipid binding is enough to maintain the native properties of rhodopsin. As Hong and Hubbell discussed [21], a hydrophobic interaction between rhodopsin and lipid (or digitonin) may be important in maintaining a special conformation of opsin which reveals the native properties of rhodopsin. We observed that the absorption and CD spectra are modified by detergent exchange. The results of far ultraviolet CD showed that the conformation of opsin depends on detergent. On irradiation, the helical structure changed in the rhodopsin-cholate complex but not in the rhodopsin-digitonin complex. This fact may explain the difference in regenerability between the two samples.

In lipid-depleted rhodopsin, metarhodopsin I had a very long lifetime. This is the only property unchanged after exchanging cholate for digitonin and it may due to a reason different from the hydrophobic interaction which affected CD, thermal stability and regenerability. Applebury et al. [22] reported that the conversion of metarhodopsin I to metarhodopsin II was blocked in the lipid-free, detergent-free rhodopsin aggregates. Van Breugel et al. [23] also reported that the conversion of metarhodopsin I to metarhodpsin II was blocked in the rod outer segment treated with phospholipase C. They suggested that the lateral aggregation of rhodopsin in the membrane caused by lipid hydrolysis resulted in the block of the transition of metarhodopsin I to metarhodopsin II. Our ultracentrifugal analysis showed that the lipid-depleted rhodopsin was in 3—4 polymer groups in the cholate solution and that rhodopsin surely aggregated. However, the s value of the lipid-depleted rhodopsin ($s_{20,w} = 6.8$) was close to that of the lipid-recombined rhodopsin ($s_{20,w} = 5.4$) in which metarhodopsin I had a short lifetime. The results of the ultracentrifugal analysis suggested that the aggregation of rhodopsin molecules is not responsible for blocking the transition of metarhodopsin I to metarhodopsin II. Fisher and Oakenfull [24] suggested that the interior of a cholate micelle was more rigid than that of the micelles of long-chain detergents. Addition of moderate amounts of lipid could relax the micelles. We think that the fluidity of lipid around the rhodopsin molecule play an important role in the transition.

At present, we can routinely prepare a large amount of rhodopsin with the highest purity by the method of ammonium sulfate fractionation. This rhodopsin preparation has some properties which are not compatible with the recombination of opsin with 11-*cis*-retinal after bleaching. It also can not be used for experiments at low pH because cholate is insoluble below pH 7. These weak points are easily overcome by exchanging cholate for digitonin by simple

dialysis. The detergent-exchanged rhodopsin preparation had good thermal stability, optical purity and regenerability.

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References

1 Kühne, W. (1978) Unters. Physiol. Inst. Heidelberg 1, 15—104
2 Wald, G. and Brown, P.K. (1952) J. Gen. Physiol. 35, 797—821
3 Heller, J. (1968) Biochemistry 7, 2906—2920
4 Crescitelli, F., Mommaerts, W.F.H.M. and Shaw, T.I. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 1729—1734
5 Shichi, H., Lewis, M.S., Irreverre, F. and Stone, A.L. (1969) J. Biol. Chem. 244, 529—536
6 Ebrey, T.G. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 713—716
7 Stubbs, G., Smith, H.G. and Litman, B.J. (1976) Biochim. Biophys. Acta 426, 46—56
8 Makino, M., Hamanaka, T., Orii, Y. and Kito, Y. (1977) Biochim. Biophys. Acta 495, 299—311
9 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 45, 29—79
10 Suzuki, T., Makino, M. and Azuma, M. (1977) Biochim. Biophys. Acta 494, 115—125
11 Wald, G. and Brown, P.K. (1954) J. Gen. Physiol. 37, 189—200
12 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—921
13 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
14 Martin, J.B. and Doty, D.M. (1949) Anal. Chem. 21, 965—967
15 Kier, L.C. (1952) J. Lab. Clin. Med. 40, 755—765
16 Osborne, H.B., Sardet, C. and Helenius, A. (1974) Eur. J. Biochem. 44, 383—390
17 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133—170
18 Chen, C., Yang, J.T. and Martinez, H.M. (1972) Biochemistry 11, 4120—4131
19 Ebina, Y., Nagasawa, N. and Tsukahara, Y. (1974) Jap. J. Physiol. 24, 93—100
20 Anderson, R.E. and Maude, M.B. (1970) Biochemistry 9, 3624—3628
21 Hong, K. and Hubbell, W.L. (1973) Biochemistry 12, 4517—4523
22 Applebury, M.L., Zuckerman, D.M., Lamola, A.A. and Jovin, T.M. (1974) Biochemistry 13, 3448—3458
23 Van Breugel, P.J.G.M., Geurts, P.H.M., Daemen, F.J.M. and Bonting, S.L. (1978) Biochim. Biophys. Acta 509, 136—147
24 Fisher, L. and Oakenfull, D. (1979) Aust. J. Chem. 32, 31—39