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# Characterization of Bovine Rod Outer Segment G-protein*

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**A simple modified procedure is described for isolating and purifying peripherally bound membrane proteins from bovine rod outer segment disks. The methods yield milligram quantities of G-protein and cGMP phosphodiesterase which are suitable for reconstitution with membranes containing visual pigments. The properties of cGMP phosphodiesterase have been previously characterized (Baehr, W., Devlin, M. J. & Applebury, M. L. (1979) *J. Biol. Chem.* 254, 11669-11677). Here we report the characterization and properties of the rod outer segment G-protein, a complex of three subunits $\alpha$, $\beta$, and $\gamma$.**

**Protein labeling studies of bovine retina show that G-protein is synthesized and transported to the outer segment at rates comparable to those of the integral membrane protein, rhodopsin. G-protein co-sediments with rhodopsin-containing membranes throughout fractionation procedures for isolating rod outer segment disks. Washing bleached disk membranes with low ionic strength buffer removes all peripheral protein except G-protein; washing with buffer containing >3 $\mu$M GTP elutes G-protein in high purity. GTP or its nonhydrolyzable analog $\beta,\gamma$-imidoguanosine 5'-triphosphate elutes G-protein equivalently indicating that GTP hydrolysis is not required for release from the membrane surface. The association constant for $\beta,\gamma$-imidoguanosine 5'-triphosphate binding to G-protein is $0.5 \times 10^7$ M$^{-1}$ with 1 mol of nucleotide bound/mol of G-protein (assuming 80,000 $M_r$ for G-protein).**

**G-protein is nonhomogeneous in solution and can be separated into two species by native gel electrophoresis. Gel electrophoresis and analytical ultracentrifugation identify one species to be $(G\text{-}\alpha)_2$ and the other to be $(G\text{-}\beta\gamma)_2$; the latter has a tendency to form tetramers or higher aggregates at moderate ionic strength. Peptide maps and isoelectric points indicate G-$\alpha$ and G-$\beta$ are distinctly different proteins. Purified G-protein can be reconstituted with bleached depleted membranes in the absence of GTP. In this system, the membranes exhibit a GTP hydrolytic activity inherent to G-protein. The turnover number is ~1 mol of GTP/mol of G-protein/min. Binding saturation of disk membranes is reached at 1 mol of G-protein to 4-5 mol of rhodopsin.**

A complex system of enzymes and regulatory components has recently been shown to be associated with rod outer segment disk membranes (1). Two of these components, G-protein (a GTP binding, regulatory protein) and cGMP phosphodiesterase, mediate the hydrolysis of cGMP which is activated upon absorption of light by the photoreceptor visual pigment (2-6). The light-activated sequence of events starts with photon absorption by rhodopsin producing an activated rhodopsin; this activated membrane receptor enables the G-protein to exchange GTP for GDP and the GTP protein complex in turn activates the cGMP phosphodiesterase (6-8). Such a cascade organization provides a two-step amplification mechanism whereby the absorption of one photon may lead to the hydrolysis of $10^5$-$10^6$ cGMP molecules/s (2, 3, 6, 9).

Though some elements of this amplification system are defined, the mechanisms by which molecular interfaces are established to achieve activation, and disrupted to restore the unactivated state are little understood. Such mechanistic details first require a basic biochemical characterization of each component. We have isolated the cGMP phosphodiesterase from bovine rod outer segments and reported its biochemical properties previously (10). In the isolation procedure developed at the time, we also purified the G-protein which we termed 80K protein according to the approximate sum of molecular weights of its subunits. We report here our studies of the molecular properties of G-protein and characterize this component as a peripheral membrane bound protein of rod outer segment disks.

To facilitate discussion throughout this work, we have adopted the term G-protein[1] for the heterogeneous complex of three polypeptides ($\alpha\beta\gamma$) which control the activation of the photoreceptor cGMP phosphodiesterase. "G-protein" (11) is the name which has been given to the regulatory GTP-binding protein that serves a similar role in the hormone receptor-adenylate cyclase system (12, 13). The nomenclature seems appropriate because both proteins lack GTPase activity in the absence of membranes (14, 15), exchange cytoplasmic GTP for bound GDP in the presence of stimulated membrane receptors (16-18), and return to the basal inactive state by GTP hydrolysis (17, 19). Moreover, at least two of the subunits have similar molecular weights (14).

## EXPERIMENTAL PROCEDURES[2]

## RESULTS

*Association of Accessory Proteins with ROS Membranes*—

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[1] G-protein has been variously termed GTPase (8, 15), 80K protein (10), transducin (6), guanine nucleotide-binding protein (17), and Γ (48). G-$\alpha$ subunit has been termed "G" component, and G-$\beta\gamma$ "H" (helper) component (4).

[2] Portions of this paper (including "Experimental Procedures" and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-2496, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Dark-adapted ROS[3] membrane fragments may be fractionated by sedimentation in a sucrose step gradient and isolated in high purity relatively free of mitochondrial or synaptosomal contamination (29, 32). SDS-PAGE analysis of the proteins in fractions throughout the gradient indicate that a number of polypeptides sediment in association with ROS membranes which are identified by their marker protein rhodopsin (Fig. 6, *track 3*). Those which have been identified are indicated in Fig. 6 and consist of the subunits of cGMP phosphodiesterase (phosphodiesterase-$\alpha$, phosphodiesterase-$\beta$, and phosphodiesterase-$\gamma$) (10), G-protein (G-$\alpha$, G-$\beta$, and G-$\gamma$) (7, 16, 24), and an integral membrane protein of high molecular weight (*I*) (33). The subunits of phosphodiesterase are tightly associated with ROS fragments (Fig. 6, *track 3*), whereas the G-protein subunits appear more loosely bound and are found throughout the gradient. This loss of G-protein can be minimized by preparing membranes under normal room light from light adapted eyes (results not shown).

The sensitivity of analysis can be enhanced by labeling photoreceptor proteins *in vitro* with [$^{35}$S]methionine (Fig. 6, *tracks 5–8*). Detection of polypeptides by autoradiography shows that rhodopsin, cGMP phosphodiesterase, and G-protein subunits readily incorporate methionine (particularly PDE-$\gamma$), whereas the integral membrane protein I is poorly labeled either due to a low methionine content or due to differences in rate of synthesis. The degree of labeling indicates the peripherally membrane bound proteins must be synthesized at a rate similar to the rate of biosynthesis of rhodopsin. Association of the proteins with isolated rod outer segment fragments indicates that the newly synthesized, peripherally membrane bound proteins are transported from the inner segment site of synthesis to the outer segment along with the integral membrane protein rhodopsin.

Protein labeling with [$^{35}$S]methionine in the presence of tunicamycin, an antibiotic which inhibits protein glycosylation (34, 35), leads to dramatic changes in the mobility of rhodopsin due to lack of carbohydrate chains (see *arrow* in Fig. 6, *track 9*). No effect on the mobility of phosphodiesterase and G-protein subunits is observed which suggests that neither protein is a glycoprotein. This agrees with the report that peripherally membrane bound protein subunits do not stain for carbohydrate with the periodic acid Schiff base reagent (30).

*Release of G-protein from ROS Membranes by Titration with GTP and GMP-PNP*—G-protein becomes tightly bound to bleached membranes (membranes in which rhodopsin is light activated) but can be eluted by washing with GTP (24). Fig. 7 illustrates that the GTP or GMP-PNP concentration that elutes 50% of the G-protein is the same (~0.75 $\mu$M for these experimental conditions) and shows that hydrolysis of GTP is not necessary for release. For this experiment, membranes containing only the integral protein opsin (bleached rhodopsin) and peripherally bound G-protein were prepared as described under "Experimental Procedures," *i.e.* ROS membrane fragments were bleached, and stripped of phosphodiesterase and other proteins by washing with hypotonic buffer. Titration of the membranes over a range of $10^{-8}$ M to $4 \times 10^{-5}$ M GTP or GMP-PNP shows that 3 $\mu$M of either guanine nucleotide elutes more than 90% of the bound G-protein under conditions of low ionic strength (Fig. 7). All three subunits of the G-protein complex are equivalently eluted (Fig. 1), although only the G-$\alpha$ subunit has been implicated to bind GTP or GMP-PNP (6).

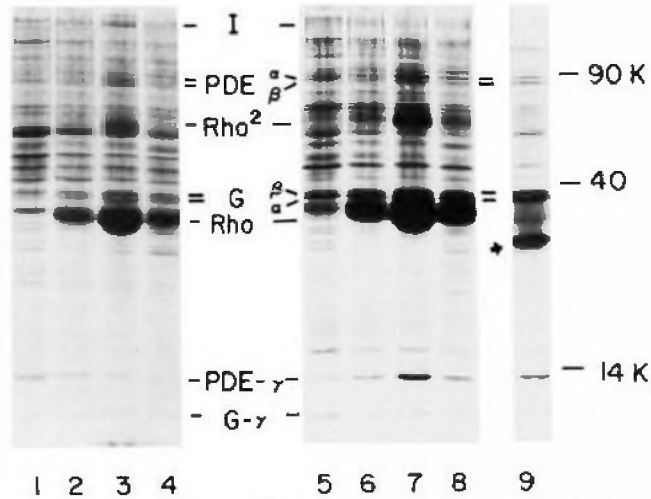


FIG. 6. **SDS-PAGE of $^{35}$S-labeled retinal proteins fractionated by a sucrose step gradient.** *Tracks 1–4*, Coomassie stained, $^{35}$S-labeled polypeptides harvested at the interfaces 1.0/1.10; 1.10/1.11; 1.11/1.13; 1.13/1.15 g/ml of a sucrose step gradient. 10 $\mu$l of the membrane suspension were loaded into a slot of a low cross-linked gel. *Tracks 5–8*, autoradiography of the gel *tracks 1–4*. *Track 9*, 1.11/1.13 g/ml interface of a gradient loaded with membranes which had been labeled in the presence of tunicamycin. The subunits of phosphodiesterase are labeled *PDE-α*, *PDE-β*, and *PDE-γ*, the subunits of G-protein *G-α*, *G-β*, and *G-γ*. Other identified proteins are *I*, an integral membrane protein of $M_r$ 240,000, *Rho*, rhodopsin, and *Rho*$^2$, dimer of rhodopsin.

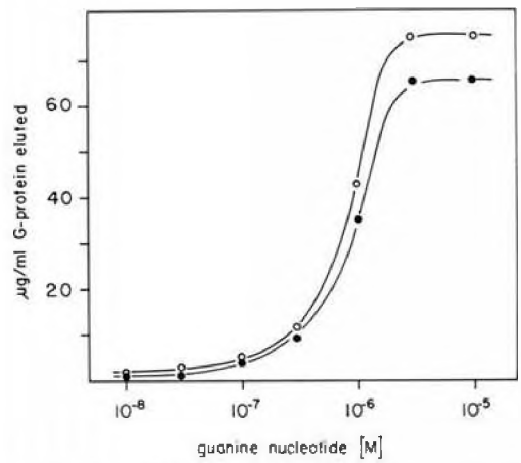


FIG. 7. **Elution of G-protein with guanine nucleotide (GTP or GMP-PNP).** Bleached ROS membranes containing 1 mg of opsin in 450 $\mu$l of hypotonic buffer (depleted of all proteins except G-protein) were incubated at 0 °C for 10 min with the indicated concentrations of guanine nucleotide by adding 50 $\mu$l of a 10 × concentrated stock solution of GTP (○——○) or GMP-PNP (●——●). The membranes were collected by centrifugation in an Eppendorf centrifuge, and the released G-protein in the supernatant determined by a Coomassie G-250 assay. Supernatants from membranes with no nucleotide added were subtracted as blanks.

*Stoichiometry and Binding Constant for the GMP-PNP/G-protein Complex*—Following elution of G-protein from ROS membranes with 40 $\mu$M [$^3$H]GMP-PNP excess nucleotide may be removed by chromatography of the protein containing supernatants on DE52 cellulose. As shown in Fig. 8*a*, [$^3$H]GMP-PNP remains tightly bound to the protein indicating a low dissociation constant for the G-protein-nucleotide complex. Analysis indicates 1.0 ± 0.2 mol of [$^3$H]GMP-PNP is bound/mol of G-protein where the latter is defined to be a

[3] The abbreviations used are: ROS, rod outer segments; GMP-PNP, $\beta,\gamma$-imidoguanosine 5′-triphosphate; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate; Rho, rhodopsin.

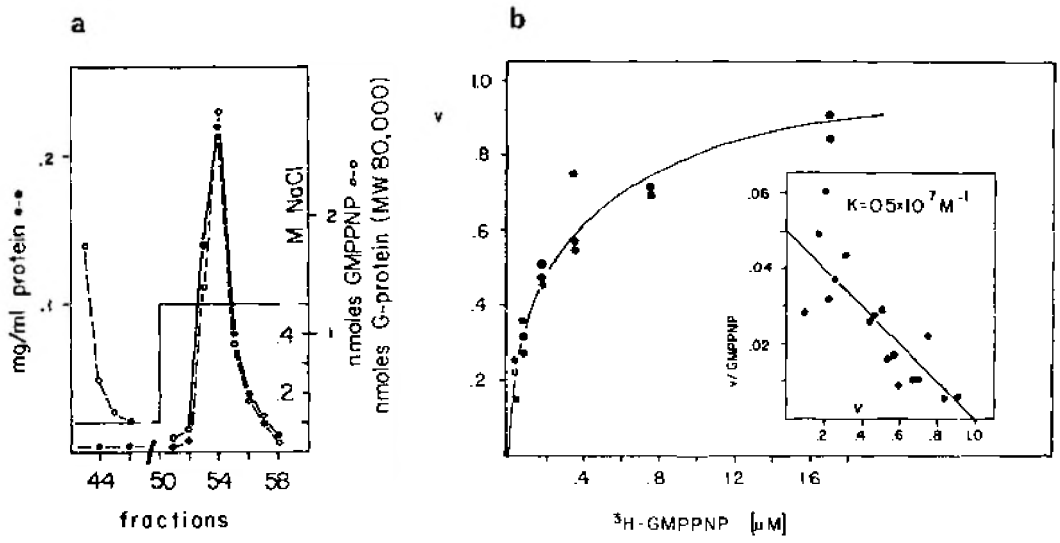


FIG. 8. **Elution of G-protein with [$^3$H]GMP-PNP (*a*) and binding constant for the nucleotide/protein complex (*b*).** *a*, depleted bleached membranes containing 10 mg of opsin were extracted 3 × with 40 μM [$^3$H]GMP-PNP and the combined supernatants chromatographed on DE52 cellulose (Fig. 3). After elution of excess nucleotide with 0.1 M NaCl, G-protein was eluted with 0.5 M NaCl. The peak fractions are analyzed for G-protein by a Coomassie G-250 assay and converted to nmoles protein based on a minimal $\bar{M}_r$ of 80,000. *b*, direct plot of υ (moles of bound [$^3$H]GMP-PNP/moles of G-protein) *versus* micromolar [$^3$H]GMP-PNP added as determined by a Millipore filter assay. The *inset* shows the same data in a Scatchard plot (36).

complex of G-$\alpha\beta\gamma$ of 80,000 $\bar{M}_r$.

The affinity of GMP-PNP for G-protein may be measured by taking advantage of the observation by Godchaux and Zimmerman (17) that G-protein, together with its bound nucleotide, does not pass through nitrocellulose filters, whereas unbound nucleotide can be washed out. Figure 8*b* shows binding data of [$^3$H]GMP-PNP to G-protein with a Scatchard analysis (36) of the same data (Fig. 8*b*, *inset*). The binding constant is determined to be $0.5 \times 10^7$ M$^{-1}$ and the stoichiometry approaches 1.0 mol/mol. The affinity and stoichiometry values determined are independent of ionic strength (data not shown).

*Reconstitution of G-protein with Depleted Membranes: Saturation of Membranes with G-protein*—The elution of G-protein from ROS membranes is reversible and depleted membranes may be reconstituted with subunits of the G-protein complex (5, 8, 17). Reconstitution affords a way to examine the stoichiometry of G-protein binding to ROS membranes and a way to examine the mechanism of interaction between activated rhodopsin and G-protein. As indicated in Fig. 9*a* and earlier reports (5, 8, 17), G-protein exhibits no GTP hydrolytic activity in the absence of membranes, but catalytic activity is restored by addition to membranes containing bleached rhodopsin. The steady state GTPase activity increases in proportion to G-protein concentration; the activity is linearly proportional over much of the range and does not show an apparent saturation. Even at 7-fold molar excess of G-protein (80,000 $\bar{M}_r$) compared to rhodopsin the activity is still nearly proportional to enzyme concentration. Moreover, the rate of GTP hydrolysis does not change significantly at 10-fold lower or 10-fold higher rhodopsin concentration. The activity is independent of ionic strength; the level is the same whether assayed at moderate ionic strength (0.1 M NaCl) where G-protein would reassemble with membranes or low ionic strength where G-protein would elute. These observations emphasize the catalytic role of rhodopsin. The rate-limiting step in the GTP hydrolysis mechanism cannot involve a one to one complex of G-protein and rhodopsin since there is no saturation of activity for a given concentration of rhodopsin with increasing addition of G-protein. The data may be used, however, to calculate the specific GTPase activity. From the region of the initial linear slope in Fig. 9*a* the activity is 1.0 mol of GTP/mol of G-protein/min.

Quantitation of G-protein in fractionated photoreceptor membranes has indicated that the molar ratio of G-protein to integral rhodopsin in the membrane is low (10, 17). In view of the fact that G-protein may be lost during ROS fractionation and washing membranes (Figs. 1 and 6) it is of interest to determine how much G-protein can be bound under known conditions. For this purpose, G-protein at increasing concentrations was equilibrated with bleached ROS membranes containing opsin (at 0.6 nmol/ml) in isotonic buffer minus GTP. Nonbound G-protein was removed from the membranes by centrifugation and washing. Residual bound G-protein was then determined by two independent methods: assay of GTPase activity of bound G-protein and densitometric analysis of Coomassie stained G-protein subunits on SDS gels. Figure 9*b* illustrates that above 0.15 nmol of G-protein/ml there is no increase in bound GTPase activity of bound G-protein; thus, binding saturation of ROS membranes is reached at a ratio of G-protein to rhodopsin of ~1:4 where G-protein is defined as a minimal molecular weight complex of 80,000 and rhodopsin of 35,000. The specific GTPase activity determined for G-protein which is membrane bound is equal to that determined above (Fig. 9*a*) within experimental error.

*Separation of G-protein Subunits into G-α and G-βγ*—Analysis of purified G-protein by native gel electrophoresis (Fig. 10) and by analytical ultracentrifugation (Fig. 11) reveal a striking nonhomogeneous behavior. Gel electrophoresis at pH 9.5 separates[4] G-protein into a faster moving species with the mobility of a protein of approximately 80,000 $\bar{M}_r$ and a larger slower moving species of 95,000 $\bar{M}_r$. Both bands appear somewhat diffuse as compared to the sharp bands of phosphodiesterase (170,000 $\bar{M}_r$), and bovine serum albumin (68,000 $\bar{M}_r$) as standards. Second dimension SDS-PAGE reveals that the faster moving species consists exclusively of G-α, and the slower one of both G-β and G-γ. We took advantage of this property of G-protein to separate G-α from G-βγ by prepara-

[4] This separation occurs both for G-protein eluted from dark-adapted membranes in the absence of GTP or for G-protein eluted from bleached membranes in the presence of GTP.

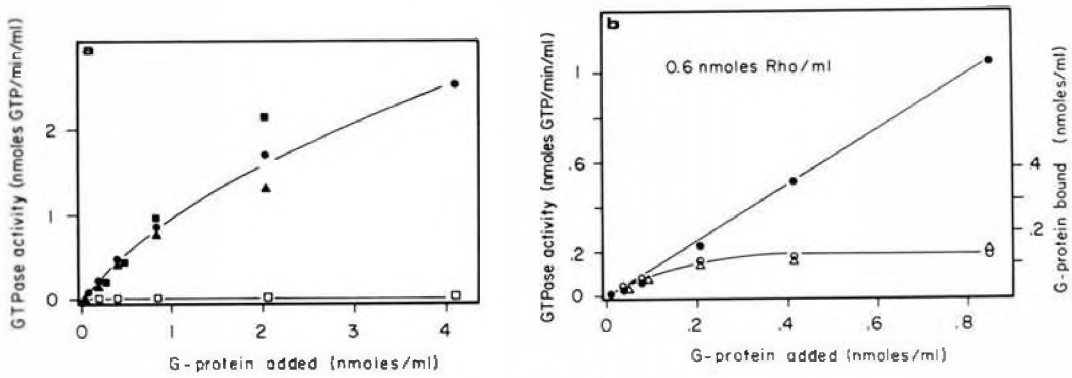


FIG. 9. **GTPase activity of G-protein in the presence of catalytic amounts of ROS membranes (*a*) and reconstitution and saturation of G-protein with depleted ROS membranes (*b*).** *a*, GTPase activity (nmoles of GTP hydrolyzed/min/ml) of the indicated amounts of G-protein (0.04 to 4.2 nmol/ml) under the following conditions: (●), hypotonic, and (▲) isotonic in the presence of 0.6 nmol of rhodopsin/ml. (■), Hypotonic in the presence of 0.06 nmol of rhodopsin/ml. (□), no rhodopsin added. *b*, GTPase activity (○) of ROS membranes reconstituted in the presence of the indicated amounts of G-protein. Membrane-bound G-protein (△) was independently measured by densitometric scanning of stained G-protein bands after separation from rhodopsin on SDS polyacrylamide gels.

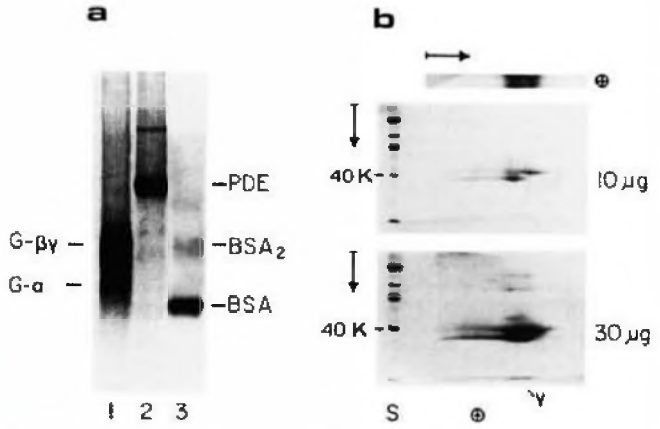


FIG. 10. **Native gel electrophoresis (*a*) and two-dimensional SDS-PAGE characterization of purified G-protein (*b*).** *a*, 10 μg of purified G-protein electrophoresed at pH 9.5 in 50 mM Tris-glycine (*track 1*) are compared with phosphodiesterase holoenzyme (170,000 $\bar{M}_r$, *track 2*) and bovine serum albumin (68,000 $\bar{M}_r$, *track 3*). *b*, two-dimensional SDS-PAGE analysis of G-protein first separated by native gel electrophoresis as in part *a*, *track 1*. A gel containing 10 μg of G-protein was incubated in a buffer containing 0.1 M Tris-HCl, pH 6.8, 2% SDS, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, polymerized horizontally into a stacking gel and the proteins separated (10% Laemmli gel). A second gel loaded with 30 μg of protein was prepared to demonstrate co-migration of G-γ with G-β. *S*, marker proteins as described previously (10). ***PDE***, phosphodiesterase.

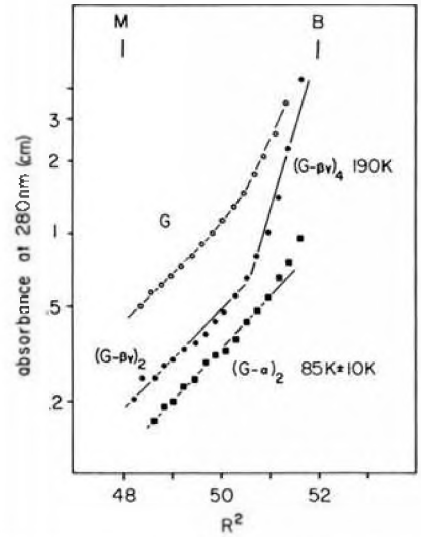


FIG. 11. **Sedimentation equilibrium analysis of purified G-protein, G-α and G-βγ.** Absorbance at 280 nm (measured in centimeters per deflection) is plotted in a logarithmic scale *versus* the square of the distance to the center of rotation, *R*. *M*, meniscus of the sample. *B*, bottom of cell.

tive native polyacrylamide gel electrophoresis (Fig. 4).

*Molecular Weight and Aggregation of Purified G-protein Subunits G-α and G-βγ*—Upon electrophoresis under denaturing conditions (0.1% SDS), purified G-α migrates as a single polypeptide of 37,000-dalton mobility on low cross-linked gels, (or 41,000 on conventional cross-linked gels, see Fig. 5). Sedimentation equilibrium in isotonic buffer (0.1 M NaCl) (Fig. 11) indicates G-α behaves homogeneously as the dimeric structure $(G\text{-}\alpha)_2$ which is consistent with the observed mobility of G-α in native gels. The apparent molecular weight was calculated to be 85,000 ± 10,000. Purified G-βγ exhibits 2 bands in low cross-linked denaturing gels of 39,000 (or 35,000 on conventional gels) and <10,000. Analysis by analytical ultracentrifugation shows that in isotonic buffer (0.1 M NaCl), G-βγ exists as dimers and tetramers (Fig. 11). The apparent molecular weights were calculated to be 85,000 ± 10,000 and 190,000 ± 20,000, respectively. In comparison, G-protein itself exhibits a molecular weight distribution ranging from 80,000 to 160,000. With increasing salt concentrations (0.5 M NaCl) dimers and tetramers of G-α, and G-βγ, disaggregate (data not shown). The behavior of the subunits analyzed by ultracentrifugation is the same in the presence of bound GMP-PNP, thus hydrolysis of GTP does not effect dissociation of G-α from G-βγ.

*Peptide Mapping of G-protein Subunits in Comparison with Rhodopsin*—Limited proteolysis of excised bands of G-α and G-β was carried out according to the technique of Cleveland *et al.* (27) and reveals a strikingly different peptide map for the two polypeptides (Fig. 11). (Note that G-β would co-migrate with rhodopsin in conventional SDS gel electrophoresis, but the peptide maps of G-β and rhodopsin are strikingly different as expected.) The protease used in the experiments shown in Fig. 12 was *Staphylococcus aureus* V8 protease which is specific for cleavage at glutamic or aspartic residues; other proteases (thermolysin, papain) give similar results (data not shown). The peptide maps of G-α and G-β are sufficiently different to indicate that the two polypeptide chains do not differ due to post-translational modifications and are unlikely to stem from a common polypeptide ancestor.

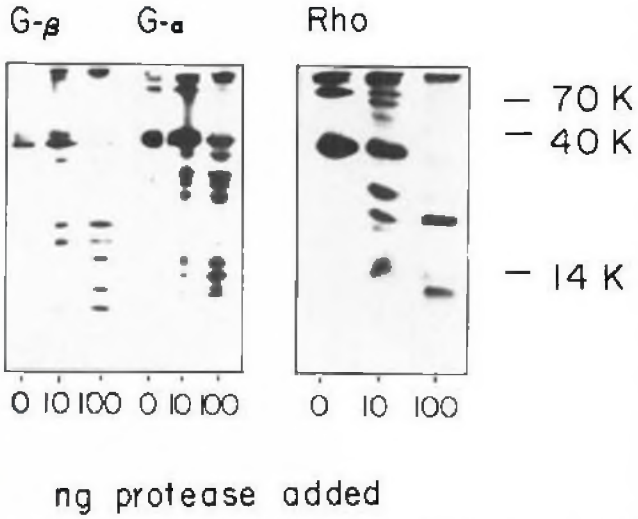


FIG. 12. **Limited proteolytic digestion of G-protein subunits and rhodopsin with *S. aureus* V8 protease.** Proteolytic digestion was performed as described under "Experimental Procedures" with the indicated amounts of *S. aureus* V8 protease. Separation of proteins was performed on 15% Laemmli gels.

*Isoelectric Points of Purified G-protein Subunits*—G-protein dissociates into its three respective subunits when subjected to electrophoresis in a pH 3–9 gradient in presence of 8 M urea according to the conditions of O'Farrell (37). This allows us to determine the characteristic isoelectric point of each subunit (data not shown). These values may be independently confirmed by analyzing the individual G-$\alpha$ and G-$\beta\gamma$ proteins purified as described under "Experimental Procedures" (Fig. 4). The isoelectric points are 5.3 (G-$\alpha$), 5.9 (G-$\beta$), and 5.2 (G-$\gamma$).

## DISCUSSION

Three of the macromolecules that function in the vertebrate photoreceptor cell to achieve light-activated cGMP hydrolysis are now well characterized, and include the membrane-embedded photoreceptor rhodopsin and the two peripheral membrane proteins G-protein and cGMP phosphodiesterase. These proteins are present in sufficient quantities in photoreceptor cells such that they can be isolated, characterized, and reconstituted to study their membrane organization. The isolation of G-protein and phosphodiesterase is made possible because these proteins interact with ROS disks at moderate ionic strength (0.1 M NaCl) strongly enough that they can be fractionated along with the membrane disks following disruption of the intact cell. Subsequently, they can be eluted from the membranes in a relatively pure form. This advantageous property was used for the isolation of phosphodiesterase (10), and we here extend and modify our procedure to include isolation of G-protein (see "Experimental Procedures," Figs. 1–5).

The association of G-protein and cGMP phosphodiesterase with fragmented ROS disks is demonstrated by analysis of protein subunits in various fractions throughout the isolation procedure for ROS membranes (Fig. 6). The results are consistent with earlier studies which demonstrated that cGMP phosphodiesterase activity (38) and GTP binding activity (17) cofractionate with ROS membranes. Protein synthesis occurs in the inner segment of the photoreceptor cell and proteins such as rhodopsin are transported to the outer segment (39–41). Labeling studies with [$^{35}$S]methionine indicate the subunits of G-protein and phosphodiesterase readily incorporate the radioactive marker in a period of 5 h. Since the newly synthesized subunits are associated with the rhodopsin-containing outer segment membranes, even after vigorous homogenization and cell fractionation, they must be synthesized at rates comparable to those for rhodopsin and cotransported (or transported in parallel) to outer segments. Comparative labeling studies carried out in the presence or absence of tunicamycin, an antibiotic which inhibits oligosaccharide addition to newly synthesized proteins (35, 42), suggest that none of the subunits of G-protein or phosphodiesterase are glycoproteins (Fig. 6, *track 9*). Rhodopsin is a glycoprotein with two oligosaccharide chains attached near the $NH_2$-terminal end (43, 44) and the action of tunicamycin is demonstrated by the dramatic change in opsin mobility on SDS gels compared to the native glycoprotein. The lack of oligosaccharide does not appear to affect the transport of rhodopsin to the outer segment. Since G-protein and phosphodiesterase are not glycoproteins, attachment of carbohydrate does not appear to be a signal which might help the cell sort and direct the transport of molecules to the outer segment.

The specificity of the G-protein and phosphodiesterase binding to ROS membranes is not well understood. Associations of both proteins with dark-adapted membranes are stabilized by moderate ionic strength (0.1 M NaCl and 5 mM $MgCl_2$) and disrupted by low ionic strength (10 mM Tris-HCl) (10, 24). Such behavior may be indicative of predominantly hydrophobic interactions which are stabilized at moderate ionic strength but a thermodynamic study of the binding is necessary to confirm this. The salt-stabilized association for phosphodiesterase is strong since there seems to be little release of either activity or subunits into the supernatants during washing of the membranes with isotonic buffer (Fig. 1). The interactions are disrupted in low salt and phosphodiesterase is eluted by hypotonic buffer; elution is similar for dark-adapted or bleached membranes (10, Fig. 1).

The behavior of G-protein remains more puzzling. There is minimal but continual "leakage" of the G-protein subunits into moderate ionic strength buffers used for washing membranes; the observation is difficult to quantitate by GTPase assay (by readdition to depleted membranes) because the specific activity is so low. Our preliminary observations and those of Godchaux and Zimmerman (17) indicate that physiological concentrations of approximately millimolar GTP in moderate ionic strength buffers enhances elution from dark-adapted membranes. It remains to be determined whether this is due to very low levels of bleached rhodopsin (1 part in 100) or an allosteric or secondary role of GTP. Once the rhodopsin is bleached, G-protein is more tightly bound, which supports the postulate that a complex is formed between activated rhodopsin and G-protein. Addition of GTP to washing buffers of moderate ionic strength is partially effective in release, but the most effective condition, which fully elutes G-protein from the bleached membranes, is washing with GTP in low ionic strength buffers (24, Fig. 1). Further controlled and quantitative studies are necessary to truly elucidate the nature of G-protein associations with ROS membranes.

GTP and GMP-PNP are equally effective in releasing G-protein from ROS, thus GTP hydrolysis is not necessary to achieve release (Fig. 7). Moreover, both nucleotides release all three subunits (G-$\alpha$, G-$\beta$, G-$\gamma$) simultaneously. One mole of GMP-PNP per mol of G-protein (assuming an 80,000 minimal molecular weight for G-$\alpha\beta\gamma$) is sufficient to achieve elution and a 1:1 molar complex may be isolated following elution with [$^3$H]GMP-PNP. A Scatchard analysis of binding data for GMP-PNP and G-protein indicates the association constant is $0.5 \times 10^7$ M$^{-1}$ ($K_d$, $2 \times 10^{-7}$ M) and the stoichiometry is 1 mol of GMP-PNP bound/mol of G-protein (Fig. 8). The dissociation constant of $2 \times 10^{-7}$ M for GMP-PNP is roughly

equivalent to the concentration of GTP that is necessary to maximally activate phosphodiesterase (0.3 μM) as measured by Yee and Liebman (3) and slightly higher than the concentration of GTP required for half-maximal activation of phosphodiesterase (0.07 μM) according to Wheeler and Bitensky (8). Godchaux and Zimmerman (17) have indicated there are two binding sites, one specific for GTP and one which accepts both GTP and GDP. If there is a second site, it is not exchangeable for GMP-PNP (or GTP) or the binding constant is $>10^4$ $M^{-1}$ as observed under our experimental conditions.

To examine the stoichiometry and properties of G-protein binding to membranes, we have reconstituted purified G-protein with depleted ROS membranes. Addition of G-protein to membranes containing light-activated rhodopsin restores GTPase activity. If a membrane-bound state, *e.g.* a G-protein-rhodopsin complex, were necessary for this activity, the dependence of steady state activity on G-protein concentration should be hyperbolic, saturating when the membrane site (rhodopsin) available for binding becomes limiting. Studies shown in Fig. 9*a* illustrate that no saturation of GTPase activity is observed even at concentrations of G-protein 7-fold greater than the rhodopsin present. Rough calculations (assuming G-protein to be a sphere of ~60Å diameter and 25,000 rhodopsins/$\mu m^2$ (45)) indicate that one G-protein per rhodopsin is the maximal number that could physically associate with the membrane surface as a single layer. Thus, there must be a constant exchange of free and bound G-protein molecules (or one of its subunits) on the surface of the membrane. These results emphasize that the interaction of G-protein with the membranes is transient at low or moderate ionic strength; moreover light-activated rhodopsin must play a catalytic role as was suggested by Godchaux and Zimmerman (17) and the rate-limiting step in GTP hydrolysis does not involve a complex of G-protein and rhodopsin. These same studies yield a turnover number for the GTPase activity of G-protein of 1 mol of GTP/mol of G-protein/min. This very low "activity" is comparable to that found for the G-protein which plays a similar role in the catecholamine stimulated-adenylate cyclase system of turkey erythrocyte membranes (19).

The actual stoichiometry of G-protein which binds to membranes containing light-activated rhodopsin at moderate ionic strength may be determined in the absence of GTP. Fig. 9*b* indicates a maximal value of 1 G-protein per 4 or 5 rhodopsins (0.13 ± 0.02 μM G-protein for 0.6 μM rhodopsin) can be reconstituted with depleted bleached membranes. Preliminary data indicate this ratio is also appropriate for dark-adapted membranes.[5] Membranes harvested from sucrose gradients have a maximal ratio of 1:10 (data not shown) and a value of 1:16 has been reported previously (17). The appropriate value for the intact cell is yet to be determined but a lower limit must be 1:10.

Throughout this paper we have used a minimal molecular weight of 80,000 for purified G-protein which corresponds to the sum of the three distinct, unrelated polypeptide chains. The purified G-protein may be further separated into G-$\alpha$ and G-$\beta\gamma$ subunits by preparative native gel electrophoresis (Fig. 4) or high pressure liquid chromatography (6). Sedimentation equilibrium data indicates that both G-$\alpha$ and G-$\beta\gamma$ (assuming $\beta\gamma$ to be a unit) behave as dimers, although G-$\beta\gamma$ tends to aggregate; moreover a mixture of G-$\alpha$ and G-$\beta\gamma$ does not reveal formation of a mixed higher order complex. Thus, we conclude that in solution G-protein exists as two proteins of approximately 80,000 $\bar{M}_r$ each. It is possible that these species were formed upon elution, but if so, the formation of homodimers is very specific and does not allow mixed aggregates. One can only speculate about the true molecular aggregation of G-$\alpha$ and G-$\beta\gamma$ on the membrane surface. G-protein may form oligomeric complexes of $Rho_m$ $(G\text{-}\alpha\beta\gamma)_n$ where rhodopsin serves as an anchor (46) and activator protein which dissociates the complex following the absorption of a photon. The existence of such oligomeric complexes between glucagon receptor and G-protein which regulates cyclase activity in the liver cell have been suggested to have molecular weights of up to $1.3 \times 10^6$ (47). The problem of determining the true molecular order of the species on the membrane surface is made difficult by the transient nature of the G-protein-membrane interaction and indicates a good deal of work lies before us before we can define the molecular nature of the functional complex.

[5] W. Baehr, unpublished data.

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SUPPLEMENTAL MATERIAL

to

Characterization of Bovine Rod Outer Segment G-protein

by

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## EXPERIMENTAL PROCEDURES

### General Methods

**Materials:** Bovine eyes were obtained within 30 minutes post mortem from a local slaughterhouse, transported on ice in a light-tight container, and dissected in the dark to obtain retinas. Cyclic nucleotides and nucleotide triphosphates were purchased from Sigma and Boehringer Mannheim. [$\gamma$-$^{32}$P]GTP was synthesized according to Glynn and Chappell (20) with the modification of Walsh et al. (21) to a specific activity of 1-2 Ci/mmole. [$\alpha$-$^{32}$P]cGMP-PNP was obtained from Amersham. Materials for gel electrophoresis were from Bio-Rad. Protease (S. aureus V8) was purchased from Miles and tunicamycin from Calbiochem-Behring.

**Buffers:** The buffers used throughout these procedures are defined as follows: Isotonic buffer (moderate ionic strength buffer): 10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01 mM PMSF; hypotonic buffer (low ionic strength buffer): 10 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01 mM PMSF; storage buffer: 50% glycerol, 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA; and column equilibration buffer: 20 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM dithiothreitol, 0.1 mM EDTA.

**Protein Assays:** Protein concentrations were determined with a Coomassie G250 assay using bovine serum albumin as a reference (22). The concentration of rhodopsin in membranes was determined from the absorbance of an aliquot solubilized in 1% Ammonyx-LO as described earlier (10).

**Phosphodiesterase Assay:** The hydrolysis of cGMP is followed by assay of 5'-GMP production as quantitated by the batch thin layer chromatography method of Zuzman (23). The final reaction mixture contains 40 mM Hepes pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 mM cGMP (radiolabelled with [8-$^{14}$C]guanosine 3',5'-cyclic monophosphate of specific activity 0.1 Ci/mol) and 1 to 10 $\mu$g/ml of protein. The reaction is started by addition of 5 $\mu$l of solution containing enzyme to 20 $\mu$l of assay mix which has been preincubated 1 min at 37°C. 8 $\mu$l aliquots are withdrawn to follow the time course and quenched by immersing the aliquot in a 95°C bath for 1 min. Small volumes are collected by spinning 5 sec in an Eppendorf centrifuge and 5 $\mu$l are spotted on a 0.5 x 2.5 $cm^2$ polyethyleneimine cellulose sheet. The sheets are washed in 12 mM LiCl three times for 10 min, dried and counted for cGMP production in a toluene-based scintillation mixture. Retention of cGMP is monitored in each experiment by spotting controls of 5 %l [8-$^{14}$C]guanosine 5'-monophosphate. One unit of activity is 1 nmol cGMP produced/min.

**GTPase Assay:** Activity is measured in a 100 $\mu$l reaction mixture of 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, depleted membranes containing 2-4 $\mu$g rhodopsin, 2.0-3.5 $\mu$M [$\gamma$-$^{32}$P]GTP and 1-10 $\mu$g G-protein at 30°C. Aliquots of 25 $\mu$l were taken at different times (3,6,9 min for low concentrations of G-protein, 30,60,90 sec for high concentrations) and quenched in 200 $\mu$l of an ice-cold suspension of Norit in 10% trichloroacetic acid containing 1 mM $NaH_2PO_4$. After 5 min at 0°C the slurry was centrifuged (15 min) in an Eppendorf 5412 centrifuge and an aliquot of the supernatant (212 $\mu$l) counted for Cerenkov radiation (17,24). The specific activity of [$\gamma$-$^{32}$P]GTP was determined the same way on the day of the experiment. Nanomoles of GTP hydrolysed/min were graphically extrapolated from the linear slopes of GTP hydrolyzed vs. time. Background activity of depleted membranes and background of inorganic phosphate (usually 2-5%) were subtracted for each point. One unit of activity represents 1 nmole $P_i$ released/min.

**[$^3$H]cGMP-PNP Binding Assay:** Purified G-protein (10 pmoles or 3 $\mu$g) and dark depleted membranes containing rhodopsin (200 pmoles or 8 $\mu$g) were incubated in 100 $\mu$l of buffer used for GTPase assays at 30°C for 5 min to hydrolyze any endogenous GTP. The assay mix was chilled to 0°C and 3.5 to 175 pmoles of [$^3$H]cGMP-PNP (11.4 Ci/mmol) added. After 10 min at 0°C the assay mix was diluted with assay buffer to 900 $\mu$l, filtered on nitrocellulose filter circles (Schleicher and Schuell BA85), and washed with 3 x 5 ml assay buffer. The filter circles were dried at 70°C for 5 min, agitated in 3 ml Aquasol overnight, and the radioactivity counted.

**Electrophoresis:** Polyacrylamide gel electrophoresis in the presence of 0.1% SDS (SDS-PAGE) and native PAGE were carried out as described earlier (10). SDS-PAGE was run in either 10% Laemmli gels (10% acrylamide, 0.27% bisacrylamide) (25) or in low crosslinked gels (15% acrylamide, 0.08% bisacrylamide) (10).

**Labelling of Retinal Proteins:** Retinal proteins were labelled in Dulbecco's modified Eagle medium (minus methionine, with L-glutamine added) as described by O'Brien (26). For each retina incubated in 5 ml of medium, 1 mCi of [$^{35}$S]methionine was added and the mixture was exposed to a gentle stream of $O_2/CO_2$ (95/5) for 5 h at 37°C in complete darkness. Special care was taken to excise a retina in one piece. Only fresh eyes were used; these were put on ice in a light-tight container about 5-10 min after slaughter of the animal and stored for no more than 6 h. For labelling in the presence of tunicamycin, 20 $\mu$g/ml was added to the incubation medium, and [$^{35}$S]methionine was added after 1 h of preincubation and oxygenation at 37°C. After the 5 h incubation period the retina was collected by centrifugation in a 1-20 rotor (5 min at 18,000 rpm), homogenized in 30% sucrose buffer (10) and ROS membranes isolated as described earlier with a preparation scaled down for 1 to 5 retina (10). The final sucrose step gradient was carried out in a 4.5 ml SW65 tube with 1 ml steps. The gradients were run at 20,000 rpm for 45 min at 4°C.

**Peptide Mapping:** Limited proteolysis of protein subunits was carried out according to Cleveland et al. (27) with the following modification: labelled polypeptides were resolved on a 0.8 mm SDS slab gel, stained 5 min, dried under low heat and autoradiographed. Radioactive bands were excised and rewollen in 0.1 M Tris pH 6.8, 1 mM dithiothreitol, 0.1% SDS, 1 mM EDTA, 30% glycerol for 30 min. Rewollen bands were loaded in a slot of 15% Laemmli gel (1.2 mm thick), overlaid with various amounts of protease, and electrophoresed. Electrophoresis was interrupted for 1 h in the stacking gel to allow proteolytic digestion to proceed. Gels were stained, destained and fluorographed (28).

**Analytical Ultracentrifugation:** Sedimentation equilibrium studies were carried out in a Model E ultracentrifuge (Beckman Instruments) equipped with a photoelectric scanner and multiplexer. One hundred microliter samples of protein (0.2 to 0.6 $A_{280\ nm}$) in isotonic buffer were centrifuged at 9,945 rpm for 18 h at 4°C. Molecular weights were calculated as described earlier (10) assuming 0.74 ml/g as partial specific volume.

### Isolation and Purification of cGMP Phosphodiesterase (PDE) and G-Protein

**Elution of Peripheral Proteins from ROS Membranes:** The following procedure is described for a preparation starting with 100 fresh, dark adapted retinas. Rod outer segment (ROS) fragments are isolated using the sucrose flotation method of Papermaster and Dreyer (29) as described earlier (10). The membranes are washed with 50 ml isotonic buffer and collected by centrifugation (18,000 rpm/30 min, Beckman 3-20 rotor) up to five times. This procedure removes a number of loosely bound polypeptides. As shown in Fig. 1, there is some loss of both G-$\alpha$ and G-$\alpha$ (Fig. 1, supernatants 1.1-1.5).

In a modification of the procedure of Kühn (24,30), the membranes are then bleached in isotonic buffer at 0°C. The SDS-PAGE analysis of supernatant 1.6 demonstrates that G-$\alpha$ is no longer released whereas small amounts of G-$\alpha$ continue to elute, perhaps indicating a weaker binding of G-$\beta$. Subsequent extractions with 50 ml aliquots of hypotonic buffer are performed in normal room light at 4°C illuminating the membranes for at least 5 min after each resuspension and collecting them by centrifugation (30,000 rpm/30 min, Beckman Ti40 rotor). This treatment released PDE as the predominant protein (Fig. 1, supernatants 2.1-2.3) and only traces of G-protein subunits (with G-$\beta$ in excess of G-$\alpha$). The three G-protein subunits are then extracted by adding GTP to a final concentration of 40 $\mu$M (Fig. 1, supernatant 3.1).

The membranes are collected as just described and the extraction is repeated twice. Due to the presence of 40 $\mu$M GTP and low protein concentrations, GTPase or GTP binding activity cannot directly be assayed.

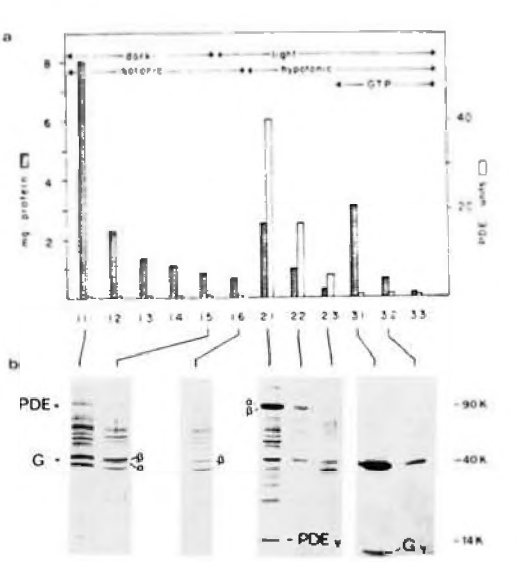


Fig. 1. (a) Elution of ROS membrane proteins with isotonic buffer (1.1-1.5), hypotonic buffer (2.1-2.3), and hypotonic buffer + 40 $\mu$M GTP (3.1-3.3). Matched columns: total protein eluted from membrane fragments of 100 retinas. Protein assayed by Coomassie G250 with bovine serum albumin as reference (22). Light columns: PDE activity. (b) Analysis of the supernatants by SDS-PAGE.

**Isolation of Phosphodiesterase by DE52/A-100 Column Chromatography:** A narrow layer of DE52 cellulose placed on top of a A-100 column serves to bind and concentrate PDE. Proteins not binding to DE52 are washed off, PDE is then eluted with 0.5 M NaCl in equilibration buffer, and subsequently purified by gel filtration (Fig. 2). Pressurized Sephadex G-100 is equilibrated in a 1.6 cm x 42 cm column (bed volume 84 ml) using a hydrostatic pressure of 50 cm. The column is overlaid with 1 cm pressurized DE52 (bed volume 2 ml) and 150 ml hypotonic supernatants containing 3-4 mg protein are applied (2.1-2.3 in Fig. 1). The column is washed extensively with equilibration buffer (5 column volumes) and PDE eluted with a step gradient of 0.5 M NaCl in equilibration buffer. PDE ($M_r$ 170,000) elutes in the void volume whereas low molecular weight proteins are retarded. Fractions (2 ml) are assayed for activity and analyzed by SDS-PAGE. Peak fractions are pooled, concentrated to ~2 ml, dialyzed against 50% glycerol buffer, and stored at -20°C. Yield of purified PDE is 0.8-1.0 mg/100 retinas. The specific activity varies from 8-12 units/mg.

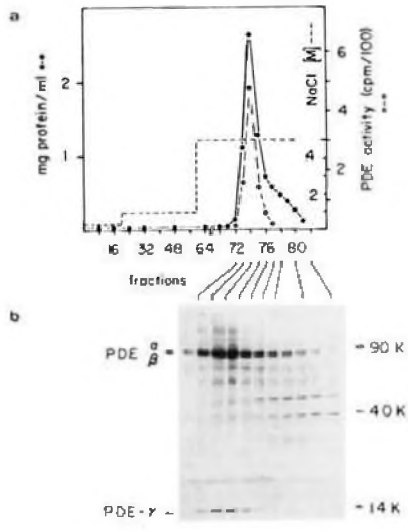


Fig. 2. (a) Chromatography of combined hypotonic supernatants on a DE52/A-100 column. Protein is assayed as in Fig. 1. (b) Peak fraction analyzed by SDS-PAGE.

**Isolation of G-protein:** DE52 column chromatography concentrates G-protein and removes excess, non-bound guanine nucleotides. Combined GTP extracts of bleached ROS (supernatants 1.1-1.3 in Fig. 1) containing 1-3 mg of protein are applied to a 3-4 ml equilibrated DE52 cellulose column (Fig. 3). After application, the column is washed with equilibration buffer until no more GTP is eluted. The G-protein subunits are then eluted with 0.5 M NaCl in equilibration buffer (1 ml fractions) and assayed for GTPase activity. Fractions with peak activity are pooled, concentrated, dialyzed against storage buffer, and kept at -20°C. This pool is referred to as purified G-protein. The final yield is ~1 mg G-protein/100 retinas.

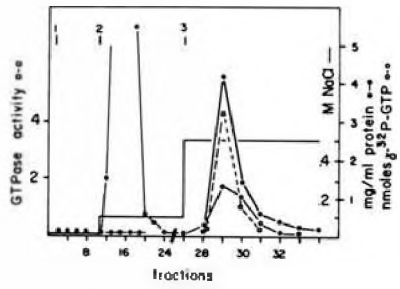


Fig. 3. DE52 column chromatography of G-protein extracted with 40 nM [$\gamma$-$^{32}$P]GTP. 1. Combined supernatants 3.1-3.3 (Fig. 1) loaded. 2. Equilibration buffer containing 0.1 M NaCl applied for washing. 3. G-protein eluted with equilibration buffer containing 0.5 M NaCl. Protein assayed as in Fig. 1. Analysis of peak fraction shows that 84% of the stoichiometric amount of GTP initially bound (1 mole GTP/ mole G-protein) is hydrolyzed. G-protein is defined as having a molecular weight of 80,000.

Separation of G-protein into G-$\alpha$ and G-$\beta\gamma$ by Preparative Native Gel Electrophoresis: G-protein subunits may be separated by a preparative native gel (11) which allows continuous elution of proteins during electrophoresis. An 11 x 13 x 0.3 $cm^3$ slab gel is polymerized over a 1 cm layer of gel buffer containing 30% glycerol. Hypodermic needles (22 gauge) are inserted into this 1 cm elution slot through each of the spacers. One side is connected to an elution buffer (50 mM Tris-glycine pH 9.5, 0.1 mM dithiothreitol) reservoir and the other to a fraction collector allowing one to fractionate the electrophoresing proteins. The electrophoresis is carried out at 4°C and the contents of the upper reservoir are continuously circulated through an external 2 liter container of electrode buffer.

A trace of both Bromophenol Blue and Xylene Cyanole FF are added as tracking dyes to 500 µg of G-protein in storage buffer. The sample is pipetted into a 10 cm wide loading slot and electrophoresis is allowed to proceed at 35 - 50 $V/cm^2$. G-$\alpha$ comigrates with Xylene Cyanole FF, a dye which can be removed by dialysis. The elution buffer is pumped through the elution slot at a flow rate of 1 ml/min and proteins are collected in 5 ml fractions for analysis by SDS-PAGE (Fig. 4). As shown in Fig. 4, there is minimal overlap of G-$\alpha$ and G-$\beta\gamma$. Non-overlapping fractions are pooled, concentrated and dialyzed against storage buffer.

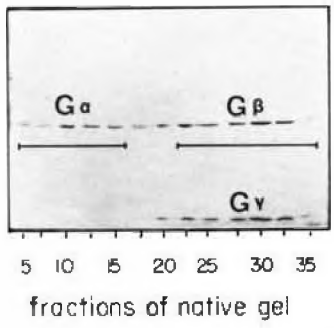


Fig. 4. SDS-PAGE analysis of fractions containing G-$\alpha$ and G-$\beta\gamma$ separated by native gel electrophoresis. The bars mark the fractions pooled.

Characterization of G-protein and PDE by SDS-PAGE: A comparison of the mobilities of the subunits of G-protein and PDE in conventional Laemmli gels (25) and low crosslinked gels (10) is shown in Fig. 5. Low crosslinked gels were used because they resolved (1) the $\alpha$ and $\beta$ subunits of PDE, (2) the $\gamma$ subunits of PDE and G from the front, and (3) G-$\beta$ and opsin, which comigrate on conventional gels. Note that in low crosslinked gels, the mobilities of G-$\alpha$ and G-$\beta$ are reversed.

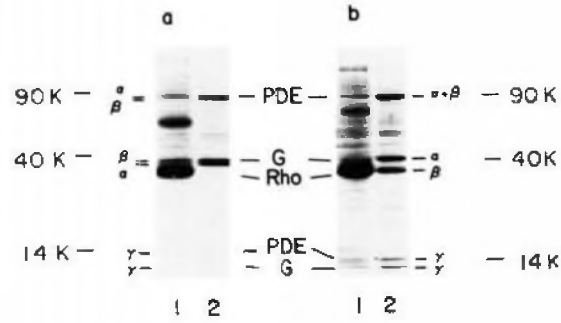


Fig.5. Relative mobilities of PDE and G-protein subunits on low crosslinked gels (a) and conventional gels (b). 1, ROS membranes corresponding to track 3 in Fig. 3. 2, hypotonic supernatants from dark adapted membranes (10).

Reconstitution of G-protein with Depleted Membranes

Preparation of Dark Depleted Membranes: Dark depleted ROS membranes were prepared as described earlier (10) by washing ROS membranes with hypotonic buffer and hypotonic buffer containing 1 mM EDTA until no significant amount of protein eluted. G-protein and PDE elute together in the dark without the addition of GTP. Depleted membranes were stored at 2-4 mg rhodopsin/ml in storage buffer at -20°C. Membranes negatively stained with uranyl acetate appear to be disks of 0.8 ± .2 µm as observed by electron microscopy. Storage in glycerol does not change their appearance or size distribution.

Reconstitution of Membrane Bound G-protein: Depleted ROS membranes (stored dark and unbleached at -20°C until used) and purified G-protein at the concentrations indicated in Fig. 9 were incubated in 200 µl of 100 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA for 1 min at 30°C and 10 min at 0°C. The reconstitution mixtures were then centrifuged at 4°C in an Eppendorf centrifuge (15 min). The pellet was resuspended in 400 µl isotonic buffer, centrifuged, and the washed pellet resuspended in the original assay volume (200 µl). For analysis of bound G-protein, this membrane suspension was subdivided into two 100 µl aliquots. One part was assayed for GTPase activity, the other one was analyzed densitometrically after separation by SDS-PAGE. To assess quantitative binding of G-protein to the membranes under the conditions used above, we extended the incubation times at 0°C from 10 min to 1 h and 4 h. Such preliminary experiments confirmed that better than 95% saturation was reached under the conditions used above.