

Purification, Reconstitution on Lipid Vesicles, and Assays of PDE6 and Its Activator G Protein, Transducin

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Summary

PDE6 in rod and cone photoreceptors is the principal effector of phototransduction. It is kept at a very low activity level in the dark, and in the light it is strongly activated by the guanosine 5'-triphosphate-bound form of the α -subunit of the G protein, transducin. Both transducin and PDE6 are peripheral proteins, and understanding both their interactions with one another and the roles of lipids in their function requires reconstituting purified proteins on the surfaces of defined lipid bilayers. We describe here methods for purifying the proteins, reconstituting them with vesicles, and assaying catalytic activity and binding.

Key Words

G proteins; phosphodiesterase; membrane reconstitution; cyclic nucleotides; phototransduction; retina; vision.

1. Introduction

The guanosine 3',5'-cyclic-monophosphate (cGMP) phosphodiesterase (PDE) of vertebrate vision, PDE6, is a heterotetrameric membrane protein found on disk membranes of rod and cone photoreceptors. It is regulated by binding of a G protein, transducin (G_{out}) in its guanosine 5'-triphosphate (GTP) form. Formation of this activated GTP-bound form of G_{out} is the pivotal reaction catalyzed by rhodopsin, the primary photon receptor, after its photoconversion to metarhodopsin II (R^*). Although the transducin-PDE6 couple seems to be unique to photoreceptor cells, there are many features that this complex shares with other G protein-effector systems, so it can serve as a model for this very common type of signal transduction mechanism.

Both transducin and PDE6 are peripheral membrane proteins with covalently attached lipids (*I*). G_{out} has one of four fatty acids (C12:0, C14:0, C14:1, C14:2)

attached to its N-terminal glycine in an amide linkage (2–4). The initial translation products of the catalytic subunits of PDE6 in rods, PDE6 α and PDE6 β , have CAAX boxes at their carboxyl termini. These are substrates for cellular enzymes that remove the last three amino acids by proteolysis, attach either a 15-carbon farnesyl (PDE6 α) or a 20-carbon geranylgeranyl (PDE6 β) isoprenyl group to the cysteine via a thioether linkage (5), and convert the C-terminal carboxyl group into a methyl ester. These hydrophobic modifications are thought to help keep the proteins tethered to the disk membranes on which phototransduction occurs.

The lipid surface on which PDE6 activation occurs is very important for the interaction with G_{out} (6,7). Although some activation of PDE6 by transducin can be observed in solution, it is very weak compared with what is observed on membranes, requiring a large molar excess of activated G_{out} in order for substantial PDE6 activation to be observed. By contrast, on membranes under optimal conditions, a nearly stoichiometric binding of G_{out} with PDE6 can be observed when both are present at nanomolar concentrations. The lipid head group has a strong influence on the strength of the activation, and the side chains of the phospholipids may play a role as well.

To probe the interactions of G_{out} with PDE6 without interference from endogenous membrane proteins in rod outer segment (ROS) disk membranes, and to define the effects of lipid composition on the protein–protein interactions, it is highly useful to purify the proteins and reconstitute them on the surface of lipid vesicles with well-defined composition and structure. We provide here methods for preparing G_{out} and PDE6 in highly purified form; for preparing and characterizing well-defined large unilamellar vesicles; for reconstituting G_{out} in, and PDE6 on, their surface; and for assaying protein binding to, and enzymatic activity on, the vesicle surfaces.

2. Materials

2.1. Biological Materials

2.1.1. Bovine ROSs

ROSs are conveniently purified from commercially available frozen dark-dissected cattle retina. Papermaster and Dreyer (8,9) provide an excellent protocol for preparing them.

2.1.2. Lipids

Phospholipids and other membrane lipids of high quality are available in both powder and solution (chloroform or some other organic solvent) from Avanti Polar Lipids. Both synthetic and natural lipids can be used in the reconstitution procedures described here. Other suppliers of biochemicals also have

phospholipid (e.g., Sigma, St. Louis, MO), including some not available from Avanti. Lipids should be stored at -80°C under argon.

2.1.3. Nucleotides

1. cGMP (Sigma G-6129; sodium salt, molecular weight = 367.2 g/mol).
 - a. To prepare a 100 mM cGMP solution, dissolve the solid cGMP in Milli-Q water with vortexing. Adjust the pH to 8.0 with 0.1 M NaOH.
 - b. Filter through a 0.22- μm syringe nitrocellulose filter.
 - c. Use absorbance at 254 nm to determine the concentration of cGMP. Measure the absorbance at 254 nm in a quartz cuvet and use Beer's law to determine the concentration of cGMP: $\text{GMP } \epsilon_{254} = 12,950 \text{ M}^{-1} \text{ cm}^{-1}$.
 - d. Make an aliquot (20–50 μL) of cGMP and store at -20°C .
2. 100 mM GTP (Sigma G5884; lithium salt, formular weight [FW] = 523.2 g/mol) or GTP γS (Sigma G8634 tetralithium salt, FW = 563 g/mol) stock solution to extract transducin from ROSSs. Dissolve GTP or GTP γS in Milli-Q water, adjust the pH with NaOH to 7.2 (*Caution:* if the pH has not been adjusted in the solution of GTP, it will denature or inactivate transducin owing to low pH during the GTP/low-salt wash steps), filter through a 0.2- μm nitrocellulose filter, and then determine GTP concentration by spectrophotometry using an extinction coefficient of 13,700 $\text{M}^{-1} \text{ cm}^{-1}$.
3. Guanosine 5'-diphosphate (GDP) (Sigma G7127; sodium salt, FW = 443.2 g/mol) stock: This is made up in the same way as GTP.

2.1.4. Proteins

1. Trypsin (Sigma T1426; TPCK treated, Type XII): Prepare small aliquots of 10 mg/mL of trypsin with filtered Milli-Q water. Store the aliquots at -20°C .
2. Soybean trypsin inhibitor (Sigma T9003; Type I-S from soybean): Prepare small aliquots of 50 mg/mL of soybean trypsin inhibitor with filtered Milli-Q water. Its molecular weight is about the same as that of trypsin, so 10X by mass excess over trypsin mass is nearly a 10X molar ratio also. Store the aliquots at -20°C .

2.2. Buffers

2.2.1. General Considerations for All Buffers

All buffers are filtered through filters with 0.2- μm pores. The filters must be made of nitrocellulose to maximize protein binding. All buffers (except pH assay buffer) should contain, in addition to the ingredients detailed in **Subheadings 2.2.2.–2.2.8.**, the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT). DTT is normally added from a 1 M stock just before filtering and using, and PMSF is added in solid form after filtering (except for high-performance liquid chromatography [HPLC] buffers, in which case the buffer with added solid PMSF is allowed to stir for 30 min just before filtration and use). PMSF is poorly soluble in water and hydrolyzes

fairly rapidly; a few suspended crystals of PMSF thus serve as a reservoir of intact PMSF, slowly releasing it into solution as the pool in the solution hydrolyzes. Unless otherwise indicated, buffers are stored and used at 4°C. Buffers used for chromatography are degassed under vacuum, and this is especially important if buffers are used at a higher temperature than the one at which they were stored. Vacuum filtration through 0.2- μ m filters is a very efficient degassing method.

2.2.2. Buffers for Extraction of Proteins From ROSs

1. Moderate-salt buffer: 10 mM 3-(N-Morpholino)propane sulfonic acid 4-Morpholino propane sulfonic acid (MOPS), pH 7.4, 30 mM NaCl, 60 mM KCl, 2 mM MgCl₂. Prepare 1.5 L of moderate-salt buffer for three washes (65 mL for each Ti45 ultracentrifuge bottle, six bottles for each spin, and a total of three washes). Another 1 L is needed the day of transducin extraction.
2. Low-salt buffer: 5 mM Tris-HCl, pH 7.2, 0.5 mM MgCl₂. Prepare 1 L for two washes of PDE on the day it is extracted, and an additional 1.5 L for low-salt wash and two low-salt/GTP transducin extractions for the day transducin is extracted.

2.2.3. Phosphate Buffers for Hydroxylapatite Chromatography

1. 1 L of 30 mM sodium phosphate buffer, pH 7.2, 50 mM NaCl.
2. 500 mL of 80 mM sodium phosphate buffer, pH 7.2.
3. 200 mL of 150 mM of sodium phosphate buffer, pH 7.2.
4. 200 mL of 300 mM of sodium phosphate buffer, pH 7.2.

2.2.4. Buffers for DEAE HPLC Chromatography of PDE6

Prepare 500 mL each of buffer A and buffer B:

1. Buffer A: 20 mM Tris-HCl, pH 7.4.
2. Buffer B: 20 mM Tris-HCl, pH 7.4, 1 M NaCl.

2.2.5. Buffer for HPLC Gel Filtration of PDE6

500 mL of 30 mM sodium phosphate buffer, pH 7.2, 300 mM NaCl.

2.2.6. Buffers for Transducin Chromatography on Hexylagarose

1. 1 L of buffer A₀: 5 mM Tris-HCl, pH 7.4, 0.5 mM MgCl₂.
2. 1 L of buffer B₀: 10 mM MOPS, pH 7.4, 2 mM MgCl₂.
3. 500 mL of buffer B₇₅: 10 mM MOPS, pH 7.4, 2 mM MgCl₂, 75 mM KCl.
4. 500 mL of buffer B₃₀₀: 10 mM MOPS, pH 7.4, 2 mM MgCl₂, 300 mM KCl.
5. 500 mL of buffer B₅₀₀: 10 mM MOPS, pH 7.4, 2 mM MgCl₂, 500 mM KCl.

2.2.7. Buffers for Separation of Transducin Subunits on Blue Sepharose

1. 1 L of buffer KCl₀: 10 mM MOPS, pH 7.4, 5 mM MgCl₂, 0.1 mM EDTA.
2. 500 mL of buffer KCl₁₀₀: 10 mM MOPS, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA.

3. 500 mL of buffer KCl₇₅₀: 10 mM MOPS, pH 7.4, 750 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA.

2.2.8. Buffers for PDE Activity Assays and for Preparation and Reconstitution of Vesicles

Two different buffer systems are used, depending on the total PDE activity and concentration of cGMP in the experiments. DTT and PMSF are omitted from buffers to be used only in assays. They should be included, as for other buffers (*see Subheading 2.2.*), when used for preparative purposes, such as the preparation of vesicles. In addition, for the preparation of sucrose-loaded vesicles, the buffer should be supplemented with 170 mM sucrose during the vesicle freeze-thaw and extrusion cycles.

1. MOPS buffer: This is routinely used for pH assay. There is not much buffering capacity at pH 8.0 because it is far from its pK_a (MOPS buffer $pK_a = 7.2$), so the assay is very sensitive to pH changes owing to cGMP hydrolysis. 5X pH assay MOPS buffer (40 mL): 100 mM MOPS, pH 8.0, 750 mM KCl, 10 mM MgCl₂. Filter with a 0.2 μM nitrocellulose membrane, and then aliquot to 1 mL/tube and store at -20°C .
2. Tris-HCl buffer: This is used only for high PDE activity or high concentrations of cGMP. Tris $pK_a = 8.1$. When less buffering capacity is needed, a lower Tris concentration buffer is used. We use 10 mM Tris-HCl (final; 5X is 50 mM) for PDE activity $<25 \mu\text{M}\cdot\text{s}^{-1}$ and 25 mM Tris-HCl (final, 5X is 125 mM) for PDE activity in the range of 25–160 $\mu\text{M}\cdot\text{s}^{-1}$.

2.3. Chromatography and Filtration Media

1. Nitrocellulose filters: Syringe filters, 0.2 μm nitrocellulose (other materials should not be used); 45-mm, 0.2- μm , or 0.45- μm nitrocellulose filters and vacuum filtering apparatus for filtering large buffer volumes.
2. Hydroxylapatite (HAP): HAP Bio-Gel HTP Gel (cat. no. 130-0420, Bio-Rad, Hercules, CA). Each milliliter of packed HAP will bind about 5 mg of total protein, so we routinely use a 30- to 40-mL packed HAP column for 300–450 retinas' worth (two to three preparations of ROSs) of extracts. To prepare the column, add 1 part of HAP powder to 6 parts of phosphate buffer (30 mM sodium phosphate buffer, pH 7.2), swirl gently, then allow to settle for 5–10 min. Decant the supernatant containing fines. Repeat at least twice until most of the fines are removed. Degas and then pour into a 2.5 cm i.d. \times 10 cm column.
3. DEAE HPLC column: The Protein-Pak™ DEAE-5PW is an anion (diethylaminoethyl)-exchange column from Waters (cat. no. WAT088044). The recommended flow rate is 0.5–1 mL/min (*do not exceed 1.2 mL/min*), and the maximum backpressure is 500 psi.
 - a. Do not exceed 20% organic content in the mobile phase.
 - b. Do not use sodium azide, sodium dodecyl sulfate (SDS), or any anionic detergents in the mobile phase.

- c. Do not change the flow rate faster than increments of 0.5 mL/min.
 - d. Do not freeze the column.
 - e. Other ion-exchange columns also work for PDE purification, including Mono Q, AP, or QMA.
4. HPLC gel filtration column. Bio-Sil[®] SEC 250-5 is a size-exclusion column from Bio-Rad. The bed volume of this column is 14 mL, so the maximum sample volume is 0.7 mL (1–5% of bed volume is recommended). The operating flow rate is 1 mL/min and maximum pressure is 1500 psi. One can also use other compatible size-exclusion columns if available.
5. Gel filtration standards: Bio-Rad gel filtration standard (cat. no. 151-1901):
- a. Thyroglobulin (5 mg, 670 kDa).
 - b. Bovine gamma globulin (5 mg, 158 kDa).
 - c. Chicken ovalbumin (5 mg, 44 kDa).
 - d. Equine myoglobin (2.5 mg, 17 kDa).
 - e. Vitamin B12 (0.5 mg, 1.35 kDa).
- Add 0.5 mL of Milli-Q water to the lyophilized standard, swirl gently to mix, and allow the vial to stand for 2 to 3 min, the final 36-mg components/mL. Centrifuge the standard solution at 16,000g for 15 min at 20°C before application to remove any fine particulates.
6. Hexylagarose (cat. no. H-1882; Sigma): This comes as a suspension in 500 mM NaCl. The capacity of the packed hexylagarose is about 10–12 mg/mL of total proteins based on binding of bovine serum albumin (BSA), so we routinely use a 10- to 15-mL column for two preparations of ROSs.
- a. Degas, pour, and equilibrate the column. The flow rate for this column usually is 0.5 to 0.6 mL/min.
 - b. Hexylagarose contains 0.5 M NaCl; ten column volumes of buffer A₀ is necessary to completely equilibrate the column in zero NaCl.
7. Reactive Blue 2 Sepharose CL-6B (Sigma): Each gram of resin can make 5 mL of the column. We routinely use 20–30 mL of column to isolate Gt-subunits from the Gt extract of two ROS preparations. At room temperature, swell the resin in buffer KCl₀, decant the fines, degas the media under vacuum, and pour the column. Then transfer the column to a cold room and equilibrate the column with 5 column vol of buffer KCl₀. If a used column is available, regenerate the column with 10 column vol of 10 mM MOPS (pH 7.4), 2 M KCl or with 6 M urea (in most cases, using 2 M KCl in MOPS buffer is sufficient); then equilibrate the column with 10 column vol of buffer KCl₀.
8. Polycarbonate filters for vesicle extrusion and tubes for freeze-thaw:
- a. Poretics[®] polycarbonate filters, 0.1 μm (cat. no. K01CP02500, Osmonics).
 - b. Corex[®] centrifuge tubes, for drying, hydrating, freezing, and thawing lipids.

2.4. Assay Reagents

2.4.1. Phosphate Assay Reagents

1. 0.1 N H₂SO₄: concentrated.
2. 70% HClO₄, as supplied.

3. Ammonium molybdate ($[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]$) (Sigma A1343, tetrahydrate, FW = 1235), 2.5% (w/v) solution.
4. Ascorbic acid (Sigma A0278), 10% (w/v) free acid form. This solution should be less than 7-wk old, stored at 4°C, and nearly colorless.
5. Phosphate standard (Sigma 6661-9).

2.4.2. Protein Assay Reagent

This is used for the Coomassie blue binding assay of Bradford (**10**). The reagent is available premixed with instructions from Pierce or Bio-Rad.

2.5. Apparatus

2.5.1. Spectrophotometer

An ultraviolet (UV)/visible spectrophotometer is needed for determining phosphate, nucleotide, and protein concentrations. Use plastic cuvetts for protein Bradford assays and phosphate assays, and quartz cuvetts for measuring the absorbance of nucleotide solutions.

2.5.2. Ultracentrifuge, Rotor, and Bottles

1. Beckman L7-55 ultracentrifuge (or equivalent).
2. Type 45-Ti rotor and bottles.
3. TL-100 or equivalent tabletop ultracentrifuge.
4. TLA-100.3 fixed-angle ultracentrifuge rotor or equivalent.
5. Tubes for TLA-100.3, both polycarbonate tubes and microcentrifuge-style polyallomer tubes with adapters.

2.5.3. Syringes and Pipetors

1. Standard air displacement adjustable volume micropipetors.
2. Glass/stainless steel/Teflon microsyringes (e.g., Hamilton). Standard biological air-displacement pipets (e.g., Pipetman, Eppendorf) can be used for aqueous solutions but must not be used for organic solvents. The solvent degrades the plastic materials and the high vapor pressure interferes with accurate volume measurement.
3. Positive displacement micropipetors or glass microcapillary pipets for strong acids; these can also be used for organic solvents.

2.5.4. Mixers and Homogenizers

1. Potter-Elvehjem (glass vessel, round-end Teflon pestle) homogenizer, for washing ROS membranes during extraction of PDE and transducin.
2. Glass rod with rubber policeman on the end, for resuspending ROS membranes.

2.5.5. Chromatography and Electrophoresis

1. Standard setup for low-pressure chromatography: peristaltic pump, tubing, fraction collector. A UV detector is useful but not essential.

2. HPLC or fast protein liquid chromatography (FPLC) system: Either standard stainless-steel systems, or glass-Teflon, or titanium-based systems can be used. To avoid heavy metal contamination do not use porous stainless steel “sinkers” on the intake ends of the solvent supply tubes in the reservoirs. For standard HPLC systems, use seals designed for use with aqueous salt solutions, and thoroughly clean out all parts of the system before and after use with HPLC-grade water to avoid contact of salt solutions with organic phases.
3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE): A standard setup for protein electrophoresis, either “minigel” or standard size, is essential for checking the purity of protein fractions and assaying protein binding to vesicles.

2.5.6. Vesicle Extrusion Device

Nitrogen pressure extruder supplied by Lipex Biomembranes: This requires an N₂ tank and a high-pressure regulator. See the manufacturer’s instructions for details of high-pressure connections.

2.5.7. pH Assay Assembly

1. Microtiter plate (e.g., Dynatech 001-012-9205; order from PGC 05-6124-24).
2. Magnetic stir plate: If the plate is warm to the touch, a thermostated plate must be placed between the stir plate and the microtiter plate to ensure constant temperature. We use one scavenged from an old spectrophotometer with tubes for water inlet and outlet connected to a circulating water bath.
3. Flea-sized magnetic stirring bars (cat. no. 37119-0005, Bel-Art products).
4. Microelectrode: MI410 Combination pH electrode (provided by Microelectrodes).
 - a. When ordering the electrode, make sure the connectors match those on the pH meter, e.g., BNC.
 - b. Check the electrode before the experiment.
 - c. Remove the sleeve from the electrode fill hole; check that the outer reference chamber is full to just below the fill hole. If it is not at the proper level, it must be refilled with 3 M KCl/saturated AgCl solution (provided by Microelectrodes).
 - d. Calibrate the electrode with pH 7.0 and 10.0 standards prior to the experiment.
5. pH meter with voltage output.
6. Chart recorder and/or analog-to-digital converter in PC.

3. Methods

3.1. Extraction of PDE and Transducin From Bovine ROSs

3.1.1. General Strategy

Bleached ROSs are sequentially washed with buffers varying in ionic strength to produce extracts that are highly enriched in either PDE6 or transducin (*II*). This protocol is written for ROSs prepared from 300 to 450 (two to three preparations of ROSs) retinas, but it is easily scaled up or down.

1. Wash three times with moderate-salt buffer to remove the soluble and not tightly bound proteins.
2. Wash two times with low-salt buffer to extract PDE from the ROS membrane.
3. Freeze the membranes overnight (or longer) while the PDE extract is processed (alternatively, if enough personnel are available, one person can extract the transducin while another processes the PDE).
4. Wash two additional times with moderate-salt and once with low-salt buffer to remove proteins released by the freeze-thaw cycle (can be skipped if transducin is extracted immediately without freezing the membranes).
5. Wash two times with low-salt buffer containing GTP or GTP γ S to extract transducin from the ROS membrane.

The total time from thawing the ROSs to finishing the second GTP/low-salt wash is about 7–9 h if both PDE and transducin are extracted in 1 d, and it is important to process the PDE immediately. Therefore, in general, if it is not necessary to extract both in one day, the pellet after the second low-salt wash is frozen at -80°C , and when transducin extract is needed, the low-salt-washed pellets must be washed twice with moderate-salt buffer and once with low-salt buffer before the GTP/low-salt buffer is used to extract transducin, because contaminating proteins from ROSs are released from the freeze/thaw step.

3.1.2. General Cautions

PDE6 is exceptionally sensitive to proteolysis. Therefore, it is very important that any surface that will touch the protein-containing extract be kept clean, cold, and covered. Wear gloves all the time during the preparation to protect the extracts from personal proteases. Filter all the buffers with a $0.2\text{-}\mu\text{m}$ nitrocellulose membrane. Transducin is extremely sensitive to oxidation; DTT or other reducing reagents are important for extraction and purification of transducin.

3.1.3. Selective Extractions of Proteins From ROSs

1. Thaw and bleach the ROSs on ice (remove aluminum foil in room light). Occasional inverting to mix the ROSs will ensure that all of the rhodopsin is activated to R* and help the ROSs thaw quickly.
2. While the ROSs are thawing, turn on the ultracentrifuge and the vacuum, and prechill all the Ti45 bottles, homogenizer, and a 500-mL beaker on ice.
3. Carry out the first isotonic wash (moderate-salt wash) as follows:
 - a. Combine all ROSs (two to three preparations) in a 500-mL beaker, and dilute the ROSs to a final volume of about 390 mL with moderate-salt buffer; add solid PMSF to the diluted ROSs, and mix with a rubber policeman.
 - b. Add about 60 mL of diluted ROSs to the homogenizer each time; homogenize the ROSs with a Teflon pestle until no macroscopic particles are visible. Because proteins denature at air-water interfaces, avoid foaming and minimize the number of times the pestle is pulled completely out of the solution during

- homogenization. Keep the homogenizer surrounded by ice, but do not let any ice contaminate the sample.
- c. Transfer the homogenized ROSs to six Ti45 bottles. Make sure the Ti45 bottles are well balanced. Clean the outside of the bottles with a paper towel, and then load into a Ti45 rotor. The Ti45 bottles must be filled to the lip (point of narrowing). If partially full Ti45 bottles are spun, there is a high probability that they will collapse.
 - d. Centrifuge with the Ti45 rotor at 150,000g for 20 min at 4°C. Watch the speed of the rotor until it comes up to 150,000g before leaving the centrifuge.
 - e. After centrifugation, remove the supernatant from the Ti45 bottles with a plastic tube (iv tubing) attached to a 60-mL syringe. Pool and store the supernatants from each spin on ice until the assays are complete to make sure they do not contain PDE or transducin by SDS-PAGE or pH assay. Then discard the supernatant.
4. Carry out the second isotonic wash as follows:
 - a. Add moderate-salt buffer to the pellet (final volume of 65 mL/tube), use the rubber policeman to resuspend the pellet, and then place in the homogenizer.
 - b. Homogenize as for the first wash.
 - c. Spin again in the Ti45 bottles at 150,000g for 20 min at 4°C.
 - d. Save all the supernatants and keep at 4°C until the assays are completed.
 5. For the third isotonic wash, repeat **steps 4a–d**.
 6. Carry out the first hypotonic wash, to extract PDE from the ROSs, as follows:
 - a. Add low-salt buffer to each pellet in a final volume up to 65 mL, use the rubber policeman to resuspend the pellet, and then put into the homogenizer.
 - b. Homogenize as in **step 3b**.
 - c. Spin again in the Ti45 bottles at 150,000g for 40 min at 4°C, because pelleting is much less efficient in low salt. Take care when removing the supernatant to avoid contamination with the pellet.
 - d. Pool the first hypotonic wash containing 75–90% of total PDE in a 500-mL bottle, and add fresh DTT (2 mM) and solid PMSF to the extract.
 - e. Measure the PDE activity in the two isotonic washes (*see Subheading 3.7.2.*) and the first hypotonic wash. Determine the amount of PDE in the hypotonic wash (should be about 1 to 2 mg/300 retinas), and start loading it to the HAP column.
 7. Carry out the second hypotonic wash as follows:
 - a. Repeat **steps 6a–e**.
 - b. Pool the second wash in another 500-mL bottle, and add solid PMSF and fresh DTT to the PDE extract. Because the second one only contains 10–25% of total PDE, it is not recommended that the first and second washes be combined until one checks the PDE activity or runs a gel.
 - c. PDE extract can be further concentrated for use as a crude extract or for chromatographic purification as described in **Subheading 3.2.2**. If the PDE is going to be purified the next day, keep the extract on ice in a cold room or cold box

- after adding DTT and solid PMSF. If the PDE cannot be purified PDE immediately, try to concentrate the extract (the extract should be subjected to ultracentrifugation first, to remove traces of membrane that will clog ultrafiltration filters in concentrators); add glycerol (40% [v/v]), fresh DTT, and solid PMSF to the crude PDE; and then keep it at -20°C for a short time (a couple of weeks). Long-term storage is not recommended because the crude extract may still be contaminated with proteases.
8. Carry out the first GTP/low-salt wash, to extract transducin from ROSs, as follows:
 - a. Add low-salt buffer to each pellet with a final volume to 65 mL, use the rubber policeman to resuspend the pellet, and then transfer to the homogenizer.
 - b. Homogenize as for **steps 6a–e**.
 - c. Transfer the homogenized ROSs to Ti45 bottles, add 65 μL of 100 mM GTP (pH 7.2) to each bottle (final: 100 μM GTP), and mix by inverting several times. Then balance the bottles and load into the rotor. Timing is important in this step, because transducin will hydrolyze GTP to GDP and rebind to R^* at a rate of approx 2/min. In our typical prep, R^* is diluted to approx 5–10 μM and transducin is approx 0.25–0.5 μM , so it will take 100–200 min to hydrolyze 100 μM GTP.
 - d. Spin in the Ti45 rotor at 150,000g for 40 min at 4°C .
 - e. Pool the first GTP/hypotonic wash that contains most of the transducin in a 500-mL bottle, and add fresh DTT and solid PMSF to the extract.
 - f. Concentrate the transducin with an Amicon pressure concentrator at 4°C . After concentration, either one can add fresh DTT to 2 mM with solid PMSF and 40% (v/v) glycerol and then store at -20°C , or one can purify further by chromatography as described in **Subheading 3.3**.
 9. Carry out the second GTP/low-salt wash as follows:
 - a. Repeat **steps 8a–f**.
 - b. The second GTP/low-salt wash usually contains little or no transducin. Check the second wash on SDS-PAGE, and if it contains sufficient amounts of transducin, the second wash can be pooled with the first one and concentrated together.
 - c. Resuspend the final pellets in a minimum volume of the low-salt buffer, pool them in a 50-mL conical tube, and store at -80°C for further purification of other ROS proteins (e.g., RGS9-1, R9AP, PKC).
 10. Save a 1-mL aliquot of each supernatant for use in gel samples (100–200 μL of each supernatant + 100–200 μL of 10% trichloroacetic acid [TCA]), PDE activity pH assay (25–50 μL), and Bradford assay (100–200 μL).

3.2. Purification of PDE6 From Low-Salt Extracts of ROSs

3.2.1. Overview

This protocol is for use with extracts from ROSs equal to 300–450 retinas (two to three preparations of ROSs). PDE isolated from low-salt washes can be sequentially purified in HAP (7), DEAE, and gel filtration columns (12),

depending on the requirements of the experiments. HAP chromatography is particularly useful for separating PDE6 from HSP-90, arrestin, and other contaminating ROS proteins. The DEAE HPLC column is a weak anion-exchange column, and elution is with an ionic strength gradient. It removes proteins not removed by HAP but will not separate PDE from HSP-90. High-resolution DEAE HPLC will separate PDE $\alpha\beta$ from PDE $\alpha\beta\gamma$. After sequential HAP and DEAE chromatography, the purity of PDE is usually more than 90%. If even more pure PDE is required, PDE can be further purified by gel filtration.

3.2.2. HAP Chromatography of PDE6 Using Step Elution

All the purification steps should be performed in a cold room (4°C) to stabilize PDE activity and minimize protease activity. This step-elution method is routinely used to purify PDE from ROS low-salt extract, but a gradient method also can be used.

1. Equilibrate 40-mL of packed HAP column with 400 mL of 30 mM sodium phosphate (NaPi), pH 7.2. The flow rate for the HAP column usually is 0.5–1.5 mL/min under gravity. If one needs to use the pump, determine the flow rate of HAP column under gravity first, and do not exceed the flow rate under gravity with the pump.
2. Add 300 mM sodium phosphate, pH 7.2, to PDE extract to make the final concentration of NaPi 30 mM. Add a few crystals of PMSF.
3. Load the PDE to HAP column under gravity and save the flowthrough. It is recommended that the SAFETY-LOOP be set up and the PDE be loaded overnight on the day of PDE extraction. The PDE extract is about 390 mL for each wash and the flow rate in the HAP column is 1 mL/min, so one needs at least 7 h to complete the loading. Begin to load the first low-salt extract as soon as possible, even while the second one is in progress.
4. Wash the column with 300–400 mL (about 10 column vol) of 80 mM NaPi (pH 7.2), 1 mM DTT, and PMSF, and collect and save the flowthrough. Most of the contaminating protein in this fraction is arrestin; occasionally, a little PDE will elute in this fraction too.
5. Elute PDE with 150 mM sodium phosphate (pH 7.2), 1 mM DTT, and PMSF. Collect 5 mL/fraction with the fraction collector. During the elution, monitor the PDE by an UV monitor, a mini Bradford assay (add 100 μ L of 1X Bradford reagent and 15–20 μ L of each fraction to each well in a 96-well plate), or PDE activity (2–10 μ L for each fraction).
6. Elute (optional) with 300 mM sodium phosphate. This step has very little PDE but most of the HSP-90.
7. Run a 10% acrylamide SDS-PAGE: 0.5 mL for loading flow through (FT) and wash FT fractions and 20–100 μ L of each elution fraction. After running the gel, the HAP can be discarded, and it is not recommended that the column be regen-

erated and reused again, because HAP crystals can gradually break down to fines that block the column.

8. Pool and concentrate the PDE (from **step 5**) based on the gel or PDE activity. If this is the only column one is going to use to purify the PDE, make the concentrate 40% in glycerol, add a few crystals of PMSF, and store at -20°C . Otherwise, during the concentration, change the buffer to 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and then purify in a DEAE-5PW column.

3.2.3. DEAE HPLC Chromatography of PDE6

1. Connect a Protein-PakTM DEAE-5PW column to an HPLC or FPLC system, and set the maximum pressure at 400 psi and the flow rate to 1 mL/min. Set up the instrument software for injection, washing, and gradient elution according to the conditions listed in **steps 4–6**. HPLC can be carried out at room temperature, although operation at 4°C probably helps the protein stability, and fractions should be covered and placed on ice immediately after elution.
2. Equilibrate the DEAE column with 10% buffer B and 90% buffer A (i.e., starting condition is 100 mM NaCl) for 20 min at a flow rate of 1 mL/min.
3. Spin the concentrated PDE (from **step 5** in **Subheading 3.2.2.**) at 82,000g for 20 min at 4°C using a TL100 centrifuge to remove the insoluble proteins.
4. Inject the PDE into the sample loop and column. Save the flowthrough.
5. Wash the column with 10% buffer B and 90% buffer A for 10 min. Save the wash fraction.
6. Elute the PDE from the DEAE column with a gradient from 10% buffer B to 100% buffer B in 50 min. Collect fractions of 0.5–1 mL.
7. Check the purity of the PDE by analyzing 10 μL of each fraction by 10% acrylamide SDS-PAGE.
8. Pool the pure PDE fractions and dialyze against 20 mM Tris-HCl (pH 7.4), 2 mM MgCl_2 , 1 mM DTT, and PMSF at 4°C .
9. Concentrate the PDE with Centri-prep 50 or another concentrator. If this is the last column to be used to purify PDE, make the concentrate 40% in glycerol, add a few crystals of PMSF, and store at -20°C . Otherwise, during the concentration, change the buffer to 30 mM sodium phosphate buffer, pH 7.2, and 300 mM NaCl for gel filtration column.

3.2.4. Gel Filtration Chromatography of PDE6

1. Set the maximum pressure at 1300 psi and the flow rate to 1 mL/min in HPLC. See **Subheading 3.2.3.1.** for comments on temperature.
2. Equilibrate the gel filtration column with 30 mM NaPi (pH 7.2), 300 mM NaCl for >30 min at a flow rate of 1 mL/min.
3. Test the column with gel filtration standard at least twice. Inject 20 μL of gel filtration standard onto the column, run HPLC at 1 mL/min, and record the retention times.
4. Spin the concentrated PDE (from DEAE fractions) at 82,000g for 20 min at 4°C with a TL-100 ultracentrifuge.

5. Inject the supernatant of PDE onto the column and elute the PDE with equilibration buffer (30 mM sodium phosphate buffer, pH 7.2, and 300 mM NaCl) at a flow rate of 1 mL/min. Collect 0.5- to 1-mL fractions.
6. Check the purity of the PDE by SDS-PAGE; load 10 μ L for each fraction to 10% acrylamide gel. It usually is very pure (about 99%), as evidenced by a lack of detectable contaminating proteins on the gel.
7. Pool the pure fractions, concentrate and dialyze the PDE, add glycerol up to 40% (v/v) with a few crystals of PMSF, and store at -20°C .
8. After purification, wash the size-exclusion column extensively, using at least 10 column vol, with the equilibration buffer to remove small-molecule contamination. Then rinse with several column volumes of HPLC-grade water, and replace the mobile phase with 0.05% sodium azide or 5% methanol in water.

3.3. Purification of Transducin by Hexylagarose Chromatography

3.3.1. Overview

This protocol is for use with extracts from our standard 300 retinas (two preparations of ROSs). It is easily scaled up or down. Transducin is extracted from low-salt-stripped ROSs with low-salt buffer and GTP and then purified on hexylagarose using steps of increasing NaCl (**11,13**). In general, multiple washes with low-salt buffer, before stripping with low-salt buffer and GTP, will eliminate 95% of the proteins and the crude transducin extract will already be very clean. The hexylagarose column primarily concentrates transducin and cleans up lipids and a few contaminating proteins. If highly pure transducin is required, transducin can be further purified with a DEAE column (*see Subheading 3.2.3.*). All the purification steps should be performed in a cold room (4°C) to stabilize transducin activity and minimize protease activity.

3.3.2. Hexylagarose Chromatography of Gt

1. Hexylagarose comes as a suspension in 500 mM NaCl. The capacity of the packed hexylagarose is about 10–12 mg/mL of total proteins based on binding of BSA, so we routinely use a 10- to 15-mL column for two preparations of ROSs. Degas the resin under vacuum, and then pour and equilibrate the column. The flow rate for this column usually is 0.5 to 0.6 mL/min. Ten column volumes of buffer A_0 are necessary in order to completely equilibrate the column in zero NaCl.
2. Equilibrate the column with 10 column vol of buffer A_0 . If one needs to use the pump, determine the flow rate of hexylagarose column under gravity first and do not exceed the flow rate under gravity with the pump.
3. Add a shake of PMSF and fresh 1 mM DTT to the GTP/low-salt extract (*see Subheading 3.1.3.8.*) and load the extract to the column under gravity or with the pump turned on. After the first extract is available, start to load the extract, which contains more than 90% transducin, onto the column; there is no need to wait and mix with the second extract. It is recommended that a “safety-loop” be set up in the column and that the Gt extract be loaded overnight starting immediately on extraction of Gt. Save the flowthrough.

4. During the loading, look at the column; occasionally, if an extract has been contaminated with ROSs, the column will be orange. If this happens, do not reuse the column.
5. Wash the column with 10 column vol of buffer B₀, and save the flowthrough, because Gt occasionally does not bind to the column owing to a problem with either the column or the buffer.
6. Wash the column with 30 mL of buffer B₇₅, and collect 3-mL fractions with a fraction collector (using a 12 × 75 mm plastic tube). It is very important to monitor these fractions carefully, because sometimes Gt will start to bleed off in this step.
7. Elute the Gt with buffer B₃₀₀, and collect 3-mL/fractions. Gt usually elutes in fractions 8–10 in this step.
 - a. On some occasions, two peaks may be seen at this step, the first of which contains a contaminating protein that runs just above Gt on SDS-PAGE and the second of which contains most of the Gt. If this happens, remove the buffer “head” carefully before changing the step buffer.
 - b. Sometimes G α t and G $\beta\gamma$ t are separated in different fractions. If this happens, monitor the fractions carefully and pool fractions of G α t and G $\beta\gamma$ t together.
8. Run a 12% acrylamide SDS-PAGE; load 20–100 μ L for each fraction on the gel (precipitate the protein with TCA prior to adding SDS-PAGE sample application buffer to obtain a manageable volume). Always run a purified holo-Gt standard on the gel and run the gel long enough to separate the α and β transducin subunits.
9. Pool the Gt based on the gel and dialyze the Gt against the low-salt buffer (10 mM Tris-HCl, pH 7.4; 2 mM MgCl₂; 1 mM DTT; and solid PMSF). Do not concentrate the Gt before dialysis because it tends to precipitate in high- or moderate-salt buffer during the concentration.
10. Concentrate the Gt with a concentrator and make the concentrate 40% in glycerol; add a few crystals of PMSF, 2 mM DTT, and 50 μ M GDP; and store at –20°C.
11. Wash the column with buffer B₅₀₀. This step usually does not elute a significant amount of Gt but can clean up and regenerate the column. Finally, wash the column with 10 column vol of buffer B₀ containing 5 mM EDTA and 0.05% NaN₃.
12. If the Gt is going to be purified further with a DEAE column, after dialysis and concentration, add NaCl (100 mM final concentration) to the Gt. Spin at 82,000g for 20 min at 4°C with a TLA-100.3 tabletop ultracentrifuge rotor. The supernatant of the Gt is now ready to load onto a DEAE column in HPLC, using essentially the same procedure as described for PDE (*see Subheading 3.2.3.*).

3.4. Separation of G α t- and G $\beta\gamma$ t-Subunits by Blue Sepharose Chromatography

3.4.1. Overview

Transducin subunits bind tightly to Cibacron blue dye (Reactive Blue 2) on blue Sepharose columns and are individually eluted with “steps” of increasing concentrations of KCl, with G $\beta\gamma$ t eluting before G α t (**14**). This protocol (**7,15**)

is used to separate the Gt-subunits from the Gt extract of two pooled preparations of ROSs, and can be used to separate either GTP γ S-bound or GDP-bound G α t from G $\beta\gamma$ t-subunits.

3.4.2. Purification of Gt-Subunits

1. Reactive Blue 2 Sepharose CL-6B (Sigma): Each gram of resin can make 5 mL of the column. We routinely use 20–30 mL of column to isolate Gt-subunits from the Gt extract of two ROS preparations. At room temperature, swell the resin in buffer KCl₀, decant the fines, degas the media under vacuum, and pour the column. Then transfer the column to a cold room and equilibrate the column with five-column vol of buffer KCl₀.
2. If a used column is available, regenerate the column with 10 column vol of 10 mM MOPS (pH 7.4), 2 M KCl or with 6 M urea (in most cases, using 2 M KCl in MOPS buffer is sufficient). Then equilibrate the column with 10 column vol of buffer KCl₀.
3. Prepare the extract for Blue Sepharose column as follows:
 - a. To separate GDP-bound G α t from G $\beta\gamma$ t-subunits, use either a concentrated low-salt/GTP-stripped extract from ROSs, or hexylagarose-purified Gt. In the latter case, the Gt must be dialyzed against buffer KCl₀ before being applied to the blue sepharose column. Add GDP (50 μ M final concentration, pH 7.4) to the Gt just prior to loading onto the column.
 - b. To obtain activated G α t (GTP γ S-G α t), it is recommended that the Gt be stripped from the ROSs with GTP γ S/low-salt buffer directly. Otherwise, G α t can be activated with R*. Briefly, mix the Gt with urea-washed ROSs containing rhodopsin at a ratio of 1 rhodopsin for 100 Gt in the presence of GTP γ S in a darkroom. Then incubate the mixture on ice under room light for 5–10 min to convert R into R* and activate the Gt. ROS membranes can be removed by centrifuging at 150,000g at 4°C for 40 min in low-salt buffer. The supernatant should contain GTP γ S-G α t and G $\beta\gamma$ t.
 - c. Measure the extract volume. We found that cleaner and more efficient separation of transducin subunits occurs when the total volume of extract is approximately equivalent to 1 column vol. Therefore, it is helpful to adjust the volume by concentrating or adding buffer. Loading a large volume of Gt extract is not recommended.
4. If the column has not been equilibrated, equilibrate it with 10 column vol of buffer KCl₀ under gravity. If the pump must be used, determine the flow rate of the blue Sepharose column under gravity first and do not exceed the flow rate under gravity with the pump, or the beads will be crushed and the flow obstructed.
5. Add 50 μ M GDP to the concentrated Gt extract in order to isolate GDP-G α t from G $\beta\gamma$ t. It is not necessary to add GDP to the GTP γ S-activated Gt extract.
6. Adjust the concentration of MgCl₂ to 5 mM in the Gt extract, because transducin binds much better to the column in 5 mM MgCl₂.
7. Let the equilibration buffer run down to the top of the column bed or remove the “head” of the buffer (to avoid diluting the extract), load the extract onto the

column with a pipet, and run the column by gravity. Collect and save the flowthrough.

8. After the sample has been completely loaded and allowed to drain to the top of the resin bead, add a head of buffer KCl_0 to the column (but without disturbing the media), and then wash the column with 100–150 mL of buffer KCl_0 (3–5 column vol).
9. Elute the $\text{G}\beta\gamma\text{t}$ with buffer KCl_{100} . Remove the head of the buffer KCl_0 , add a new head of buffer with buffer KCl_{100} , and then elute the $\text{G}\beta\gamma\text{t}$ with 4 to 5 column vol of buffer KCl_{100} . Collect 3-mL fractions in 12×75 mm plastic tubes. The $\text{G}\beta\gamma\text{t}$ should begin to elute following one-third of the column volume in the buffer KCl_{100} elution step. Check the last fraction with a Coomassie-Blue binding (**10**) assay to determine whether a detectable amount of protein is still coming off after elution with 5 column vol, and, if so, use more buffer KCl_{100} to elute the proteins until no more protein can be detected in the eluent.
10. Elute the $\text{G}\alpha\text{t}$ with buffer KCl_{750} . Remove the head of the buffer KCl_{100} , add a new head of buffer with buffer KCl_{750} , and then elute the $\text{G}\alpha\text{t}$ with 4 to 5 column vol of buffer KCl_{750} . Collect 3 mL fractions in 12×75 mm plastic tubes.
11. Run a 12% acrylamide SDS-PAGE gel to determine which fractions to pool. Load 20–100 μL /fraction onto the gel. Include a holo-Gt as a standard so that each subunit can be properly identified. Often a little $\text{G}\beta\gamma\text{t}$ bleeds out with the KCl_0 wash, and some $\text{G}\alpha\text{t}$ bleeds out at the end of the KCl_{100} elution.
12. Pool the fractions of pure $\text{G}\beta\gamma\text{t}$ and $\text{G}\alpha\text{t}$ separately. Dialyze the pooled fractions with low-salt buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl_2 , 1 mM DTT, and solid PMSF). Do not concentrate Gt-subunits before dialysis, to avoid losses owing to precipitaton.
13. After dialysis, concentrate Gt-subunits with Amicon or Centricon ultrafiltration devices. Then make the concentrate 40% in glycerol, add a few crystals of PMSF, 2 mM DTT, and 50 μM GDP, and store at -20°C .
14. Clean the column with 10 column vol of a buffer containing 10 mM MOPS (pH 7.4), 2 M KCl, and 6 M urea, then wash with 10 column vol of buffer KCl_0 ; and, finally, store the column in buffer KCl_0 supplemented with 1 mM EDTA and 0.05% NaN_3 . Seal the column top and bottom with the caps and store at 4°C . The column can be reused many times.
15. $\text{G}_{\alpha\text{t}}\text{-GTP}\gamma\text{S}$ prepared using this procedure is pure enough for most experiments. However, if highly pure $\text{G}_{\alpha\text{t}}\text{-GTP}\gamma\text{S}$ is required—e.g., if high concentrations will be used so that small traces of PDE become a concern— $\text{G}_{\alpha\text{t}}\text{-GTP}\gamma\text{S}$ can be further purified by DEAE HPLC chromatography and/or gel filtration using essentially the same procedures as those described for PDE6 in **Subheadings 3.2.3.** and **3.2.4.**)

3.5. Preparation of Lipid Vesicles

3.5.1. Overview

This procedure (**16,17**) results in a preparation of vesicles that predominantly have a single bilayer, and a narrow size distribution in the hundred-nanometer

range, in addition to a well-defined lipid composition. Thus, it is possible to know with some accuracy the total lipid surface available for binding proteins, and to eliminate the degree of curvature of the membrane surface as an uncontrolled variable. Fairly homogeneous small unilamellar vesicles can also be prepared by sonication, but their small radius of curvature is not optimal for reconstitution of the G_{out} complex (7). The basic idea is to form a thin layer of lipid by drying, hydrate it uniformly by multiple freeze-thaw cycles, and then to force it multiple times through well-defined submicron pores to produce uniform vesicles. This last step relies on a commercially available extrusion device using high-pressure nitrogen, but *see* **Note 1**.

3.5.2. Preparation of Vesicles

1. Clean a Corex tube and gastight (e.g., Hamilton) syringes with chloroform.
2. Remove the desired volume of each lipid solution from stock, under argon, and place in the tube. Cover the stocks with an argon blank, reseal, and store at -80°C . For example, to make 20 mg of vesicles containing 40:39:20:1 (molar ratio) phosphocholine (PC):phosphoethanolamine (PE):phosphoserine (PE):rhodamine-labeled PE, remove (403-2 μL of 20 mg/mL PC, 372 μL of 200mg/mL PE, 207-7 μL of 20 mg/mL PS, and 34.2 μL of 10 mg/mL rhodamine PE. The rhodamine-labeled PE is included to facilitate separation of the supernatant and pellet in sedimentation experiments and is optional if such procedures are not to be carried out.
3. Dry lipid to a thin homogeneous film using a gentle stream of argon or nitrogen in a fume hood. Holding the tube in one hand, tilt it so that the lipid solution spreads up the sides of the tube. While rotating the tube, direct a very gentle stream of gas into the tube. It is very important that the lipid surface be smooth, with no trapped solvent. If the surface after drying does not look smooth, redissolve in a small amount (200–500 μL) of chloroform and carefully repeat the drying procedure.
4. Extensively clean the syringes with chloroform immediately after using.
5. Place the tube in a vacuum chamber (e.g., a vacuum desiccator or a stoppered side-arm flask), and dry under vacuum for at least 2 h (it is convenient to keep under vacuum overnight, but no longer, to ensure complete removal of solvent. Longer periods under vacuum can lead to contamination with oil from the vacuum system).
6. Add 1–10 mL of buffer, depending on the final concentration desired. Remember that some lipid will be lost during subsequent procedures. For PDE assays, a good buffer is pH assay buffer (*see* **Subheading 2.2.8.**). It should be supplemented with 170 mM sucrose if sedimentation experiments are planned (17). The lipids should be covered with an argon blanket and the tube covered with parafilm. Vortex the sample for 1-min intervals every 5 min over a 15- to 30-min period, until the surface film is no longer apparent and the suspension has a uniform “chalky” appearance. The lipids are now hydrated in large multilamellar

structures, but with uneven distribution of aqueous solution throughout the lamellae.

7. Subject the lipids to six freeze-thaw cycles. These involve immersing in liquid nitrogen (or, if unavailable, a dry ice–acetone slurry) until frozen solid, then placing in water until thawed. This procedure enhances the distribution of aqueous solution within the lamellae. To prevent contamination from acetone or water, cover the tubes with Parafilm pierced with two or three tiny holes.
8. Extrude the lipids through submicron pores in two stacked polycarbonate filters to produce uniformly sized unilamellar vesicles. This procedure is most conveniently carried out using a nitrogen pressure extruder supplied by Lipex Biomembranes (*see Note 1*). The directions supplied by the manufacturer should be followed carefully. The filters used must be polycarbonate filters supplied by Osmonics. These have much more uniform size than normal commercial filters. They are available in a range of pore sizes, but 0.1- μm pores are recommended, because they produce predominantly unilamellar vesicles with a narrow and stable size distribution. Load the lipid suspension into the sample compartment, and then seal. Apply pressure until all the liquid is eluted into a collection tube. Relieve the pressure, and load the sample again, until 10 extrusion cycles have been carried out. At this point, the lipid suspension should no longer be “chalky” but, rather, should be transparent with an opalescent appearance.
9. To remove unwanted solutes (e.g., sucrose) from the external solution, dialysis can be used. Alternatively, if the vesicles are sucrose loaded, they may be diluted (at least fivefold) with the desired buffer and pelleted by ultracentrifugation before resuspending in the desired buffer. Spinning for 60 min at 10,000g in a polycarbonate tube using a TLA-100.3 rotor is sufficient. Gently but thoroughly resuspend the pellet in the desired final buffer. Longer or harder spinning may collapse some vesicles and release trapped solution. To prepare vesicles for the binding assay described in **Subheading 3.6.**, after resuspension, spin the vesicles again at 52,000g in microfuge-style TL-100 tubes (with adapters) for 20 min. The vesicles collected from the pellet at this step can be relied on to be pelleted by the assay spin at 86,000g.
10. To accurately measure the amount of phospholipid collected in the vesicles after the procedures, it is necessary to carry out an assay of total phosphate; this step is critical for comparing the effects of different lipid mixtures. This is a colorimetric assay based on the formation of molybdate blue (**18**). Sensitivity is in the range of 5–100 nmol/assay sample (e.g., 5–100 μL of 1 mM, 0.76 mg/mL egg PC). Samples and phosphorous standards covering the expected range of phosphate are prepared in triplicate by pipetting 1–100 μL into 13 \times 100 mm glass tubes washed with phosphate-free detergent and rinsed with Milli-Q water. To each sample add 20 μL of 70% HClO_4 and 20 μL of concentrated H_2SO_4 using microcapillary glass pipets or positive displacement pipets or syringes. Add enough water to each sample so that the total volume is now 140 μL . Vortex thoroughly. Heat overnight in a fume hood at 110–120°C, or heat for 3 h at 150°C. Use safety goggles or a face shield to protect against hot acid splashes. Samples char and then become clear when

hydrolysis is complete. Add 500 μL of water and 400 μL of the following freshly prepared solution: 2 vol of Milli-Q water, 1 vol of 0.1 N H_2SO_4 , 1 vol of 2.5% (w/v) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. Add 10 μL of 10% (w/v) ascorbic acid, free acid form; that solution should be less than 7 wk old, stored at 4°C, and colorless. Heat tubes for 60 min at 37°C. Measure the absorbance at 820 nm. Standards should give a linear response in the range of 5–65 nmol of phosphorus, and the phosphorus content of the lipid samples is determined by comparison to standards. Correct the results for any lipids lacking phosphate or containing extra phosphates (e.g., phosphoinositides) to determine the total lipid content.

3.6. Vesicle-Binding Assays

3.6.1. Overview

Binding assays are facilitated by loading sucrose (170 mM) during the vesicle formation step (17). The increased density allows the vesicles to sediment in an ultracentrifuge under conditions in which proteins remain in the supernatant fraction. The assay is also facilitated by incorporating a dye-labeled lipid (e.g., lissamine-rhodamine B sulfonyl-PE) into the vesicles during preparation to check for completeness of pelleting. Vesicles and proteins to be reconstituted should be exchanged into the proper buffers for the binding assays beforehand using the methods described in **Subheading 3.5.2.9.** or using gel filtration.

3.6.2. Procedure

Proteins (e.g., G_{act} -GTP γ S and PDE6) are mixed with vesicles in microfuge-style polyallomer tubes for the TLA-100.3 or similar fixed-angle tabletop ultracentrifuge rotor and spun at 86,000g for 30 min. Typical concentrations in a final volume of 200 μL are 0–5 nM G_{act} -GTP γ S, 1 nM PDE6, and 0–15 μM (total phospholipid) sucrose-loaded vesicles. The sucrose-loaded vesicles under many conditions do not form a tight pellet. A procedure for separating the supernatant and pellet that does not rely on flawless manual technique is to remove only the top 150 μL of the sample. A 150- μL volume of buffer is added to the pellet plus 50 μL of the supernatant, and these “75% supernatant” and “pellet plus 25% of supernatant” fractions are analyzed to determine the amount of specific proteins in each and the tracer fluorescent lipid is analyzed by measuring the fluorescence of 10- μL aliquots diluted to 200 μL in 1% SDS.

Protein quantification depends on the proteins to be analyzed. If antibodies are available, standard SDS-PAGE immunoblotting procedures with standards from the same stocks used to prepare the samples can be used to determine the fraction of protein in the supernatant and pellet fractions. If the G_{act} -GTP γ S is prepared using GTP $\gamma^{35}\text{S}$, then the radioactivity can easily be measured by scintillation counting. Total PDE in each fraction can be determined by mea-

suring PDE activity using the assay described in **Subheading 3.7.2.** after trypsin activation. If fairly large amounts of proteins are used (at least 200 ng/protein subunit/sample), Coomassie staining of gels followed by densitometry can be used to quantify each protein.

3.7. Assays of PDE Activity

3.7.1. Overview

The assay described here, derived from a method developed by Liebman and Evanczuk (19), is based on pH measurements over a range in which proton release increases linearly with cGMP hydrolysis. It is very convenient, provides activity readouts within a few minutes, and allows multiple additions to be made to a single sample and the activity recorded after each one. The disadvantages are that it is not tremendously sensitive (at least 100 μL of a few nanomolar PDE6 is required) and that each sample takes several minutes. Thus, if one wants to assay a large number of samples, each with much lower levels of PDE6 activity, then assays based on monitoring hydrolysis of radiolabeled cGMP are preferable.

The general principle of the assay is that the $\text{p}K_a$ of 5'-GMP is lower than the $\text{p}K_a$ of cGMP, so that hydrolysis of cGMP in a pH range of 7.6–8.2 liberates nearly one proton per cGMP hydrolyzed, and in this narrow pH range, pH decreases in a nearly linear fashion with protons released. A standard pH meter records this as a potential change, and this changing voltage can be recorded as a good approximation to cGMP hydrolysis using a chart recorder or a computer with an analog-to-digital converter card. With time as the x -axis, and cGMP hydrolysis as the y -axis, the slope at any point (as analyzed by computer or simply by using a ruler to draw a straight line on the chart) is proportional to the rate of hydrolysis. The proportionality constant is determined by multiplying the slope in (arbitrary y units/s) times the total y excursion, in the same units, resulting from hydrolysis of a known concentration of cGMP (e.g., 2 mM).

The assay is conveniently carried out in wells of 96-well microtiter plates containing magnetic stir fleas that just fit the wells. Loss of protein to surfaces is minimized by having the assay solution contain 0.1% (w/v) ovalbumin, or by coating the wells with such a solution before use. Microelectrode (these are “micro” by conventional pH electrode standards, but enormous by comparison with those used for cellular recording) volumes in the range of 100–200 μL can be used routinely. Use of a 5X buffer stock allows other components to be added as needed, with water making up the remainder of the volume, so that the buffer capacity does not vary significantly. In the same sample, one can sequentially assay basal activity, activity stimulated by one or more concentrations of an activator such as $\text{G}\alpha\text{t-GTP}\gamma\text{S}$ or a vesicle

suspension, and maximal activity stimulated by trypsin. Trypsin rapidly degrades the inhibitory PDE γ -subunit and thus gives rise to the highest observable level of PDE6 activity, which can be used to normalize the G protein-stimulated activity.

3.7.2. pH Assay for PDE6 Catalytic Activity

1. Clean the electrode, microtiter plate, and stir flea with 0.1 *N* HCl, and then wash with Milli-Q water.
2. Condition the electrode with pH assay buffer, pH 8.0, at least 30 min before planning to start the assay. Start the recorder and observe the drift and noise level. Measuring PDE basal activity accurately requires a flat baseline. To minimize drift, do the following:
 - a. Be sure to clean up traces of trypsin in the electrode between each assay with 0.1 *N* HCl and washing with Milli-Q water.
 - b. Keep the Milli-Q water and cGMP aliquot at room temperature.
 - c. Make sure that the assay solution covers the reference junction, about 1.5 mm from the tip of the electrode.
 - d. Maintain a constant ionic strength.
3. Check the recorder range with solutions prepared from standards (pH 6.0 and 8.0 standards), adjust to zero, and vary the calibration knobs as necessary. The voltage output from the pH meter can be connected to a computer analog-to-digital converter card as an alternative or supplement to the chart recorder.
4. Calculate the volumes and concentrations of all planned components in the assay; we routinely use a 200- μ L volume in each assay that contains a final 1X pH assay buffer: 2 mM cGMP, with 0.5–20 nM PDE and transducin, and at least a 1000-fold excess of total phospholipids over PDE. The final trypsin concentration is typically 0.1 mg/mL.
5. Put a clean stir flea into the microtiter well and start the stirring motor. Add 40 μ L of 5X pH assay buffer and an appropriate volume of Milli-Q water (calculated to give the desired 200- μ L volume after the other components are added) to the stir bar-containing well. Lower the electrode to the solution. The tip of the electrode should not touch the stir bar or the side of the well.
6. After the pH is stable at 8.0, start the chart recorder.
7. Add the assay components in the desired order, labeling the chart paper after each addition with a line pointing to the position (time) of the addition.
8. Between assays, wash the electrode with Milli-Q water, 0.1 *N* HCl, and then Milli-Q water.
9. At the end of the assay, extensively wash everything used with Milli-Q water and 0.1 *N* HCl, and then wash with Milli-Q water. Store the electrode probe in pH 4.0 standard.
10. Calculate PDE activity. The calculations are based on the assumption that the concentration of cGMP is much greater than the K_m (PDE K_m for cGMP is \sim 10 μ M), the initial rate of cGMP hydrolysis is at its maximum, and it is directly proportional to the amount of PDE present in the assay. Raw data from a typical

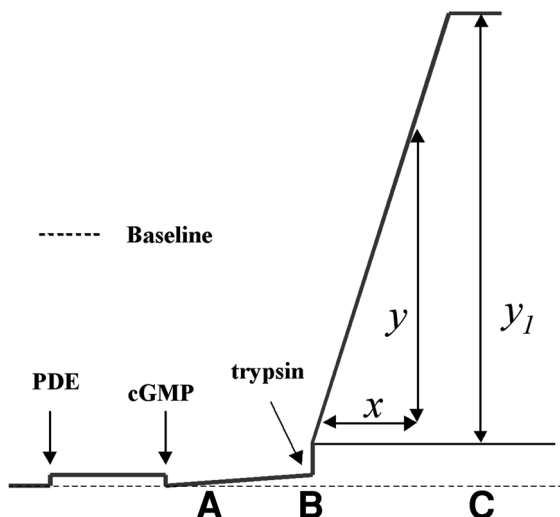


Fig. 1. (A) The data is the slope of the line (rise over run; y distance [cm]/ x distance [cm]) that represents the initial velocity, change in pH/time, following each addition. (B) This is proportionality constant between y excursion and cGMP hydrolysis, based on the change in the y_1 direction after complete hydrolysis of 2mM cGMP. It does not include the change owing to trypsin solution alone. (C) The speed of the chart; set up typically as 1 cm/60 s.

PDE assay are shown in **Fig. 1**. Use the following equation to determine PDE activity:

$$\text{PDE activity } (\mu\text{M s}^{-1}) = \frac{y \text{ cm}}{x \text{ cm}} \cdot \frac{2000 \mu\text{M CGMP}}{y_1 \text{ cm}} \cdot \frac{1 \text{ cm}}{60 \text{ s}}$$

The data in the first part of the equation represent the slope of the line (“rise over run”; y distance [cm]/ x distance [cm]) that represents the initial velocity, change in pH/time, following each addition. The second part of the equation represents the proportionality constant between y excursion and cGMP hydrolysis, based on the change in the y_1 direction after complete hydrolysis of 2 mM cGMP. It does not include the change owing to trypsin solution alone. The last part of the equation represents the speed of the chart, set up typically as 1 cm/60 s.

4. Note

1. For vesicle extrusion (see **Subheading 3.5.2.**), Avanti supplies a manual extruder in which the pressure is supplied via hand pressure on microsyringes. In our experience, an earlier version of this extruder works, but at the expense of multiple

broken microsyringe barrels, and it is not recommended. A more recent version includes an extruder stand/stabilizer block that may alleviate this problem and is less expensive than the Lipex device. Another alternative if an HPLC system is available involves the use of a high-pressure stainless steel filter holder supplied by Millipore for use with 2.5-cm filter disks. It can be connected to the injection port of an HPLC system in place of a column, with the outlet directed into a collection tube instead of the detector. The sample is injected via the system's injection loop and extruded using the same buffer used for hydrating the lipids, supplied by the system's solvent delivery pump. The flow rate setting should be initially set to 0.1 mL/min and slowly increased until the lipid suspension begins to elute from the filter holder. A pressure limit of 800 psi should be used. As soon as the injected volume is eluted the flow is stopped, and the eluted lipids are reinjected. A total of 10 cycles of extrusion is sufficient. Note that the volume unavoidably increases a little each time, and this dilution must be taken into account.

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