

## Cyclic Guanosine 5'-Monophosphate Binding to Regulatory GAF Domains of Photoreceptor Phosphodiesterase

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### Summary

Of the 11 families of mammalian cyclic nucleotide phosphodiesterases (PDEs), 5 contain regulatory domains capable of binding cyclic guanosine 5'-monophosphate (cGMP). The best understood of the GAF-containing PDEs is the family of rod (PDE6R) and cone (PDE6C) photoreceptor PDEs. Binding of cGMP to the rod PDE6 catalytic dimer ( $\alpha\beta$ ) allosterically regulates the affinity of the inhibitory subunits of PDE6 ( $\gamma$ ) for the enzyme. Two nonidentical, high-affinity cGMP-binding sites exist on the nonactivated mammalian PDE6R holoenzyme ( $\alpha\beta\gamma\gamma$ ). One of the sites does not readily exchange with free cGMP when the catalytic dimer is complexed with  $P\gamma$ . On dissociation of  $\gamma$  from the catalytic dimer, one of the two cGMP-binding sites undergoes a transition from high to low affinity. This chapter describes techniques to quantify cGMP binding to PDE6 in order to study the regulatory significance of the GAF domains. For high-affinity cGMP binding sites on PDE6, membrane filtration is the method of choice because of its speed, simplicity, and sensitivity. However, lower-affinity cGMP-binding sites require a method that does not perturb the equilibrium between bound and free ligand. The use of ammonium sulfate solutions during filtration extends to lower-binding affinities the useful range of membrane filtration. However, a centrifugal separation technique that minimizes perturbation of the cGMP-binding equilibrium is also presented for measuring lower-affinity cGMP-binding sites. These methods are applicable to understanding the regulatory mechanisms regulating other GAF-containing PDEs as well.

### Key Words

Photoreceptor; phosphodiesterase; cyclic guanosine 5'-monophosphate; GAF domain; transducin; membrane filtration; ligand binding.

### 1. Introduction

Binding of a ligand to its cognate receptor induces conformational changes in the protein receptor that alter its structure and function. Changes in the

intracellular concentration of cyclic guanosine 5'-monophosphate (cGMP) can cause changes in cGMP binding to specific cGMP receptor proteins, including the cyclic nucleotide-gated ion channel, cGMP-dependent protein kinase, and cGMP-binding PDEs. In the former case, the relatively low affinity of cGMP ( $K_D > \mu M$ ) for this class of ion channel precludes the use of traditional binding assays, and electrical recordings of channel activity are the preferred approach for studying this class of cGMP receptor (e.g., *see* **ref. 1**). For cGMP-binding kinases and PDEs, a number of cGMP-binding assays have been used to characterize these regulatory cGMP-binding sites. Quantitation of cGMP-binding to receptor proteins can be carried out using equilibrium or nonequilibrium methods.

The choice of the optimal method for assaying cGMP binding depends on several factors, the most important of which is the intrinsic dissociation rate of cGMP from its receptor protein. If the rate of cGMP dissociation is slow relative to the time needed to separate bound from free ligand in the binding assay, then nonequilibrium binding assays (e.g., membrane filtration) are usually the method of choice, because they are rapidly performed and exhibit high sensitivity and low nonspecific binding. If significant dissociation of bound cGMP can occur during the separation of free from bound cGMP, then an equilibrium binding method (e.g., equilibrium dialysis, ultrafiltration) is required to prevent underestimating the extent of cGMP binding.

This chapter presents both nonequilibrium (membrane filtration) and equilibrium (centrifugal separation) methods for quantitating cGMP binding to rod PDE6.

## 2. Materials

1. Photoreceptor cell extracts containing PDE6 or purified PDE6 (*see* Chapter 10 on PDE6 purification).
2. Recombinant bovine rod inhibitory  $\gamma$ -subunit expressed in *Escherichia coli* harboring the pET11a/ $\gamma$  expression plasmid (2).
3. PDE6 storage buffer: 10 mM Tris-HCl, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, pH 7.5, supplemented with 2 mM dithiothreitol and 0.1 mM Pefabloc.
4. 100 mM zaprinast (cat. no. Z0878; Sigma Chemical Corporation, St. Louis, MO) dissolved in 1-methyl-2-pyrrolidinone or dimethyl sulfoxide.
5. Millipore MF membrane, 0.45- $\mu$ m pore size, 25-mm diameter (cat. no. HAWP 025 00; Millipore, Bedford, MA).
6. Hoefer FH 225V 10-place filter manifold or Millipore 1225 sampling manifold.
7. Gast vacuum pump.
8. Buffer A: 60 mM KCl, 30 mM NaCl, 20 mM HEPES, 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA, pH 7.5.
9. [<sup>3</sup>H]cGMP (cat. no. NET-554; Perkin Elmer) prepared as a 10X stock solution in buffer A containing 100  $\mu$ M zaprinast or 10 mM EDTA. Adjust the cGMP

concentration and specific activity with nonradioactive cGMP as needed for particular experiments.

10. Ultima Gold and Ultima Gold XR scintillation fluids (Packard).
11. 95% Buffered ammonium sulfate (BAS): First dissolve 353.3 g of ammonium sulfate in 500 mL of water chilled to 4°C, and then mix with 35 mL of 200 mM Tris-HCl, pH 7.5.
12. 2% Sodium dodecyl sulfate (SDS): Add 10 g of SDS to 500 mL of distilled water.
13. Scintillation counter with automatic quench correction and disintegration-per-minute calculations.
14. Silicone oils: Dow Corning 550 fluid (density: 1.07 g/mL) and Dow Corning 200 fluid (density: 0.818 g/mL, viscosity: 1.0 cP) (William F. Nye, New Bedford, MA).
15. Tabletop air-driven ultracentrifuge (Beckman Airfuge) with A-110 rotor.
16. Airfuge centrifuge tubes (5 × 20 mm, polyallomer, no. 342630).

### 3. Methods

The methods described outline preparation of various forms of PDE6 for assays of cGMP binding, a general membrane filtration assay for cGMP binding to photoreceptor cell extracts or purified PDE6, the use of ammonium sulfate to stabilize cGMP bound to PDE6 during the membrane filtration assay, a centrifugal separation assay that sediments membrane-associated PDE6 through a silicone oil layer to separate cGMP bound to PDE from free cGMP, and guidelines for the analysis of ligand-binding data to extract equilibrium binding constants and kinetic rate constants.

#### 3.1. Preparation of PDE6 for cGMP-Binding Assays

##### 3.1.1. Purification of PDE6 From Photoreceptor Cells

Methods for isolating rod and cone PDE6 from retinal photoreceptors are described in Chapter 10. Because PDE6 is the only high-affinity cGMP-binding protein detected in rod and cone cells (3–5), assays of cGMP binding to PDE6 can be performed in crude photoreceptor cell extracts as well as with highly purified PDE6 with similar results (see **Note 1**). For storage periods of more than 2 mo, purified PDE6 is stored in PDE storage buffer containing 50% glycerol and kept at –20°C. For longer storage, PDE6 is frozen in this solution at –80°C.

##### 3.1.2. Depletion of Endogenous cGMP From GAF Domains

Because of the high affinity with which PDE6 binds cGMP, care must be taken to remove endogenous, bound cGMP prior to addition of radiolabeled cGMP. Repeated washing of rod cell membranes, or chromatographic purification of soluble PDE6, will remove unbound cGMP. Bound cGMP is allowed to dissociate from the GAF domains by incubating nonactivated PDE6 for 1 to 2 h at 30°C in the absence of PDE6 catalytic site inhibitors (4,6). In this way,

cGMP that dissociates is hydrolyzed and thus prevented from rebinding to the GAF domains. PDE6 catalytic dimers lacking bound  $\gamma$ -subunits are more readily depleted of endogenous cGMP (0.5 h at 30°C) because the cGMP dissociation rate is accelerated when  $\gamma$  is not present. The state of occupancy of PDE6 can be ascertained either by radioimmunoassay measurements of PDE6 cGMP content or by empirically determining the maximum stoichiometry of cGMP binding ( $B_{\max}$ ) at a saturating (1  $\mu\text{M}$ ) concentration of [ $^3\text{H}$ ]cGMP (see **Subheading 3.2.1.**).

### 3.1.3. Inhibition of Enzymatic Activity to Prevent Ligand Destruction

Complicating cGMP-binding assays is the fact that the ligand of the binding assay is also the substrate for the active site of PDE6. Unless precautions are taken to prevent catalysis by inclusion of active-site inhibitors, added cGMP will be consumed and reduced cGMP binding will be observed. The extent to which [ $^3\text{H}$ ]cGMP is broken down should be ascertained under the experimental conditions of the binding assay using a standard cyclic nucleotide radiotracer assay method (7). Either of the following conditions is effective in preventing the breakdown of cGMP during the time needed for PDE6 to reach cGMP-binding equilibrium at its GAF domains: (1) 10 mM EDTA preincubated for 10 min prior to nucleotide addition to PDE6  $\alpha\beta$  catalytic dimers (see **Note 2**); (2) 0.1 mM zaprinast, a PDE5/6-selective inhibitor (see **Note 3**).

## 3.2. Membrane Filtration Assay of cGMP Binding

Receptor-binding studies often use membrane filtration to separate ligand bound to its receptor protein from unbound ligand. Membranes composed of mixed esters of cellulose (typically nitrate and acetate) are the most popular, because they are hydrophilic, have a high capacity for binding proteins, and usually exhibit low levels of nonspecific binding of the ligand. For multiple samples, a vacuum filtration manifold should be used. High-throughput receptor-binding assays rely increasingly on 96-well dishes to which various membrane filters are attached.

The following section describes the general membrane filtration assay for quantitating cGMP binding to PDE6. The validity of the method depends on meeting the following criteria: (1) quantitative retention of protein on the filter, (2) low nonspecific binding of the ligand to the membrane, and (3) no ligand dissociation from the receptor protein caused by the separation of free from bound ligand.

### 3.2.1. Membrane Filtration Assay for cGMP Binding to PDE6

1. Incubate nucleotide-depleted PDE6 with [ $^3\text{H}$ ]cGMP in buffer A (supplemented with zaprinast or EDTA) until binding equilibrium is attained (see **Note 4**).

2. Apply membrane filter disks to the filter manifold.
3. Just before applying the sample to the membrane, prewet the filter disks with 1 mL of cold filter wash buffer, and open the vacuum to draw the wash solution through the filter.
4. Apply the [ $^3\text{H}$ ]cGMP-PDE6 mixture (typically 20–100  $\mu\text{L}$ ) directly to the membrane with a micropipet.
5. Immediately rinse the filter three times with 1 mL of cold wash buffer with a repetitive dispensing pipet (e.g., Eppendorf Repeater Pipette). This process should be completed as quickly as possible to minimize cGMP dissociation from PDE6-binding sites.
6. Include samples that will allow determination of the extent of nonspecific binding of [ $^3\text{H}$ ]cGMP to the filter (*see Subheading 3.2.3.*). In addition, determine the total disintegrations per minute applied to the filter by adding known volumes of the incubation mixture directly to scintillation vials.
7. After drying the filter under vacuum for approximately one additional minute, place the filter in a scintillation vial, and add 4 mL of scintillation cocktail.
8. Vigorously shake the vials until the membrane filter becomes translucent.
9. Set up a scintillation counter to automatically calculate disintegrations per minute, and count each sample until sufficient disintegrations per minute have been recorded to reduce the counting error to less than 1%.

### 3.2.2. Assessment of Quantitative Recovery of PDE6 on Membrane Filter

Failure to retain all of the applied PDE6 on the MF-Millipore membrane can be assessed by comparing the enzyme activity of the PDE6 before filtration with the enzyme activity recovered in the filtrate. Because of the high sensitivity of the filter-binding method and the high protein-binding capacity of the MF-Millipore membranes, even rod photoreceptor extracts in which PDE is <1% of the total protein show no evidence for less than full recovery of PDE6 on the membrane. Quantitative binding of PDE6 to the membrane does not require PDE6 attachment to photoreceptor membranes; purified PDE6  $\alpha\beta$  dimers are also quantitatively retained on the filter membranes.

### 3.2.3. Determination of Nonspecific Binding of cGMP to Filter Membrane

One of the advantages of membrane filtration over other ligand-binding methods is the low level of nonspecific binding generally observed. Since the nonspecific binding component of the disintegrations per minute recovered on the membrane depends on the total disintegrations per minute applied to the filter, nonspecific binding controls should be performed in each membrane filtration experiment.

1. To test for nonspecific binding of the [ $^3\text{H}$ ]cGMP to the filter, perform a filter-binding assay in which the PDE6 is omitted from the incubation (*see Subheading 3.2.1., step 1*), but otherwise the sample is treated identically to the PDE6-containing samples.

2. To test for nonspecific binding of [<sup>3</sup>H]cGMP to PDE6, incubate the PDE6 with [<sup>3</sup>H]cGMP to which a >1000-fold excess of unlabeled cGMP has been added (i.e., 0.5 μM [<sup>3</sup>H]cGMP premixed with 1.0 mM cGMP). Elimination of unbound [<sup>3</sup>H]cGMP from the membrane filter can be optimized by varying the volume and number of washes of the filters.

#### 3.2.4. Assessment of Whether cGMP Dissociation From PDE6 Occurs During Membrane Filtration

During membrane filtration, the equilibrium between bound and free cGMP is perturbed, and the washing step can potentially cause bound cGMP to dissociate. The rate of dissociation of cGMP from its bound state on PDE6 is defined by the dissociation rate constant,  $k_{-1}$ , which can be experimentally measured (4). Furthermore, the  $k_{-1}$  value depends on several factors, including the activation state of PDE6 (5,6), the amount of exogenous  $\gamma$ -subunit (8), and the temperature. If  $k_{-1}$  is known, then the time needed to complete membrane filtration and washing with a loss of <10% of the bound nucleotide can be calculated as described originally by Bennett and Yamamura (9):  $T_{10\%} = 0.14/k_{-1}$ . If  $k_{-1}$  is not known, it can be evaluated by an isotopic dilution experiment in which a large excess of nonradioactive cGMP is added to an equilibrium mixture of [<sup>3</sup>H]cGMP-PDE6 and the kinetics of cGMP dissociation are monitored:

1. Incubate nucleotide-depleted PDE6 with 1 μM [<sup>3</sup>H]cGMP to saturate all of the cGMP-binding sites. Allow the binding reaction to reach equilibrium.
2. Following **steps 1–5** in **Subheading 3.2.1.**, determine the extent of bound [<sup>3</sup>H]cGMP using the standard protocol.
3. To the remaining PDE6 sample, add 1 mM nonradioactive cGMP (1/50 dilution of a 50 mM cGMP solution).
4. Immediately remove portions and perform the standard membrane filtration, noting the time at which each sample is filtered and washed.
5. Perform nonspecific binding controls as in **Subheading 3.2.3., step 2.**

The initial time course of [<sup>3</sup>H]cGMP dissociation from PDE6 should follow an exponential decay.  $k_{-1}$  can be estimated from standard curve-fitting programs and used to calculate the  $T_{10\%}$  in the previous equation. If significant cGMP dissociation from PDE6 is occurring faster than the filtration process can be completed, lowering the temperature to 4°C may retard the dissociation reaction sufficiently.

### 3.3. Ammonium Sulfate Stabilization of cGMP Binding to PDE6

Under conditions in which cGMP dissociation from the PDE6 GAF domains is occurring during the standard membrane filtration procedure, high concentrations of ammonium sulfate can be used to effectively retain bound cGMP

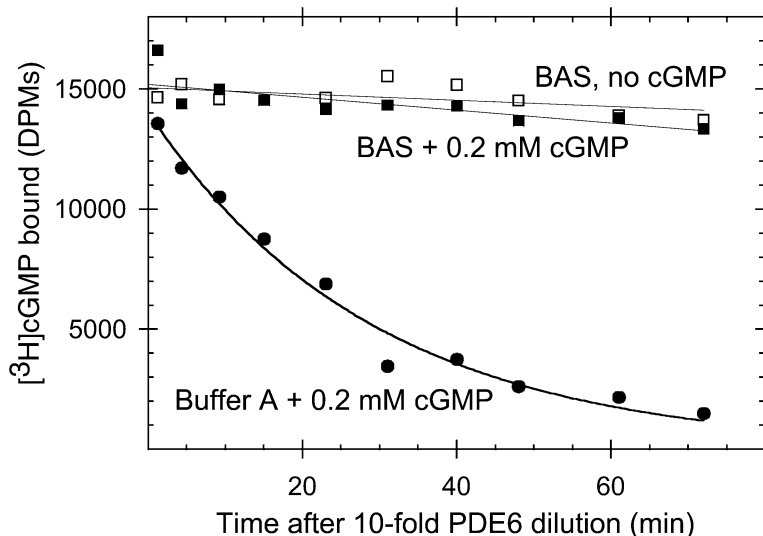


Fig. 1. Ammonium sulfate stops cGMP exchange at the GAF domain of rod PDE6. Rod PDE6 (24 nM) was incubated with 200 nM [ $^3\text{H}$ ]cGMP for 30 min at 4°C. At time zero, portions (200  $\mu\text{L}$ ) were added to 2.0 mL of the following solutions (at 4°C): an isotonic solution (buffer A) containing 0.2 mM nonradioactive cGMP (filled circles), buffered 95% ammonium sulfate (BAS) either lacking (open squares) or containing (filled squares) 0.2 mM cGMP. Aliquots were removed for membrane filtration at the indicated times. Even in the presence of a large excess of cold cGMP, the ammonium sulfate solution essentially halted cGMP exchange at the GAF domains of PDE6. In contrast, dissociation of [ $^3\text{H}$ ]cGMP from PDE6 followed an exponential decay when cold cGMP was added in Buffer A.

during filtration (*see Fig. 1*). The use of ammonium sulfate is also appropriate for dissociation kinetics experiments, in which the dissociation reaction can be effectively halted on addition of PDE6 to the ammonium sulfate solution. This approach has previously been used for studying cyclic nucleotide-dependent protein kinases (10–12), PDE2 (13), and PDE5 (14).

### 3.3.1. Halting of cGMP Dissociation from PDE6 GAF Domains Using Buffered Ammonium Sulfate Stop Solution

1. Chill microcentrifuge tubes containing 200  $\mu\text{L}$  of 95% BAS to 4°C in an ice water bath.
2. Prepare PDE6 ( $\geq 5$  nM) as described in **Subheading 3.1.**, and incubate with [ $^3\text{H}$ ]cGMP in buffer A (containing zaprinast or EDTA to block cGMP hydrolysis). Permit the binding reaction to reach equilibrium.
3. Add a 20- $\mu\text{L}$  portion of the PDE-[ $^3\text{H}$ ]cGMP mixture to the 200- $\mu\text{L}$  BAS solution and mix well.

4. After adding each of the PDE samples to the BAS stop solution, keep samples ice cold until filtration is performed.
5. Include samples to determine the extent of nonspecific binding (*see Subheading 3.2.3.*) and to quantify the total disintegrations per minute added to the BAS.
6. Prewet Millipore filters with 2 mL of BAS just before use. Allow the filters to become fully wetted before applying a vacuum.
7. Apply the 220- $\mu$ L PDE-containing sample to the prewet filters.
8. Rinse immediately with three 1-mL portions of ice-cold BAS.
9. Allow the filters to dry partially under a vacuum for approx 1 min, and then place the filters in 20-mL scintillation vials with 2.0 mL of 2% SDS (*see Note 5*). Shake the vials for 10 min.
10. Add 3.5 mL of Ultima Gold XR, and mix well.
11. Count the samples in a liquid scintillation counter.

### **3.4. Centrifugal Separation of Membrane-Associated PDE6 Through Silicone Oil**

A major drawback of membrane filtration described in **Subheading 3.2.** is that low-affinity cGMP-binding sites might not be detected, because of rapid dissociation during the filtration and washing steps. True equilibrium binding methods such as equilibrium dialysis are well suited to studying low-affinity ligand-binding sites but often have high nonspecific binding, require large quantities of binding protein, and are laborious to perform (**15**). This section describes centrifugation of PDE6 through silicone oil, in which radiolabeled cGMP bound to membrane-associated PDE6 is sedimented through the oil, while leaving unbound [ $^3\text{H}$ ]cGMP in the upper aqueous layer (**16**). This method minimizes disruption of the cGMP-binding equilibrium, while reducing greatly the nonspecific entrapment of unbound [ $^3\text{H}$ ]cGMP with the pelleted PDE6. This centrifugal separation method has been successfully used to validate the membrane filtration method, to identify a class of lower-affinity cGMP-binding sites on PDE6, and to correlate the dissociation of bound cGMP with the dissociation of the inhibitory  $\gamma$ -subunit from the PDE6 holoenzyme (**8,16**).

#### **3.4.1. Preparation of Centrifuge Tubes Containing Silicone Oil**

1. Mix the higher (1.07 g/mL)- and lower (0.818 g/mL)-density silicone oils to obtain a final density of 1.02 g/mL. The refractive index of the oils can be used to estimate the density of the oil mixture.
2. Add 100  $\mu$ L of the 1.02 g/mL silicone oil mixture to a 5  $\times$  20 mm centrifuge tube (*see Note 6*).
3. Briefly spin the centrifuge tubes to ensure that no oil adheres to the side of the tube.

### 3.4.2. Preparation of Rod Outer Segment Membranes Containing Bound PDE6 and Incubation With [ $^3\text{H}$ ]cGMP

The procedures for preparing disrupted rod outer segment (ROS) membranes and removing soluble proteins are described in **Chapter 10, Subheading 3.2.1.**

1. Treat the PDE6-containing ROS membranes as described in this chapter, **Subheading 3.1.2.** to deplete endogenous cGMP from the PDE6 GAF domains (*see Note 7*).
2. Incubate nucleotide-depleted ROS membranes containing 60 nM PDE6 with [ $^3\text{H}$ ]cGMP in buffer A supplemented with catalytic site inhibitors (as described in **Subheading 3.1.3.**). Allow the binding reaction to come to equilibrium at room temperature.

### 3.4.3. High-Speed Centrifugation of Membrane-Associated PDE6 Through Silicone Oil and Quantitation of Bound [ $^3\text{H}$ ]cGMP

1. Layer portions (10  $\mu\text{L}$ ) of ROS homogenates preincubated with [ $^3\text{H}$ ]cGMP on top of the silicone oil in centrifuge tubes.
2. Centrifuge the tubes at 90,000 rpm (110,000 $g_{\text{av}}$ ) for 3 min at room temperature. The PDE6-containing ROS membranes are sedimented through the silicone oil layer, while the aqueous layer remains above the oil (*see Note 8*).
3. Immerse the tubes in a dry ice–ethanol bath to freeze the aqueous layer.
4. Cut the tubes with a razor blade through the oil layer just above the pelleted membranes, and discard the frozen top layer.
5. Transfer the contents of the bottom portion of the tubes to a scintillation vial. Resuspend the pellets at the bottom of the tube with 50  $\mu\text{L}$  of 2% SDS, and then transfer to the scintillation vial.
6. Add 4 mL of Ultima Gold XR to the vial, mix thoroughly, and quantify the radioactivity in a scintillation counter.

### 3.4.4. Verification of Efficacy of Centrifugal Separation of ROS Membranes From Aqueous Layer

Quantitative sedimentation of the ROS membranes can be estimated by spectroscopic determination of the rhodopsin concentration (**17**) in the pellet fraction. This requires that the ROS membrane preparation and centrifugation be carried out under infrared illumination conditions. Nonspecific entrapment of unbound [ $^3\text{H}$ ]cGMP can be estimated exactly as described in **Subheading 3.2.3.** In addition,  $^{14}\text{C}$ -labeled compounds that do not bind to ROS membranes (e.g., sorbitol) can be included with the [ $^3\text{H}$ ]cGMP-binding mixture to assess the efficacy of the separation procedure (*see Note 9*).

## 3.5. Analysis and Interpretation of Radiolabeled cGMP-Binding Data

Because the type of data analysis depends on the nature of the experiment, this section presents some general guidelines that are relevant to all analyses of radiolabeled cGMP-binding experiments.

Transformation of ligand-binding data for graphic interpretation of the results using simple linear regression (e.g., Scatchard plot) should be avoided (18). With the ready availability of powerful nonlinear curve-fitting applications (e.g., Sigmaplot, Graphpad Prism, Origin), the experimental results (corrected for non-specific binding) can be fit to an exact mathematical model, and a statistical analysis of the goodness of fit can be reviewed. One specialized program, KELL (Biosoft), uses an iteratively weighted, nonlinear curve-fitting routine to specifically analyze radioligand-binding experiments (19). This program is particularly useful when the variance of the data as a function of ligand concentration is known, or when ligand depletion owing to receptor binding is significant (20). KELL also offers the advantage of several built-in statistical tests that help assess the degree to which the chosen model fits the actual data; this is important when multiple classes of ligand-binding sites may exist. However, in most instances, we have found that judicious use of standard curve-fitting programs such as Sigmaplot works well for interpreting the results of [<sup>3</sup>H]cGMP binding to PDE6.

For saturation-binding studies in which the extent of [<sup>3</sup>H]cGMP binding to PDE6 is measured as a function of the total cGMP concentration added, the binding affinity ( $K_D$ ) and the maximum binding ( $B_{\max}$ ) can be estimated from fitting the data to a two-parameter hyperbola (one class of sites) or four-parameter double hyperbola (two classes of binding sites). Various statistical tests can be applied to the results to decide which model is most appropriate for the data (21). In practice, six to eight data points covering 10–90% of  $B_{\max}$  are needed to determine accurately the affinity and density of a single class of sites. To resolve two classes of binding sites whose affinity differs by less than 100-fold, our experience is that 15–20 data points that span the range of 10–90% of  $B_{\max}$  are needed to discern accurately the individual classes of sites. A final caution is that low-affinity cGMP-binding sites are sometimes difficult to demonstrate because relatively small errors in estimating the magnitude of nonspecific binding can be mistaken for a low-affinity binding site.

Graphic analysis of the kinetics of [<sup>3</sup>H]cGMP dissociation from PDE6 GAF domains following the addition of a large excess of nonradioactive cGMP (cold chase) can be fit to a two-parameter (one class of sites) or four-parameter (two sites) exponential decay model to estimate the dissociation rate constant ( $k_{-1}$ ) and the concentration for each class of cGMP-binding sites. Similar considerations to those described for statistical analysis and experimental design of saturation-binding studies apply equally to kinetic measurements of cGMP dissociation.

#### 4. Notes

1. The state of activation, membrane attachment, and species from which PDE6 is isolated all influence the cGMP-binding properties of the enzyme. Differences also exist between rod and cone PDE6 from a given species.

- a. The state of activation of PDE6 greatly influences the affinity and stoichiometry of cGMP binding. Nonactivated PDE6 holoenzyme ( $\alpha\beta\gamma\gamma$ ) exhibits a single class of high-affinity binding sites that exchange slowly their bound cGMP with cGMP in solution (6,22). PDE6 activated by transducin or by physical removal of the inhibitory  $\gamma$ -subunits has two classes of nonidentical binding sites (5,8). Rod PDE6 catalytic dimers ( $\alpha\beta$ ) can be reconstituted with recombinant  $\gamma$  or with peptide fragments of  $\gamma$  containing a GAF-stimulatory domain (amino acids 18–41 of the  $\gamma$  sequence), thereby restoring the binding properties of the PDE6 holoenzyme (6,23).
  - b. The exchange rates of cGMP with the GAF domains of rod PDE6 are slower for PDE6 attached to cell membranes than is observed for PDE6 that is hypotonically extracted from the membrane. It is unclear whether this is owing to an effect of the isoprenyl moieties that anchor PDE6 to the membrane, or whether the procedure to release PDE6 from the membrane alters the interaction of a PDE6-binding protein that modulates cGMP affinity to PDE6.
  - c. Species differences in the cGMP-binding properties of PDE6 exist. In general, mammalian rod PDE6 has higher affinity for cGMP (6,22) than amphibian rod PDE6 (4,5,8).
  - d. Although not as well characterized as the rod enzyme, cone PDE6 appears to have the same binding stoichiometry for cGMP as rod PDE6 but binds cGMP with lower affinity (24,25).
2. EDTA acts by chelating divalent cations that are required for the catalytic mechanism of cGMP hydrolysis. EDTA treatment is most effective for PDE6 catalytic dimers lacking bound  $\gamma$ -subunits. In the case of PDE6 holoenzyme, EDTA is much less effective, presumably because the  $\gamma$ -subunit binds in the active site and prevents dissociation of the divalent cations responsible for catalysis (26).
  3. Previous studies have shown that the cGMP-binding GAF domains are highly specific for cGMP (27) and bind PDE5/6 inhibitors very poorly (26). Thus, drugs acting on the catalytic site do not greatly influence cGMP-binding properties at the GAF domains.
  4. The exact incubation conditions depend on the purpose of the experiment. To give one specific example, to measure the maximum extent to which cGMP can bind to a nonactivated PDE6 preparation ( $B_{\max}$ ), incubate 25  $\mu\text{L}$  of 6.0 nM PDE6 with 500 nM [ $^3\text{H}$ ]cGMP (specific activity =  $3.5 \times 10^{16}$  disintegrations per min, per mol) for 30 min at 4°C. Under these conditions, high-affinity cGMP-binding sites will be saturated, and specific binding will be more than 10-fold higher than the level of nonspecific binding and easily quantified in a scintillation counter.
  5. The use of 2% SDS is unnecessary in the standard membrane filtration procedure but is helpful to solubilize rapidly the radioactive cGMP when ammonium sulfate is used. Alternatively, the dried filters can be shaken for 48 h in scintillation fluid (in the absence of SDS) with similar results.
  6. To facilitate removal of the pelleted ROS membranes following centrifugation, 5  $\mu\text{L}$  of 50% glycerol can be added to the bottom of the tube prior to the addition of the oil.

7. Bovine rod PDE6 in its membrane-associated state is not easily depleted of all endogenous bound cGMP. One of the two high-affinity sites is much more easily exchangeable than the second cGMP site (6). By contrast, membrane-associated amphibian rod PDE6 can be depleted of all endogenous cGMP using the method in **Subheading 3.1.2. (4,8)**.
8. The method used to disrupt intact ROS and to prepare soluble protein-depleted ROS membranes can influence the final density of the ROS membranes used for these experiments. If inversion of the aqueous and oil layers is observed, then the density of the oil may need to be increased up to 1.028 g/mL. Incomplete sedimentation of the ROS membranes (determined by <100% recovery of rhodopsin in the pellet) will require lowering the oil density.
9. With this method, no radioactivity can be detected in the silicone oil layer itself, and less than 0.04% of the total [<sup>14</sup>C]sorbitol is found in the pellet fraction using this procedure.

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## References

1. Taylor, W. R. and Baylor, D. A. (1995) Conductance and kinetics of single cGMP-activated channels in salamander rod outer segments. *J. Physiol. (Lond.)* **483**, 567–582.
2. Artemyev, N. O., Arshavsky, V. Y., and Cote, R. H. (1998) Photoreceptor phosphodiesterase: interaction of inhibitory  $\gamma$  subunit and cyclic GMP with specific binding sites on catalytic subunits. *Methods* **14**, 93–104.
3. Yamazaki, A., Sen, I., Bitensky, M. W., Casnellie, J. E., and Greengard, P. (1980) Cyclic GMP-specific, high affinity, noncatalytic binding sites on light-activated phosphodiesterase. *J. Biol. Chem.* **255**, 11,619–11,624.
4. Cote, R. H. and Brunnock, M. A. (1993) Intracellular cGMP concentration in rod photoreceptors is regulated by binding to high and moderate affinity cGMP binding sites. *J. Biol. Chem.* **268**, 17,190–17,198.
5. Cote, R. H., Bownds, M. D., and Arshavsky, V. Y. (1994) cGMP binding sites on photoreceptor phosphodiesterase: role in feedback regulation of visual transduction. *Proc. Natl. Acad. Sci. USA* **91**, 4845–4849.
6. Mou, H., Grazio, H. J., Cook, T. A., Beavo, J. A., and Cote, R. H. (1999) cGMP binding to noncatalytic sites on mammalian rod photoreceptor phosphodiesterase is regulated by binding of its  $\gamma$  and  $\delta$  subunits. *J. Biol. Chem.* **274**, 18,813–18,820.
7. Kincaid, R. L. and Manganiello, V. C. (1988) Assay of cyclic nucleotide phosphodiesterase using radiolabeled and fluorescent substrates. *Methods Enzymol.* **159**, 457–470.

8. Norton, A. W., D'Amours, M. R., Grazio, H. J., Hebert, T. L., and Cote, R. H. (2000) Mechanism of transducin activation of frog rod photoreceptor phosphodiesterase: allosteric interactions between the inhibitory  $\gamma$  subunit and the noncatalytic cGMP binding sites. *J. Biol. Chem.* **275**, 38,611–38,619.
9. Bennett, J. P. Jr. and Yamamura, H. I. (1985) Neurotransmitter, hormone, or drug receptor binding methods, in *Neurotransmitter Receptor Binding*, 2nd ed. (Yamamura, H. I., ed.), Raven, New York, pp. 61–89.
10. Doskeland, S. O., Ueland, P. M., and Haga, H. J. (1977) Factors affecting the binding of [ $^3$ H] adenosine 3':5'-cyclic monophosphate to protein kinase from bovine adrenal cortex. *Biochem. J.* **161**, 653–665.
11. Corbin, J. D. and Doskeland, S. O. (1983) Studies of two different intrachain cGMP-binding sites of cGMP-dependent protein kinase. *J. Biol. Chem.* **258**, 11,391–11,397.
12. Doskeland, S. O. and OGREID, D. (1988) Ammonium sulfate precipitation assay for the study of cyclic nucleotide binding to proteins. *Methods Enzymol.* **159**, 147–151.
13. Miot, F., Van Haastert, P. J. M., and Erneux, C. (1985) Specificity of cGMP binding to a purified cGMP stimulated phosphodiesterase from bovine adrenal tissue. *Eur. J. Biochem.* **149**, 59–65.
14. Coquil, J. F., Franks, D. J., Wells, J. N., Dupuis, M., and Hamet, P. (1980) Characteristics of a new binding protein distinct from the kinase for guanosine 3'-5'-monophosphate in rat platelets. *Biochim. Biophys. Acta* **631**, 148–165.
15. Hulme, E. C. (1990) Receptor binding studies, a brief outline, in *Receptor-Effector Coupling, A Practical Approach* (Hulme, E. C., ed.), Oxford University Press, UK, pp. 203–215.
16. Forget, R. S., Martin, J. E., and Cote, R. H. (1993) A centrifugal separation procedure detects moderate affinity cGMP binding sites in membrane-associated proteins and permeabilized cells. *Anal. Biochem.* **215**, 159–161.
17. Bownds, D., Gordon-Walker, A., Gaide Huguenin, A. C., and Robinson, W. (1971) Characterization and analysis of frog photoreceptor membranes. *J. Gen. Physiol.* **58**, 225–237.
18. Leatherbarrow, R. J. (1990) Using linear and non-linear regression to fit biochemical data. *Trends Biochem. Sci.* **15**, 455–458.
19. McPherson, G. A. (1985) Analysis of radioligand binding experiments on a microcomputer system. *J. Pharmacol. Meth.* **14**, 213–228.
20. Munson, P. J. (1983) LIGAND: a computerized analysis of ligand binding data. *Methods Enzymol.* **92**, 543–576.
21. Munson, P. J. and Rodbard, D. (1980) LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* **107**, 220–239.
22. Gillespie, P. G. and Beavo, J. A. (1989) cGMP is tightly bound to bovine retinal rod phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **86**, 4311–4315.
23. Mou, H. and Cote, R. H. (2001) The catalytic and GAF domains of the rod cGMP phosphodiesterase (PDE6) heterodimer are regulated by distinct regions of its inhibitory  $\gamma$  subunit. *J. Biol. Chem.* **276**, 27,527–27,534.

24. Gillespie, P. G. and Beavo, J. A. (1988) Characterization of a bovine cone photoreceptor phosphodiesterase purified by cyclic GMP–Sephadex chromatography. *J. Biol. Chem.* **263**, 8133–8141.
25. Cote, R. H., Daly, A. E., Valeriani, B. A., and Vardi, N. (2002) Regulation of cone photoreceptor phosphodiesterase (PDE6C) by its inhibitory  $\gamma$  subunit and by cGMP binding. *Invest. Ophthalmol. Vis. Sci.* **43**, ARVO E-Abstract 1960.
26. D'Amours, M. R., Granovsky, A. E., Artemyev, N. O., and Cote, R. H. (1999) The potency and mechanism of action of E4021, a PDE5-selective inhibitor, on the photoreceptor phosphodiesterase depends on its state of activation. *Mol. Pharmacol.* **55**, 508–514.
27. Hebert, M. C., Schwede, F., Jastorff, B., and Cote, R. H. (1998). Structural features of the noncatalytic cGMP binding sites of frog photoreceptor phosphodiesterase using cGMP analogs. *J. Biol. Chem.* **273**, 5557–5565.