Characterization and Analysis of Frog Photoreceptor Membranes

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ABSTRACT Frog photoreceptor membranes contain 54,000 g of protein per mole of visual pigment chromophore, virtually all of it insoluble membrane protein. Acrylamide gel electrophoresis indicates one major polypeptide class, most likely the visual pigment apoprotein. Suspensions of these photoreceptor membranes accumulate calcium ions when ATP is present, a characteristic that may play a part in visual excitation.

The rod outer segments of the vertebrate retina are cylindrical organelles which contain photoreceptor membranes. Frog rod outer segments are approximately 50 μ in length and 6 μ in diameter, and are made up of 1500-2000 self-enclosed membranous discs or sacs enclosed by a plasma membrane (1, 2). These outer segments can be purified in quantity, and their composition, enzymology, and ion permeability studied in vitro. It may be possible to specify the steps which occur during the photoexcitation of these structures in vitro, and the roles of specific membrane components might eventually be determined by dissociation and reconstitution studies. This paper describes the purification and analysis of these frog photoreceptor membranes, as well as some ion movements and enzyme activities which may be pertinent to visual excitation. A subsequent paper will discuss some changes in outer segment membranes which accompany photoexcitation.

METHODS

Preparation of Frog Rod Outer Segments

For most experiments retinas were removed from five large dark-adapted bullfrogs (Rana catesbeiana), and 6 ml of Ringer solution containing Ficoll was added to each milliliter of retinal material. The solution was made by dissolving 33 g of Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) in Ringer solution to a final volume of 100 ml. The Ringer solution contained 115 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 10 mM N-2-hydroxyethylpiperazine-N’2-ethanesulfonic acid (HEPES,
Calbiochem, Los Angeles, Calif.), pH 7.5. The retinas were homogenized by slow passage through a No. 17 syringe needle whose tip had been blunted on a grinding wheel. The homogenate was divided into three portions in Beckman-Spinco 302232 centrifuge tubes, 1–2 ml of 20 % (w/v) Ficoll in Ringer solution were layered over each portion, and the tubes centrifuged for 30 min at 18,500 rpm using a Spinco SW 25.1 rotor. The outer segments floated up to form a thick "carpet" at the interface between 20 and 33 % Ficoll and were withdrawn in a minimum volume (approximately 2 ml total) by means of a syringe. The outer segments were then suspended in 10–12 ml of 20 % Ficoll, layered over 1–2 ml of 33 % Ficoll in three similar centrifuge tubes, and centrifuged again as above. Outer segments which sedimented to the interface to form a carpet were again withdrawn. When outer segments were being prepared for amino acid analysis it was necessary to remove Ficoll by suspending this second interface in 10 ml of Ringer solution and centrifuging in a Spinco 40 rotor for 10 min at 10,000 rpm. The resulting outer segment pellet was used for analysis. Unless specified otherwise, all manipulations with outer segments were performed at 4°C in dim red light which bleached less than 1 % of the rhodopsin present.

**Spectrophotometric Measurements**

Rhodopsin and porphyropsin appear to account for more than 90 % of the visual pigment present in these frog retinas (3, 4). Other pigments, derived from green rods of cones (2), could not be detected. The amount of rhodopsin and porphyropsin present in each preparation was determined using Reuter's procedures (3, 4). Outer segments derived from approximately one-fifth of a retina were dissolved in a final volume of 0.4 ml of 0.02 M hexadecylammonium chloride (Eastman). Absorption spectra were recorded before and after bleaching with an orange light (Corning No. C53-67 filter). The ratio of the absorption change at 562 nm to that at 512 nm (Δ OD562:Δ OD512) is 0.295 for pure bullfrog rhodopsin and 0.748 for porphyropsin. The relative amounts of rhodopsin and porphyropsin vary linearly with this ratio, and thus one can determine for any given mixture the amount of porphyropsin, expressed as the per cent of the 512 nm absorption due to porphyropsin. This allows one to determine from published spectra (3) the absorption at 502 nm due to rhodopsin and at 522 nm due to porphyropsin. Molar extinction coefficients of 42,000 (rhodopsin) and 30,000 (porphyropsin) are used to calculate molar quantities (3, 4). Porphyropsin accounted for up to 20 % of the visual pigment present in our preparations. Data were finally expressed as moles of retinyl group (retinal plus 3-dehydroretinal) present.

**Analytical Procedures**

Amino acid analyses of rod outer segments before and after solubilization with hexadecylammonium chloride (0.04 M, in 10 mM HEPES, pH 7.5) were performed on the Beckman Model 120 C analyzer (5). Samples were hydrolyzed for 24 hr in glass tubes which had been evacuated and sealed; longer times of hydrolysis did not produce significantly different results. The presence of hexadecylammonium chloride during acid hydrolysis did not influence the analysis. Cysteine and methionine were analyzed after performic acid oxidation according to Moore (6), and tryptophan was
measured using the procedure of Patchornik et al. (7). Moore's procedure was used to perform ninhydrin analysis after alkaline hydrolysis (8). For each preparation the data were expressed as moles of amino acid present (determined by amino acid analysis) per mole of retinyl group bound in visual pigment (determined by difference spectroscopy).

**Electrophoresis**

Weber and Osborn's buffer and gel formulations (9) were followed for acrylamide gel electrophoresis except that 0.5% (w/v) sodium dodecyl sulfate in 0.1 M phosphate buffer was used. Just before application of the sample to the gels, 3-mercaptopropionic acid (Eastman, 20 µl, 0.1 M, in sample buffer pH 7.2) was electrophoresed into the gel for 15 min using a current of 8 ma per gel. In preparation for electrophoresis rod outer segments were first washed with water twice by centrifugation at 60,000 g for 30 min, and then dissolved in 5% sodium dodecyl sulfate (SDS) in water by stirring overnight. This solution was made 1% (v/v) in 2-mercaptoethanol and 10% (v/v) in glycerol, and 5-20 µl (1 mg protein/ml) were layered on top of the gel column. Known proteins were dissolved in a standard “sample buffer” containing 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) mercaptoethanol, and 0.01 M in phosphate buffer, pH 7.2. After electrophoresis for approximately 2-4 hr at 8 ma per tube, gels were fixed for 2 hr in methanol-acetic acid-water (in the volume ratios 50:7.5:42.5). For protein staining, gels were soaked for 3 hr in this same solution containing 0.25% (w/v) Coomassie brilliant blue R-250 (Mann Research Labs., Inc., New York) and destained by stirring overnight.

Mobilities of rod outer segment components and known proteins were related to that of myoglobin. Densitometer traces of gels revealed that staining intensity was proportional to the amount of protein in single bands up to 30 µg protein/band. Gels were stained for carbohydrates by the periodic acid–Schiff technique using the procedure of Zacharius et al. (10) except that gels were fixed for 2 hr in the methanol–acetic acid–water mixture prior to trichloroacetic acid fixation. In preparation for lipid staining a saturated solution of oil red O (Allied Chemical Corp., Morristown, N.J.) in warm methanol was prepared and filtered. An equal volume of 20% trichloroacetic acid in water was added to the filtrate (11). After electrophoresis and fixation in methanol–acetic acid–water for 2 hr, gels were placed in this solution overnight, and destained by stirring in the methanol–acetic acid–water mixture. Known proteins were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Mann Research Labs., lipids and glycoproteins from Sigma Chemical Company and Pierce Chemical Co. (Rockford, Ill.). Bromegrass mosaic virus coat protein was a gift of L. Lane.

**Adenosine Triphosphatase Assay**

Outer segments, prepared as described above, were washed once with Ringer solution and twice with water to remove any nonmembrane-bound adenosine triphosphatase (ATPase) activity. Activity was measured in a 100 µl reaction mixture of outer segments which was 4 mM in MgCl$_2$, 60 mM in KCl, 60 mM in NaCl, 25 mM in Tris-acetate, pH 7.5. The reaction was started by the addition of neutralized ATP in 10 µl of water. Aliquots were removed at frequent intervals during a 30 min incubation at
37°C and assayed for ATP content using the luciferase procedure described by the Sigma Chemical Company, with slight modifications.

**Calcium Accumulation**

Active calcium accumulation by suspensions of rod outer segments was measured using $^{45}$Ca (New England Nuclear Corp., Boston, Mass.). In the experiment shown in Fig. 4 outer segments, which have very small internal volumes, accounted for less than 0.1% of the total incubation volume. Thus, only a small fraction of the $^{45}$Ca in the medium became associated with these structures. Knowing the calcium concentration in the medium one could calculate its specific activity after the addition of a trace amount of $^{45}$Ca, and then determine how many moles of calcium were taken up from this medium per mole of rhodopsin present. The incubation conditions used in the experiment shown in Fig. 4 are described in the figure legend.

In order to measure $^{45}$Ca bound or taken up by an aliquot of outer segments, 0.5 ml of a reaction mixture was diluted to 10 ml with frog Ringer solution or with 0.26 M sucrose, 10 mM in HEPES, pH 7.5. This suspension was drawn through a 25 mm Millipore HA filter (pore size 0.45 μ) and the outer segments sedimented on the filter were washed with four 5 ml portions of the wash medium without being allowed to dry. This washing procedure required less than 1 min. Both the sucrose and Ringer wash solutions yielded the same results, and varying temperature between 4° and 20°C appeared to have little effect. The Millipore filter was then put directly into 5 ml of scintillation counting fluid [0.5% (w/v) PPO, 0.01% (w/v) dimethyl POPOP, Packard Co., in toluene-Triton X-100, 2:1 (v/v)] to which 0.65 ml of 0.1 N sodium hydroxide had been added. The scintillation vials were shaken for 1 hr and then counted on a Packard Tri-Carb scintillation counter. In some experiments, $^3$H and $^{45}$Ca were determined simultaneously. All radioactive materials were purchased from New England Nuclear Corp., and $\beta,\gamma$-methylene-ATP was obtained from Miles Laboratories, Inc., Elkhart, Ind. Sigma Chemical Company provided antimycin A, oligomycin, and EGTA (ethylene glycol bis [\(\beta\)-aminoethyl ether]N,N' , N'-tetraacetic acid).

**RESULTS**

**Analysis of Frog Photoreceptor Membranes**

Microscopic examination reveals that outer segments retain their in vivo shape and birefringence after the Ficoll purification procedure but are shorter in length, having broken at least once or twice during preparation. Very few nuclei and no rod inner segments are seen. The absence of significant mitochondrial contamination is indicated by the gel electrophoresis patterns (Figs. 1 b, 2) which do not reveal protein bands characteristic of mitochondria. Membrane-bound ATPase activity is relatively low: water-washed outer segments hydrolyze 2.0 moles ATP/mole rhodopsin per min (initial rate) in the presence of 0.36 mM ATP, 4 mM MgCl$_2$, 60 mM NaCl, and 60 mM KCl. This rate increases to 16 moles ATP/mole rhodopsin per min when the ATP concentration is raised to 10 mM, near its saturation level. Outer segments lyo-
philized from a water suspension and reconstituted with water have the same or slightly lower activity. Outer segment preparations which contain more than 54,000 g of protein per mole of retinyl group (see below) have higher levels of ATPase activity.

The protein content of whole frog rod outer segments, determined by amino acid analysis of nine different preparations, is 54,000 (± 3,100) g of protein per mole of retinyl group. Outer segments prepared using McConnell's more extensive purification procedure (13) yield very similar values. If isosmotically prepared outer segments are extracted sequentially with water, 10^{-4} M EDTA, and 0.8 M NaCl, following the procedure of Rosenberg and Guidotti

\[ \text{Origin} \quad \text{direction of electrophoresis} \rightarrow \]

\text{Figure 1. Optical density at 550 nm of outer segment membrane components separated by electrophoresis and stained with Coomassie blue. Curve } a, \text{ pattern obtained when outer segments are dissolved in 1\% SDS. Curve } b, \text{ outer segments dissolved in 5\% SDS.} \]

\footnote{The number of 28,500 given in an earlier report (12) is incorrect because of an error in the calibration of the amino acid analyzer used.}
(14), no measurable amount of protein material is removed. The protein content of rods after these extractions, determined in six different preparations, is $55,200 \pm 2,200$ g of protein per mole of retinyl group. Examination of each extract by ninhydrin analysis after alkaline hydrolysis indicates that 10-15% of the total ninhydrin color is found in the first aqueous extract. At least part of this material may be polysaccharide, for some amino sugars detected during amino acid analysis are partially removed by the aqueous extraction. The residue left after extraction can be solubilized only by detergents such as hexadecyl ammonium chloride or sodium dodecyl sulfate. (This material is mainly disc membrane protein, for the plasma membrane accounts for less than 1% of the total membrane of frog rod outer segments.)

The proteins of solubilized frog photoreceptor membranes can be analyzed by acrylamide gel electrophoresis, and the protein pattern obtained depends critically on the conditions used for solubilization. Many protein bands with

![Figure 2](image-url)
apparent molecular weights varying from 39,000 to over 100,000 are seen when outer segments are initially dissolved in 1% SDS and electrophoresed in gels containing 0.1% SDS (Fig. 1a). If, however, the outer segments are first washed with water, dissolved in 5% SDS in glass-distilled water, and electrophoresed in 0.5% SDS in 0.1 M phosphate buffer, a major protein band is seen with apparent molecular weight 39,000 ± 3,000 (Figs. 1b, 2).

In different preparations this major protein band accounts for approximately 80% (± 10%) of the material reacting with the Coomassie protein stain, the balance being accounted for by both faster and slower moving components. Occasionally some material appears which has approximately twice the apparent molecular weight of this major component. Increasing the concentration of SDS used to solubilize the outer segments, addition of 5% mer-
captoethanol, or addition of 8 M urea does not produce material with a lower apparent molecular weight. The 39,000 moiety is not likely to have been derived from a larger component by proteolytic degradation, for we have used the techniques suggested by Pringle (15) to avoid such artifacts on several different gels.

In addition to the major component several slow moving bands and a fast moving band are seen (Fig. 1b). Using Swank and Munkres' procedures (16)

![Graph showing calcium accumulation and efflux](image)

**Figure 4a.** Calcium accumulation by outer segment suspensions. Outer segments were incubated in 40 separate 0.5 ml aliquots of Ringer solution containing 10^-5 M CaCl₂. At zero time trace amounts of ⁴⁵Ca were added and the aliquots made 2 mM in MgCl₂ and 2 mM in ATP. After incubation at 20°C for the times indicated, ⁴⁵Ca associated with a given aliquot was measured (O–O–O). (○–○–○) indicates β,γ-methylene-ATP used instead of ATP. •, □, and △ indicate final levels of ⁴⁵Ca accumulated in the presence, respectively, of 10^-5 M antimycin A, 10^-4 M oligomycin, and 10^-4 M EGTA.

**Figure 4b.** Loss of accumulated calcium. After active ⁴⁵Ca accumulation was complete, 0.5 ml aliquots were diluted with 20 ml of Ringer solution for the indicated times, and the ⁴⁵Ca remaining was then measured. Actual data for Fig. 4a and 4b are shown on the left ordinate. These data are used to calculate the number of moles of calcium accumulated/mole of rhodopsin present, shown on the right ordinate.
the apparent molecular weight of the fast component is between one and two thousand. The staining properties of this material are very different from those of the major protein material of the membrane; it reacts much more intensely with carbohydrate and lipid stains than with the Coomassie blue protein stain (Fig. 3), and forms a precipitate in the gel. Its electrophoretic and staining properties are similar to those of phosphatidylcholine, and other complex lipids such as phosphatidylethanolamine and sphingomyelin migrate to the same position in the gel. Extraction of rods with 99% ethanol which removes most of the phospholipids present, also removes the fast component.

Active Calcium Accumulation by Frog Photoreceptor Membranes

Frog rod outer segments suspended in Ringer solution containing low levels of calcium accumulate more calcium in the presence of ATP than in its absence (Fig. 4 a). Neither the β,γ-methylene analogue of ATP nor ADP supports this accumulation. The activity decreases with increasing age of the preparations, and differs somewhat from preparation to preparation. Stimulation of calcium accumulation varies from 2- to 10-fold under different conditions. This stimulation is optimal in 5 mM ATP, 5 mM MgCl₂, and 10⁻⁵ M CaCl₂, decreases with increasing calcium concentration, and is not seen in 10⁻⁴ M CaCl₂. As with the calcium uptake system of the sarcoplasmic reticulum (17), addition of EGTA abolishes calcium accumulation (Fig. 4 a). Oligomycin and antimycin A depress the net calcium accumulation by 20-30%.

Accumulated calcium is lost with the time course shown in Fig. 4 b if the ATP concentration is suddenly reduced by dilution. As the ATP concentration is increased from 10⁻⁵ to 10⁻³ M the number of moles of ATP bound per mole of calcium accumulated increases from approximately 0.5 to 12. (This stoichiometry is determined in an experiment using 10⁻⁴ M calcium and ⁴⁰Ca and ³H ATP of known specific activities.) When methoxy-³H-inulin is included in the reaction mixture, its binding is not significantly changed by the addition of ATP.

DISCUSSION

Preparation and Analysis of Frog Photoreceptor Membranes

The relatively simple isosmotic procedure developed for preparing outer segments yields preparations with constant ratios of protein to visual pigment prosthetic group. The outer segment suspensions appear to have relatively less ATPase activity than reported for some other preparations (18, 19). Very little protein, if any, is removed by the gentle procedures which are known to extract 50% of the protein of erythrocyte membranes (14). The components which appear in the first aqueous extract might include metabolic enzymes, which Futterman et al. (20) have characterized, and also mucopolysaccharides. Virtually all of the protein present in these outer segments is solubilized.
only by detergents which disrupt membrane structure. Acrylamide gel
electrophoresis reveals that this insoluble protein material is relatively simple,
having a limited number of polypeptide chains which fall into several discrete
molecular weight classes. Because dissociation of outer segment membranes in
5% sodium dodecyl sulfate in water yields more homogeneous material of
lower apparent molecular weight, we assume that the electrophoresis pattern
observed when 1% SDS is used (Fig. 1a) is caused by higher molecular weight
aggregates which have not completely dissociated.

The major protein fraction in these solubilized outer segments has an ap-
parent molecular weight of 39,000 and accounts for approximately 80% of the
material reacting with the protein stain. In addition to the major component
one sees a compound which moves very rapidly. While Dreyer and his col-
leagues have labeled this component from cattle outer segments a “mini pro-
tein” (21), the present experiments provide little evidence that it is a protein.
It has staining and electrophoretic properties characteristic of glycolipids or
gangliosides, not proteins (Fig. 3).

Estimates of rhodopsin’s molecular weight range from 27,000 to 40,000 g of
protein per mole of retinal (22–26), suggesting that it must account for 50–
75% of the 54,000 g of protein present per mole of retinal in whole frog outer
segments. The component which accounts for 80% of the stained material on
acrylamide gels is most likely to be opsin. The figure of 80% as the rhodopsin
content of outer segments is also suggested by the data of Hall et al. (27) who
used entirely different techniques and Rana pipiens frogs.

If 80% of the 54,000 g protein per mole retinal actually placed on the gel is
accounted for by rhodopsin (approximately 43,000 g protein per mole retinal)
one expects to see in gel electrophoresis a protein with an apparent molecular
weight of 43,000 (± 3,000). Given the errors involved, this number is very
close to the apparent molecular weight measured for the major component,
39,000 (± 3,000). (The retinyl prosthetic group is split from opsin on exposure
to sodium dodecyl sulfate and thus cannot be used as a marker. Because
standard protein treated with sodium borohydride behaves anomalously in
gel electrophoresis, we have not yet extensively studied the reduced \(N\)-retinyl-
opsin derivative of rhodopsin (28) in this system.) We are now resolving outer
segment proteins using gentle column techniques which do not split the retinyl
group from opsin and in preliminary experiments have found that frog rhodo-
psin contains 40,000 g protein per mole retinyl group. Cavanagh, in a similar
electrophoretic analysis of solubilized cattle outer segments, has estimated the
molecular weight of cattle rhodopsin to be 35,000 (29).

Active Calcium Accumulation by Frog Photoreceptor Membranes

The ATP-stimulated calcium accumulation seen in rod outer segment suspen-
sions might be due to a membrane transport mechanism, or to specific ATP-
dependent calcium binding (30), or both. The process is reversible, as shown by the loss of accumulated calcium when the ATP concentration is lowered (Fig. 4 b), and this is explicable by either mechanism. It seems likely that hydrolysis of the terminal phosphate of ATP is required, for neither the β,γ-methylene analogue of ATP nor ADP supports calcium accumulation. Maximum stimulation of calcium accumulation by ATP occurs at very low calcium levels (10⁻⁵ M) characteristic of intracellular rather than extracellular fluid. Disc membranes, generally thought to be in intracellular media in vivo, may then be performing the observed calcium accumulation. One can calculate that accumulation of 10⁻³ M calcium inside discs would result in the presence of approximately 1 free calcium ion per 30 rhodopsin molecules present. (Values used in the calculations are: water space in discs, 0.0085 μl; 2000 discs/outer segment, 5.5 × 10⁻¹⁵ moles rhodopsin/outer segment.) Thus the accumulation of 0.025 mole calcium/mole rhodopsin, if it is due to a membrane transport mechanism, might bring the calcium concentration in the disc lumen to approximately 10⁻³ M.

The fact that oligomycin and antimycin A depress net calcium accumulation by 20-30% suggests that this fraction of the observed accumulation might be due to contaminating mitochondria. Given the values published for the calcium uptake activity of mitochondria (31) (approximately 1 μmole calcium taken up per milligram of protein) this accumulation could be caused by mitochondria which account for 0.1% or less of total protein present in these outer segment preparations. The presence of mitochondrial contamination need not be assumed, however, for McConnell has reported that an oligomycin-sensitive ATPase, not associated with mitochondria, is found in cattle outer segment preparations (13). The fact that 20-30% of the calcium accumulation might be accounted for by a minor fraction of the total protein suggests the possibility that the remaining accumulation might be due not to photoreceptor membranes, but to some other contaminants present in low levels. Such a possibility is difficult to exclude.

The significance of the calcium accumulation system can only be determined if a specific role for calcium in photoreceptor membrane function and a requirement for a calcium accumulation system can be demonstrated. Yoshikami and Hagins have recently suggested that photoexcitation of disc membranes might cause the release of calcium ions which in turn interact with the plasma membrane to increase its resistance (32). If this model is correct, an active accumulation mechanism must function to restore to the discs the calcium which is lost on photoexcitation. While the experiments presented here demonstrate that such a mechanism might exist, it is most important to determine whether photoexcitation in fact causes the release of calcium from discs. We have found that illumination does not cause a dramatic release of calcium from purified outer segments in suspension, but technical problems have so far
prevented us from determining whether 10% or less of the total calcium might be released.

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