FORMATION OF DETERGENT-FREE PROTEOLIPIDS FROM BIOLOGICAL MEMBRANES: APPLICATION TO RHODOPSIN

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1. Introduction

We have repeatedly reported that aqueous lipid–protein complexes of membrane origin are extractable into hydrocarbon solvents provided the overall charge of the complex is neutralized with suitable counterions [1–6]. This lipoprotein–proteolipid transition constitutes a technical maneuver to extract lipoproteins from aqueous to non-aqueous phases for use in bilayer reconstitution studies [3,7]. We have extensively used this approach with mammalian rhodopsin [3–6].

A major breakthrough in the preparation of the rhodopsin proteolipid has been recently achieved. It consists of the direct transfer of rhodopsin from retinal-rod disc-membranes into an organic solvent. This method obviates the solubilization step of rhodopsin from the membrane into a detergent solution, thus avoiding the deleterious effect of the residual surfactant in reconstitution studies. We report here the procedure of formation and some properties of this proteolipid, which we call the 'instant proteolipid'.

2. Methods and materials

All of the procedures were performed under dim red light at 4°C unless otherwise stated. Rod-outer-segments (ROS) from dark adapted bovine retinae (Hormel Co.) were isolated by sucrose flotation and purified in a discontinuous gradient [8]. The rhodopsin concentration was determined by solubilizing an aliquot of membranes in cetyltrimethyl ammonium bromide [9] and measuring the difference in absorbance ΔA at 500 nm (ΔA_{500}) between dark and bleached samples, assuming an extinction coefficient of 40 000 $M^{-1}cm^{-1}$ [10] and mol. wt 40 000 [11]. ROS membrane samples in 0.066 M phosphate buffer, pH 7.0, containing about 0.75 mg of rhodopsin were centrifuged down at 27 000 × g for 20 min, the supernatant removed and the pellet frozen in liquid nitrogen for storage at −70°C.

The following protocol was employed to prepare 'instant proteolipids': A sample was thawed by immersing the frozen tube in a water bath at 45°C for 3 min. The pellet was resuspended in 1.0 ml of 0.1 M KCl, 0.01 M imidazole–HCl, pH 7.0 containing a dispersion of partially purified soybean phospholipids at a defined concentration. This lipid mixture contains about 37% phosphatidylcholine, 37% phosphatidylethanolamine, 8% cardiolipin and others [12]. The mixture is then transferred into a glass test tube and sonicated in a water bath sonicator (Bransonic ultrasonic cleaner, power output 100 W) for 3.5 min. Then, a defined concentration of $CaCl_2$ (in 0.1 ml) and 1 ml of hexane were added to the suspension. The tube was vigorously mixed for 4 min and the two phases were separated in a clinical centrifuge for 1 min. The hexane phase was removed and a second extraction performed with 1 ml of diethylether. The suspension was mixed for 3 min and the two phases were separated by centrifugation. Absorption spectra of 0.5 ml aliquots of the first hexane-extraction and the second ether-extraction were recorded. Samples were also analyzed for phospholipid-phosphorus according

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to Dawson [13] and for protein according to Lowry [14]. Absorption spectra were recorded in a Cary 14 spectrophotometer (Applied Physics Corp., Monrova, Calif.) using the corresponding solvent in the reference cell.

3. Results and discussion

No partition of rhodopsin into hexane or ether could be observed if ROS membranes were sonicated in the absence of added lipid. As illustrated in fig.1, the extent of extraction of rhodopsin from ROS membranes into either hexane or ether is dependent on the concentration of added lipid. The first hexane-extraction requires high lipid concentrations to achieve comparatively low yields, i.e., $\Delta A_{500} = 0.035 \pm 0.005$ with 40 mg/ml of lipid added. The measured phospholipid to rhodopsin ratio in the hexane-extract is about 2×10^4, indicating that in this first step a considerable amount of free phospholipid is partitioned into the solvent. In contrast, the yield of the second ether-extract is a $\Delta A_{500} = 0.14 \pm 0.031$ with a ratio of around 2.5×10^3 lipid molecules per rhodopsin. The efficiency of transferring rhodopsin from lipid supplemented and sonicated ROS membranes into ether is comparable to that of the second ether-extract prepared from detergent-(cetyltrimethyl ammonium bromide)-solibilized rhodopsin [6]. However, in the latter proteolipid there are about 10^3 phospholipids and 5×10^2 detergent molecules per rhodopsin. Thus, while the instant proteolipid derived from ROS membranes has never been in contact with detergents, it is enriched in accompanying lipids, whereas the proteolipid derived from detergent-solubilized rhodopsin has residual detergent [6]. It is possible, therefore, to achieve a considerable extraction of detergent-free rhodopsin into a solvent.

As previously shown lipoproteins are extractable into hydrocarbon solvents, provided the overall charge of the complex is neutralized by Ca^{2+}, the most effective counterion [2]. The extraction yield of rhodopsin from ROS membranes is enhanced by Ca^{2+} as illustrated in table 1. It is noteworthy that the data presented in fig.1 result from experiments performed at 10 mM Ca^{2+}.

It is clear, therefore, that in addition to charge neutralization, lipid and detergent, in one case, or excess lipid, in the other, are required in order to favor the partition of the complex into the solvent. This process can be schematically visualized as analo-

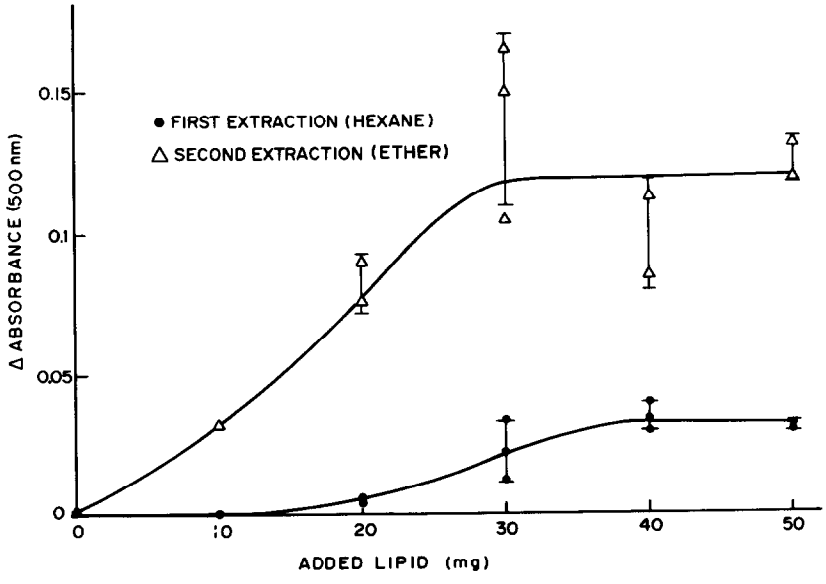

Fig.1. Extent of extraction of rhodopsin from retinal-rod disc-membranes into hexane (·) or diethylether (Δ) as a function of the concentration of added lipid. Ca^{2+} was used at a final concentration of 10 mM. Other conditions as described in the text.

Table 1
Effect of Ca^{2+}-concentration on the extraction yields of rhodopsin into organic solvents

$[Ca^{2+}]$ mM	Hexane ($\Delta A500$)	Ether ($\Delta A500$)
0	0.008	0.05
10	0.02	0.14
100	0.058	0.10

The proteolipid was prepared as described in the text. An aliquot of a concentrated $CaCl_2$ solution was added to achieve the indicated final concentration. The lipid concentration used was 30 mg/ml.

gous to the formation of inverted micelles where the polar entities (hydrophilic lipid and protein moieties) are segregated in an inner core and carried into the apolar solvent by the hydrocarbon coat provided through the lipid-acyl chains [3].

An index of rhodopsin integrity is provided by the concentration ratio of protein, as measured by standard chemical assays [14], to that of rhodopsin, as calculated from ΔA_{500}. Such an index for detergent solubilized rhodopsin is 1.75 and for the instant proteolipid is 2.32 ± 0.11 suggesting the preservation of the overall protein structure.

The instant proteolipid exhibits, in the organic solvent, spectral characteristics similar to those of native rhodopsin in the disc-membrane, namely: an absorption maximum at 500 nm which changes upon illumination to a distinct yellow colour and an isosbestic point between dark and bleached samples around 415 nm [11]. Spectral integrity is not a sufficient criterion of functional preservation. An operational criterion of the extent of rhodopsin denaturation is its regenerability: bleached rhodopsin, i.e., opsin, when incubated in the dark with 11-*cis* or 9-*cis* retinal, regenerates its color – the absorbance at 500 nm or 480 nm, respectively [15]. Regeneration occurs in digitonin but not in other detergents [16]. The assay for rhodopsin regeneration in the proteolipid is achieved by layering the ether proteolipid over a salt aqueous medium which contains digitonin (1%) and driving the ether away by bubbling nitrogen into the test tube. Thereafter, an absorption spectrum is taken, the sample is bleached and supplemented with an excess (two-fold) of 9-*cis* retinal. After a dark incubation period of 15 h the spectrum is measured. The ether proteolipid can be regenerated up to 70% of its original value. This taken altogether, is evidence that despite the partition of rhodopsin between an aqueous and a non-aqueous phase, the protein remains native.

The presence of excitable rhodopsin molecules in the instant proteolipid has also been established in interfacial layers where flashes evoke fast photoelectric signals which arise from capacitative charge displacements of oriented rhodopsin upon bleaching [6].

We have previously reported that the proteolipid from detergent-solubilized rhodopsin can be used to form planar lipid-bilayers by the method of hydrophobic apposition of two monolayers. The rhodopsin-bilayers display voltage-sensitive conductance and distinct step fluctuations in the current under voltage-clamp conditions; this has been taken as evidence for the formation of a voltage-sensitive channel by rhodopsin [3,5,17]. Bilayers prepared from the instant proteolipid also exhibit these conductance phenomena. The fact that this proteolipid has never been in contact with detergent, rules out the participation of detergent in the phenomenology described and establishes that rhodopsin is the species responsible of the membrane conductance.

The procedure to form instant proteolipids from natural membranes may have general application in bilayer reconstitution studies. This may be of major relevance in membrane preparations preferentially enriched with a protein involved in a given function, e.g., the acetylcholine receptor, the sarcoplasmic reticulum ATPase, bacteriorhodopsin and others [cf. 3,7]. In membranes containing several proteins the favorable approach is to solubilize and purify the protein of interest and then proceed to form the proteolipid [3,7].

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