PURIFIED RHODOPSIN–PHOSPHATIDYLCHOLINE COMPLEX IN HEXANE: FORMATION AND CHARACTERIZATION

A. DARSZON, L. BLAIR and M. MONTAL
Departments of Biology and Physics, University of California, San Diego, B-019 La Jolla, CA 92093, USA

Received 24 August 1979

1. Introduction

Rhodopsin, the light receptor protein of the animal retina, has been incorporated into planar lipid bilayer membranes and shown to display electrical characteristics consistent with the formation of transmembrane ion-channels [1]. The results hitherto reported have been obtained with preparations of rhodopsin–lipid complexes in hexane or ether derived from both detergent solubilized as well as from membrane-bound rhodopsin [2]. Although rhodopsin is the major protein component of rod discs (>90%) [3,4], it is conceivable that other disc proteins could contribute to the bilayer results. In addition, the lipids from the discs are also extracted into the solvent and since they are extremely unsaturated (50% of the fatty acids are docosahexanoic acid [5]) we are concerned with the possibility that their oxidation products would play a role in the formation of channels [6].

Here, we report that affinity purified and delipidated rhodopsin [7,8] recombined with a single non-oxidizable phospholipid, or a lipid mixture of known composition, is efficiently transferred as a lipid–protein complex into organic solvents. The absorption spectra, stability and regeneration capacity of rhodopsin from these preparations indicate that the overall photochemical behaviour of the protein is preserved throughout the procedure. Furthermore, planar bilayers formed from this material display channel activity with similar characteristics as those in [1].

Abbreviations: DDAO, dodecyl-dimethylamine oxide; DTT, dithiothreitol; ROS, rod outer segments; RLC, rhodopsin–lipid complexes; dPC, diphytanoyl phosphatidylcholine; bPE, bacterial phosphatidylethanolamine; ΔA_{500} (or ΔA_{485}), difference in the absorbance between dark and bleached samples at 500 nm (or 485 nm)

2. Materials and methods

The following materials were obtained from the indicated sources: dark-adapted retinas (Hormel, Austin, MN); L-α-lecithin from soybeans, 9-*cis* retinal and concanavalin A–Sepharose 4B (Sigma Chem., St Louis, MO); diphytanoyl phosphatidylcholine (Avanti Biochem., Birmingham, AL); bacterial phosphatidyl ethanolamine (Supelco, Bellefonte, PA); dodecyl dimethylamine oxide (Onyx Chem., Jersey City, NJ); dithiothreitol (Eastman Organic Chem., Rochester, NY); [^{14}C]DDAO (ICN Chemical and Radioisotope Div., Irvine, CA). All other reagents were of the highest purity commercially available.

Unless otherwise stated, all experimental procedures were conducted under dim red light at 4°C. Rod outer segments from dark-adapted bovine retinas were purified as in [3,9]. Purified ROS from 50 retinas were solubilized in 5 ml 5% (v/v) DDAO, 0.1 M acetate, 0.1 M NaCl, 1 mM DTT, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ (pH 6.0) [8] and centrifuged 20 min at 27 000 × *g*. Rhodopsin from the supernatant was purified and delipidated over a concanavalin A–Sepharose 4B column [7,8]. The peak fraction of the column contained 2.7–4.2 mg rhodopsin/ml in the detergent–buffer solution and had a $A_{280}/\Delta A_{500}=1.67$. This fraction was utilized for the extractions.

Rhodopsin–lipid vesicles were prepared by mixing 0.5 mg purified rhodopsin with 1 mg indicated phospholipid dissolved in 50 μl 5% DDAO, and incubating for 30 min. The sample was then diluted to 5 ml with 10 mM imidazole at pH 7.0, 1 mM $CaCl_2$ and 0.2 M NaCl. The detergent was removed by dialysis against 500 ml 0.1 mM imidazole–HCl (pH 7.0), 0.01 mM $CaCl_2$, 2 mM NaCl with or without 1 mM DTT, for 2–7 days with at least 3 changes of buffer. The residual detergent was measured using [^{14}C]DDAO. The resulting rhodopsin–lipid vesicles were collected by centrifugation at 180 000 × *g* for 2 h.

Rhodopsin–lipid complexes (RLC) were transferred into hexane according to the following protocol: the vesicle pellet was resuspended in 1 ml hexane containing 10 mg lipid, either partially purified soy bean phospholipid [10], or a mixture of bPE and dPC (4:6) or dPC alone. The suspension was sonicated for 6 min (Bransonic, Heat Systems Ultrasonic, Plainview, NJ, power output of 100 W). Thereafter, 0.1 ml 1 M $CaCl_2$ was added and the mixture stirred in a vortex mixer for 2.5 min. The phases were separated in a clinical centrifuge at maximum speed (4 min) and the hexane phase removed. The transfer of the RLC into hexane was not dependent on $CaCl_2$ since the extraction yield was the same in its absence. This could be explained by the presence of residual detergent [2].

Absorption spectra were recorded with a Perkin Elmer 555 spectrophotometer in 1 cm pathlength cells. Radioactivity was measured in a Packard Scintillation Counter, utilizing Scintisol (Isolab, Akron, OH). Polyacrylamide gel electrophoresis was performed according to [11] using 8% (w/v) acrylamide.

Regeneration of rhodopsin in hexane was assayed by incubating the bleached sample in the dark with an excess of 9-*cis* retinal at room temperature for 150 min [12,17]. Regeneration of rhodopsin from the hexane extracts was also assayed after solvent evaporation and resolubilization in 50 mM octyl glucoside. The extent of regeneration was determined by totally bleaching the regenerated sample, measuring the ΔA_{485} (isorhodopsin [12]) and after correcting for the difference in extinction coefficients between rhodopsin and isorhodopsin [12] compared to the initial amount of rhodopsin in the sample.

Rhodopsin containing planar bilayers derived from RLC in hexane were formed and studied as in [14].

3. Results and discussion

The optical absorbance spectrum of affinity purified and delipidated rhodopsin in DDAO is shown in fig.1A. The ratios of the absorbance at 280 nm and 400 nm both to 500 nm are 1.67 and 0.16, respectively, thus indicating the purity of preparation. These

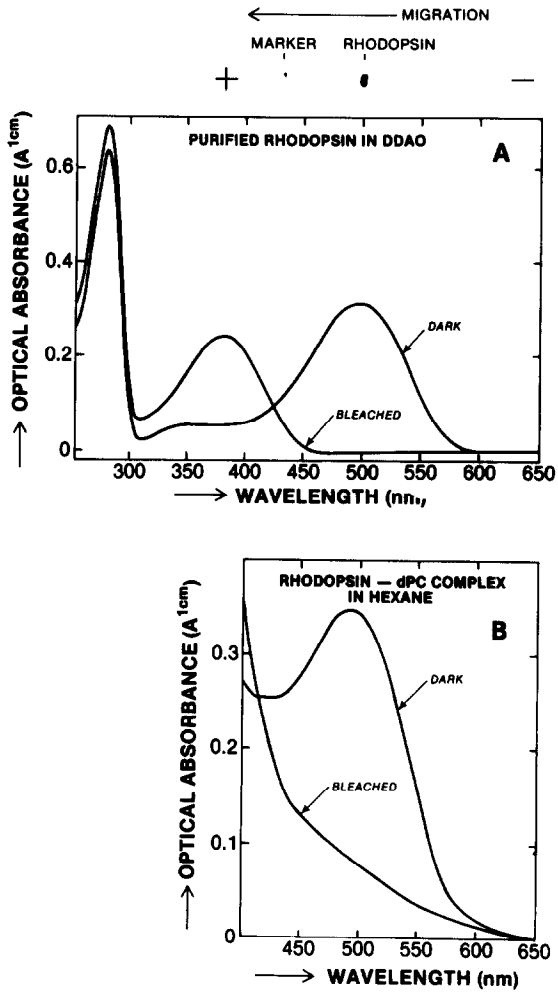

Fig.1. Optical absorbance spectra of purified rhodopsin in DDAO (A), and rhodopsin–dPC complexes in hexane (B). The upper part of the figure shows an electrophoresis gel loaded with 30 μg purified protein.

values are similar to those reported for the purest rhodopsin samples [7,15]. Polyacrylamide gel electrophoresis of this preparation showed one single band (upper part of fig.1A). The dark and bleached absolute spectra of purified rhodopsin–dPC complexes in hexane is illustrated in fig.1B. In the visible range the hexane extracts showed a peak with a λ_{max} at ∿494 nm which vanished upon illumination and an isosbestic point between dark and bleached samples at ∿415 nm [16,17]. Similar spectral characteristics were obtained when the lipid mixtures were used. Calculated difference spectra between dark and bleached samples display a λ_{max} at ∿500 nm.

The spectral characteristics of RLC in hexane are similar to those displayed by rhodopsin in retinal rod disc membranes [18]. Spectral integrity, however, is not a sufficient criterion of functional preservation. The ability of opsin to combine with 11-*cis* (or 9-*cis*) retinal is a sensitive assay of the native state of rhodopsin [12]. As illustrated in table 1, the extent of regeneration with 9-*cis* retinal was ∼50% in the 3 extraction conditions studied. Since this was lower than expected, the regeneration was also tested after the removal of hexane and resolubilization in octyl glucoside, a detergent in which rhodopsin can regenerate [13]; hexane extracts in octyl glucoside regenerated ⩽87%. The stability of the opsin–lipid complex in hexane at room temperature was assayed by repeating the regeneration–bleaching cycle several times with efficiencies of ∼80%. The spectral characteristics of the complex stored at 4°C were retained for several weeks.

Table 1 presents also the extraction yield and the ΔA_{500} of the RLC in hexane. These parameters do not vary significantly with respect to the lipids used and indicate that the purified protein is transferred (∿50%) as a lipid–protein complex into the solvent when using a single nonoxidizable lipid. However, the yield is dependent on the concentration of the residual detergent. The results shown were obtained from vesicles extracted after a 2 day dialysis period. The amount of residual detergent transferred into the solvent was 0.192% of that originally present (130 DDAO molecules/rhodopsin). If dialysis was extended to 7 days (21 buffer changes), the detergent transferred into the solvent was equivalent to 50 DDAO molecules/rhodopsin. The spectral characteristics of these extracts were the same but the extraction yield was 26±4% (n=3). Control experiments indicate that DDAO alone is almost completely dialyzable in 7 days, but in the presence of phospholipids it is retained to the same extent as in the recombined rhodopsin lipid vesicles (see also [19]).

Thus, it is possible to transfer into hexane affinity purified rhodopsin as a complex with a single lipid species which, in addition, is nonoxidizable (dPC). Rhodopsin from these complexes is photochemically active as assayed by the dark and bleached spectra and its ability to regenerate isorhodopsin.

RLC in hexane is the starting material for reconstitution in planar bilayer membranes [1]. We have shown that the steady-state conductance of rhodopsin-containing bilayers is characterized by the occurrence of defined fluctuations attributed to the presence of transmembrane ion-channels [1]. In addition, voltage-jump relaxation experiments have shown that the channels open at zero voltage and close at higher voltage of either polarity. The membrane conductance of symmetric bilayers formed by apposing two monolayers of RLC extracts derived from affinity purified rhodopsin display the two main characteristics of rhodopsin-containing bilayers, i.e., the presence of channels which fluctuate between open and closed states according to the transmem-

Table 1
Yield of extraction and regeneration of purified rhodopsin–lipid complexes in hexane

Phospho-lipid	n[a]	ΔA_{500}	Extraction yield (%)	Regeneration in hexane (%)	
Soybean	6	0.19 ± 0.09	42.2 ± 15.3	45 ± 5	(n=2)
dPC-bPE	2	0.22 ± 0.01	50.3 ± 0.3	47	(n=1)
dPC	4	0.23 ± 0.07	46 ± 14	59 ± 12	(n=2)

[a] The results are expressed as the mean ± 1 SD, and n is the number of experiments

brane voltage, being open in the vicinity of zero voltage and closing with higher voltages of either polarity.

The channel activity recorded in planar bilayers formed from purified rhodopsin and a nonoxidizable phospholipid cannot be attributed to lipid oxidation and the possibility of assigning the channel activity to a non-rhodopsin protein component has been rendered very unlikely. We favor, therefore, the interpretation that the channels are associated with the presence of rhodopsin in the bilayers. These results will be published in detail elsewhere.

Acknowledgements

We thank Carol Vandenberg for her perceptive comments. This work was supported by National Eye Institute grant no. EY-02084.

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