Guanosine 3',5'-cyclic nucleotide binding proteins of bovine retina identified by photoaffinity labeling

(8-azido-cGMP/rod outer segments/cGMP phosphodiesterase/cGMP-dependent protein kinase/cGMP-activated conductance)

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ABSTRACT Cyclic GMP-binding proteins present in membrane fractions of bovine retina and, in particular, rod outer segments (ROS) were identified by photoaffinity labeling with 8-azido-[³²P]cGMP. Two soluble proteins and two membrane-associated proteins were specifically labeled. The soluble proteins, 93 and 72 kDa, corresponded respectively to the α subunit of ROS cGMP phosphodiesterase and cGMP-dependent protein kinase. One of the two membrane-associated proteins, 53 kDa, was present in all particulate retinal fractions. Its function is unknown. It is distinct from cAMP-dependent protein kinase or the 63-kDa cGMP-activated channel from ROS. The second membrane-associated protein, 37 kDa, was present only in fractions that did not contain ROS. The molecular mass of this protein was similar to that of a cGMP-binding protein previously attributed to rod cells.

Signal transduction in vertebrate photoreceptors centers around the regulation of intracellular cGMP. Illumination of rhodopsin in rod outer segments (ROS) leads to the activation of a guanine nucleotide-binding protein that, in turn, activates a cGMP phosphodiesterase (1). The resulting hydrolysis of cGMP is thought to bring about the closure of plasma membrane cGMP-gated cation channels, leading to hyperpolarization of the cell and decreased neurotransmitter release (2). The channels reopen when cGMP levels are restored through the action of a calcium-sensitive guanylate cyclase (3). In addition, cGMP is reported to initiate calcium efflux from purified disc membranes (4) and to stimulate the phosphorylation of ROS proteins (5, 6). Understanding the role of cGMP in visual signal transduction will be greatly aided by the biochemical characterization of the cGMP-binding proteins responsible for all of these activities. To date, the cGMP phosphodiesterase and a cGMP-gated channel have been characterized. The cGMP phosphodiesterase is a hetero-oligomeric protein composed of α , β , and γ subunits (7) and is negatively regulated by its γ subunit (8). The cGMP-gated channel from the plasma membrane of bovine ROS consists of a single 63-kDa polypeptide and is thought to be the light-sensitive channel (9). However, a 39-kDa protein identified by photoaffinity labeling has also been claimed to exhibit cGMP-dependent channel activity (10).

In the present work we have used 8-azido-[³²P]cGMP to radioactively label cGMP-binding proteins present in ROS and retina. Under conditions that inhibited nonspecific incorporation of radioactivity, three cGMP-binding proteins were identified in ROS, while a fourth protein was identified in membrane fractions of the retina that did not contain ROS. These cGMP-binding proteins have been characterized in terms of their size, solubility, and nucleotide specificity and are evaluated in relationship to previously identified cyclic nucleotide-binding proteins.

MATERIALS AND METHODS

Materials. 8-Azido-[³²P]cGMP was from ICN Radiochemicals. Protease inhibitors, chymotrypsin, and cAMP-agarose were from Sigma. Frozen bovine retinæ were purchased from J. Lawson Co. (Lincoln, NE). Fresh bovine retinæ were from Arena and Sons (Hopkinton, MA). ¹⁴C-labeled protein molecular mass standards were from Bethesda Research Laboratories. A monoclonal antibody (PMc 1D1) that binds to the 63-kDa channel from bovine ROS plasma membrane (11) was provided by Robert Molday (University of British Columbia). Polyclonal antiserum against cGMP-dependent protein kinase from lung (12), as well as the kinase, were obtained from Terry Woodford and Jackie Corbin (Vanderbilt University). The regulatory subunit of cAMP-dependent protein kinase (type II) from porcine heart was the gift of Susan S. Taylor (University of California, San Diego).

Fractionation of the Retina and Preparation of ROS. ROS were isolated by using Papermaster's procedure (13) modified as follows. Phenylmethylsulfonyl fluoride (0.1 mM), aprotinin (5 μ g/ml), leupeptin (2 μ g/ml), pepstatin (2 μ g/ml), and *o*-phenanthroline (100 μ g/ml) were included in all solutions. Retinæ in homogenizing medium were disrupted by mixing in a Vortex instead of homogenization and then were centrifuged (4000 $\times$ g for 4 min). The ROS-containing supernates were diluted 1:2.5 with 10 mM Tris acetate, pH 7.4/150 mM NaCl (buffer A), and the crude ROS were collected by centrifugation (4000 $\times$ g for 6 min). The supernate was recentrifuged (40,000 $\times$ g for 30 min), and the pellets were designated "ROS-depleted homogenate." The pellets from the initial homogenization of the retina were rehomogenized for 1 min in buffer A by using a Tekmar Tissuemizer; the membranes were collected by centrifugation (40,000 $\times$ g for 30 min) and designated "ROS-depleted retina." The crude ROS pellets from fresh retinæ were resuspended and centrifuged on discontinuous sucrose gradients as described (13). Crude ROS from frozen retinæ were layered on a 1.15 density sucrose cushion, collected at the interface by centrifugation (100,000 $\times$ g for 45 min), and centrifuged on sucrose gradients. The four fractions recovered were: the material at the top of the gradient (1.10/1.11 density interface), the band 1 purified ROS (1.11/1.13 density interface), the band 2 impure ROS (1.13/1.15 density interface), and the material at the bottom of the gradient. Each fraction was diluted 1:3 with buffer A; centrifuged at 40,000 $\times$ g for 30 min; resuspended in 40% sucrose/10 mM Tris-HCl, pH 7.4; frozen in liquid N₂; and stored at -70°C. The purity of the ROS preparations was assessed by the A_{280}/A_{500} ratio and ranged from 2.1 to 2.5.

Washed ROS and retinal fractions were prepared by washing twice with buffer A, washing four times with 10 mM Tris-HCl (pH 7.4) using Potter-Elvehjem homogenization,

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Abbreviation: ROS, rod outer segment(s).

and centrifuging at $150,000 \times g$ for 30 min. Urea-washed ROS were prepared as described (14).

Photoaffinity Labeling. Membrane samples. 8-Azido- ^{32}P cGMP (4–8 Ci/mmol; 1 Ci = 37 GBq) in methanol was dried *in vacuo* in plastic centrifuge tubes. To the residue was added buffer B [10 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM MgCl_2 /0.5 mM isobutylmethylxanthine (IBMX)] with or without 2 mM competing nucleotide, followed by 75 μg of the protein in 20% sucrose (final volume = 100 μl containing 5–10 μM 8-azido- ^{32}P cGMP). The samples were incubated at 4°C for 1 hr, diluted 1:15 with buffer B without IBMX, and then pelleted ($12,000 \times g$ for 30 min). After resuspension in 100 μl of the above buffer containing 20% sucrose, the samples were made 0.5 mM in dithiothreitol and immediately irradiated on ice at 253.7 nm for 7 min with a RPR-2537A Rayonet UV lamp at a distance of 8 cm ($\approx 13 \text{ mW/cm}^2$). The samples were again diluted, pelleted, and dissolved in 100 μl of sodium dodecyl sulfate (SDS) sample buffer (2% SDS/2% β -mercaptoethanol/10% glycerol/62.5 mM Tris-HCl, pH 6.8).

Soluble samples. Soluble proteins in 0.1 μM 8-azido- ^{32}P cGMP and buffer B containing 0.05 mM IBMX with or without 0.1 mM 8-azido-cGMP were incubated and photolyzed as above.

Proteolysis. Twenty nanograms of chymotrypsin and 1 mM cAMP were added to samples directly after photolysis and incubated for 1 hr at 4°C . The digestion was stopped by addition of 0.5 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone followed by SDS sample buffer.

cAMP-Agarose Binding. Protein (2 mg) from fractions of the retina generated during the preparation of ROS was incubated overnight with cAMP-agarose (50 μl) in Tris-HCl, pH 7.5/150 mM NaCl/2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) at 4°C . The resin was extensively washed with the same buffer, and bound protein was eluted with SDS sample buffer.

Other Methods. 8-Azido-cGMP was synthesized as described by Haley (15). 8-Amino-cGMP was synthesized as described (16) and purified by DEAE-chromatography as for 8-azido-cGMP. cGMP phosphodiesterase from ROS was partially purified by chromatography on DEAE-cellulose (7). Protein was determined as described by Peterson (17). Protein immunoblotting was as described by Towbin *et al.* (18). SDS/polyacrylamide gel electrophoresis on low-crosslinked gels was as described by Baehr *et al.* (7). Gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat film with intensifying screens at -70°C for 5–8 days.

RESULTS

Photoaffinity Labeling of ROS Proteins with 8-Azido- ^{32}P cGMP. Suspensions of ROS were incubated with 8-azido- ^{32}P cGMP, unbound nucleotide was removed by diluting and pelleting the membranes, and the resuspended ROS were irradiated as described. Fig. 1A shows the Coomassie blue staining patterns of purified ROS and washed ROS on SDS/polyacrylamide gels, and Fig. 1B and C show autoradiographs of photolabeled samples.

When purified ROS were treated with 8-azido- ^{32}P cGMP, three proteins of 93, 72, and 53 kDa were specifically photoaffinity-labeled (Fig. 1B, lane 2). These proteins were not significantly labeled in nonirradiated samples or in samples incubated with an excess of unlabeled 8-azido-cGMP (Fig. 1B, lanes 1 and 3). In contrast, the radioactivity seen in rhodopsin and rhodopsin dimer (32 and 58 kDa), guanine nucleotide binding protein and β subunits (38 and 36 kDa), 88- and 220-kDa proteins, and phospholipid (migrating ahead of the dye front) was present in samples and controls alike and, therefore, appeared to result from nonspecific incorporation of the photoactivatable compound. When ROS washed free of soluble proteins were treated with 8-azido- ^{32}P cGMP,

FIG. 1. ROS proteins photoaffinity-labeled with 8-azido- ^{32}P cGMP analyzed by SDS/polyacrylamide gel electrophoresis and autoradiography. (A) Coomassie blue-stained gel of purified ROS (lane 1) and washed ROS (lane 2). (B) Autoradiograph of purified ROS treated with 10 μM 8-azido- ^{32}P cGMP. Samples were either nonirradiated (lane 1), irradiated at 254 nm (lane 2), or included 2 mM 8-azido-cGMP during the preincubation and were irradiated (lane 3). (C) Autoradiograph of washed ROS treated as in B.

only the 53-kDa band remained associated with the membranes and was specifically labeled (Fig. 1C). The 93- and 53-kDa proteins were labeled by 8-azido- ^{32}P cGMP in all preparations of ROS proteins tested; however, the appearance of the 72-kDa protein was more variable (see below). Labeling of the 53-kDa protein was very sensitive to the purity of the photoactivatable compound. No qualitative differences were seen between ROS prepared from fresh and frozen retinæ.

Nonspecific incorporation of radioactivity in ROS samples correlated, in most cases, with high-abundance proteins (rhodopsin, rhodopsin dimer, and G-protein). However, two less-abundant proteins, the 88- and 220-kDa proteins, consistently incorporated label that was not competitively blocked by nonradioactive nucleotide. It is possible that the nonspecific labeling of the 220-kDa protein reflects the presence of a nucleotide binding pocket. A protein of the same size was identified in rat ROS by using 8-azido- ^{32}P ATP (19). Our experiments showed no evidence of a 250-kDa protein reported to be specific for cGMP that was photolabeled in frog ROS by direct irradiation with ^3H cGMP (20).

Nucleotide Specificity of the Proteins Labeled with 8-Azido- ^{32}P cGMP. Specificity was determined by examining the ability of various nucleotides to inhibit the incorporation of 8-azido- ^{32}P cGMP. The autoradiograph in Fig. 2A shows the results of an experiment performed with purified ROS. Labeling of the 93-kDa protein was inhibited by excess cGMP, its C^8 and $\text{O}^{2'}$ analogs, but not the N^2 analogs. The nucleotides GMP, GDP, and GTP as well as cAMP, AMP, ADP, and ATP were not effective competitors. The 53-kDa protein exhibited a different specificity. Incorporation of 8-azido- ^{32}P cGMP was not inhibited by cGMP in purified ROS (or hypotonically washed ROS) under conditions where 8-azido-cGMP, other C^8 analogs of cGMP, and cAMP competed. However, cGMP was an effective competitor in preparations of urea-washed ROS (Fig. 2B). Furthermore, labeling in urea-washed membranes appeared to be stronger in general. Urea treatment probably served to remove tightly bound endogenous nucleotide that interfered with binding of exogenously added compounds. The nucleotide preference of the 53-kDa protein, determined by incubating urea-washed ROS with 8-azido- ^{32}P cGMP (7.5 μM) and various nucleotides in a range of concentrations (1 μM to 1 mM), was for cAMP over cGMP or 8-azido-cGMP. The 53-kDa protein also bound derivatives of cGMP in the $\text{O}^{2'}$ but not the N^2 position, cIMP and cCMP.

FIG. 2. Competition of the photoaffinity labeling of ROS proteins with 8-azido-[32 P]cGMP by various nucleotides. ROS were incubated with 10 μ M 8-azido-[32 P]cGMP in the absence or presence of the following nucleotides: 8-azido-cGMP, 8-amino-cGMP, cGMP, GMP, GDP, GTP, cAMP, 8-bromo-cGMP, 2'-O-succinyl-cGMP, N 2 -2'-O-dibutyl-cGMP, cCMP, cIMP, or cXMP; after incubation, the ROS were irradiated, electrophoresed, and autoradiographed. (A) ROS purified as in *Materials and Methods*. (B) Urea-washed ROS. (C) ROS prepared in high-phosphate buffer (21). Rho $_2$, rhodopsin dimers. Rho $_3$, rhodopsin trimers. Markers are as in Fig. 1.

The specificity of the soluble 72-kDa protein was initially difficult to assess because of its low abundance. Retention of the 72-kDa protein and soluble proteins in general depended on the method of ROS preparation used and was found to be greatest in ROS prepared in high-phosphate buffer (21). Representative competitions of ROS prepared in phosphate buffer are shown in Fig. 2C. The specificity of the 72-kDa protein appeared to be similar to that of the 93-kDa protein.

Distribution of the 93-, 72-, and 53-kDa cGMP-Binding Proteins in Fractions of the Retina. To determine whether the proteins that were photoaffinity-labeled by 8-azido-[32 P]cGMP in our experiments were ROS specific, their distribution in membrane fractions of the retina generated during the isolation of ROS from fresh retinæ was investigated. Membranes were treated with 8-azido-[32 P]cGMP with or without excess 8-azido-cGMP as described. Fig. 3A shows the Coomassie blue-stained proteins, and Fig. 3B shows the autoradiograph of the same SDS/polyacrylamide gel.

The 93- and 72-kDa proteins were photoaffinity-labeled only in rhodopsin-containing retinal fractions: purified ROS (band 1), impure ROS (band 2), and the small amount of material that did not enter the sucrose gradients. Labeling of both bands was strongest in the latter fraction, even though more 93-kDa protein was seen by Coomassie blue staining in bands 1 and 2, suggesting the presence of a high-affinity form of the protein. In contrast to the soluble proteins, photolabeling of the 53-kDa protein was not confined to the ROS. The 53-kDa protein was seen in all membrane fractions of the retina tested, apparently at greatest abundance in nonphoto-receptor-containing fractions.

Identification of a 37 kDa cGMP-Binding Protein. The experiments performed on fractions of the retina lead to the discovery of a fourth retinal cGMP-binding protein. A protein of 37 kDa was specifically labeled with 8-azido-[32 P]cGMP in

FIG. 3. Proteins in membrane fractions of the retina, generated during the preparation of ROS, photoaffinity-labeled with 8-azido-[32 P]cGMP. (A) Coomassie blue-stained proteins. (B) Radioactively labeled proteins present in the fractions in A incubated in the absence (lanes -) or presence (lanes +) of unlabeled 8-azido-cGMP and detected by autoradiography. The retinal fractions were: ROS-depleted supernate from the retinal homogenate (lane 1), material that did not enter the sucrose gradient (lane 2), ROS band 1 from the gradient (lane 3), impure ROS band 2 from the gradient (lane 4), material at the bottom of the gradient (lane 5), and ROS-depleted retinæ (lane 6). Seventy-five micrograms of protein was loaded per lane. Markers are as in Fig. 1.

the ROS-depleted supernate from the retinal homogenate and the pelleted material from the sucrose gradients (Fig. 3B, lanes 1 and 5) but was never seen in ROS-containing retinal fractions. The 37-kDa protein remained associated with membranes that were washed extensively with hypotonic buffer but could not be identified in samples that were refrozen and thawed.

The Soluble cGMP-Binding Proteins. The molecular mass and solubility of the 93-kDa protein led us to think it might be a subunit of cGMP phosphodiesterase. When the enzyme was partially purified from ROS hypotonic extracts and analyzed on gels, the α subunit comigrated with the 93-kDa protein (Fig. 4A). In addition, photolabeling the phosphodiesterase preparation resulted in specific incorporation of 8-azido-[32 P]cGMP into the α subunit (Fig. 4B). However, specificity was more difficult to demonstrate than for the enzyme in ROS, and preference for the α subunit was seen only at low concentrations of photoactivatable ligand. This suggests the possibility that light-activated turnover of the enzyme in ROS

FIG. 4. Photoaffinity labeling of partially purified ROS cGMP phosphodiesterase with 8-azido-[32 P]cGMP. (A) Coomassie blue-stained protein. (B) Autoradiograph of samples incubated with 8-azido-[32 P]cGMP in the absence (lane -) or presence (lane +) of unlabeled 8-azido-cGMP. Markers are as in Fig. 1.

may play a role in the specific incorporation of cGMP into the α -subunit binding site. The 88-kDa protein that was nonspecifically photolabeled in ROS comigrated with the β subunit of the phosphodiesterase. A 55-kDa protein was also specifically labeled in the phosphodiesterase preparation. It is probably the regulatory subunit of a soluble form of retinal cAMP-dependent protein kinase that was coeluted from DEAE-cellulose with the phosphodiesterase (5, 22).

The molecular weight and nucleotide specificity of the 72-kDa protein suggested that it might be a retinal form of cGMP-dependent protein kinase, similar to that from other tissues. When antibodies against the lung enzyme (12) were used to probe immunoblots (Western blots) of retinal fractions, a clear positive response was not seen over background. However, when the experiment was performed with material enriched in cyclic nucleotide-binding proteins using cAMP-agarose, an immunoreactive band that comigrated with cGMP-dependent protein kinase from lung was detected in all retinal fractions (Fig. 5A). Its abundance was greatest among the strictly soluble proteins. Our ability to detect the 72-kDa protein in all fractions by immunoblotting is most likely the result of using 25 times more protein than possible in the photoaffinity experiments.

The 53-kDa Cyclic Nucleotide-Binding Protein. The 53-kDa protein was characterized in terms of its possible identity with two other cyclic nucleotide-binding proteins of similar molecular mass: the cGMP-activated channel from ROS and the regulatory subunits of cAMP-dependent protein kinase.

The 63-kDa cGMP-activated cation channel from ROS is recognized by a monoclonal antibody, PMc 1D1 (11). PMc 1D1 was used to probe fractions of retinal proteins on Western blots. In contrast to the protein identified by photoaffinity labeling, the immunoreactive protein was seen only in ROS-containing fractions (band 1 and band 2) and ran with lower relative mobility (Fig. 5B).

The regulatory subunits of cAMP-dependent protein kinases contain a very susceptible hinge region that, when cleaved by proteases, yields an ≈ 37 -kDa fragment retaining bound nucleotide (23). Urea-washed ROS were photoaffinity-labeled with 8-azido-[32 P]cGMP and then incubated with chymotrypsin under conditions that resulted in limited proteolysis of added regulatory subunit from the type II heart

FIG. 5. Western blot analysis of fractionated retinal proteins. (A) Cyclic nucleotide-binding proteins present in fractions of the retina were enriched by binding to cAMP-agarose. Proteins transferred to nitrocellulose were probed with polyclonal antiserum raised against cGMP-dependent protein kinase purified from lung (12). cGMP-dependent protein kinase from lung is shown (PKG). (B) Retinal membrane fractions were probed with a monoclonal antibody (PMc 1D1) specific for the 63-kDa channel from bovine ROS plasma membrane (11). Immunoreactive proteins were detected by using alkaline phosphatase-conjugated second antibody. The retinal fractions are as in Fig. 3. Markers are as in Fig. 1.

FIG. 6. Limited chymotryptic hydrolysis of ROS proteins photoaffinity-labeled with 8-azido-[32 P]cGMP in the absence or presence of the regulatory subunit of cAMP-dependent protein kinase (type II). (A) Autoradiograph of urea-washed ROS treated with 7.5 μ M 8-azido-[32 P]cGMP in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of unlabeled 8-azido-cGMP and then treated with chymotrypsin (lanes 3 and 4). (B) Samples as in A to which regulatory subunit was added. RII, the regulatory subunit of cAMP-dependent protein kinase, type II. cRII, the major chymotryptic fragment of RII. Markers are as in Fig. 1.

enzyme (Fig. 6). The retinal protein was not degraded by chymotrypsin, even when detergent was added to disrupt membrane vesicles. The results indicate that the 53-kDa protein is not identical with cAMP-dependent protein kinases from other sources but leave open the possibility that it may be a unique form of the enzyme.

DISCUSSION

With the goal of identifying the proteins that regulate cGMP metabolism in the retina, we have investigated photoaffinity labeling with 8-azido-[32 P]cGMP. Specific labeling was accomplished by incubating protein samples with the photoactivatable ligand at high concentration, diluting, and removing unbound nucleotide prior to irradiation. Such a procedure could result in the loss of certain soluble proteins or the oversight of low-affinity cGMP-binding proteins, or both. However, this liability was far outweighed by the specificity achieved and the confidence with which cGMP-binding proteins could be identified.

Prior to the present work, a number of laboratories had carried out studies addressing the nucleotide binding and photoaffinity labeling of the cGMP phosphodiesterase from ROS. The work of Bitensky and coworkers led to the proposal that the enzyme has two types of binding sites, catalytic and noncatalytic (24). Recent work by Beavo and coworkers indicates that the ROS enzyme binds cGMP with extremely high affinity and retains 1.8–1.9 mol of cGMP per mol of purified protein (25). The question whether these sites are located on the α and/or β subunits has not been answered. When proteins from toad ROS were photoaffinity-labeled with 8-azido-[32 P]cIMP, the α and β subunits were not distinguished from one another (24). When direct UV-irradiation was used to label bovine ROS with [32 P]cGMP (26) or frog ROS with [3 H]cGMP (20), the α and β subunits were labeled to the same extent.

Our own studies show that the phosphodiesterase is labeled only on the α subunit specifically with 8-azido-[32 P]cGMP. The difference between our results and those obtained with the underivatized nucleotide could be accounted for if 8-azido-cGMP binds to one class of structurally

distinct binding sites, whereas cGMP binds to both. However, it seems equally likely that the high concentrations of nucleotide used in the studies cited above resulted in non-specific labeling. Experiments we performed at similar concentrations resulted in nonspecific incorporation of label into many proteins in addition to the α subunit, including the β subunit. Our results do not exclude the possibility, however, that the β subunit contains a very high affinity binding site that did not exchange during our experiments, since a small amount of radioactivity was consistently incorporated into the β subunit under all conditions. Specific labeling of the α subunit was best demonstrated with the enzyme in ROS, where light-activation of the visual enzymatic cascade could occur. Since ROS cGMP phosphodiesterase is predicted to exist in the dark as a complex with tightly bound cGMP (25), it seems likely that the insertion of 8-azido-[32 P]cGMP may have occurred as the enzyme turned over. If so, the α subunit may carry the catalytic binding site.

The 72-kDa protein identified in our studies as a cGMP-binding protein was concluded to be cGMP-dependent protein kinase, similar to the enzyme from other tissues. Cyclic GMP-dependent protein kinase has been identified previously in the soluble fraction of the retina by measuring activity and using antibodies (Terry Woodford and James Hurley, personal communication). In the present work, the enzyme was also found to be associated with membrane fractions of the retina, including ROS. The presence of cGMP-dependent protein kinase in photoreceptors would be highly significant, since cyclic nucleotide-dependent phosphorylation of at least two bovine ROS proteins has been reported (5, 27).

We have not yet established the identity of the 53-kDa protein labeled by 8-azido-[32 P]cGMP. This membrane-associated protein did not exhibit the proteolytic susceptibility characteristic of regulatory subunits of cAMP-dependent protein kinases, nor were its apparent molecular weight and distribution in fractions of the retina the same as that of the ROS 63-kDa cGMP-activated channel. The 63-kDa protein was not photolabeled in our experiments, even though it was detected by Western blotting. The channel should bind the photoactivatable ligand used in our studies, since it is activated by C^8 analogs of cGMP with micromolar affinity (28). Perhaps the protein simply lacks nucleophilic groups near the cGMP-binding site. The 53-kDa protein identified in our work exhibited a preference for binding cAMP. Previous studies of the retina have shown the presence of cAMP-binding sites in photoreceptor inner segments, outer nuclear layer, and outer plexiform layer by using fluorescent analogs of cAMP (29). Several nucleotide-binding proteins, such as cyclic nucleotide-dependent kinases, bind cGMP and cAMP with differing affinity. The 53-kDa was unusual in also binding cCMP. In this regard, it is similar to a cation channel present in olfactory epithelium (30).

The 37-kDa retinal protein identified in our work as a cGMP-binding protein does not originate from outer segments. It was photoaffinity-labeled in material separated from ROS by differential density centrifugation in sucrose gradients and was never observed in purified ROS. A protein of similar apparent molecular mass, 39 kDa, was previously identified in bovine ROS by Matesic and Liebman with 8-azido-[32 P]cGMP (10). Recent work indicates that the 39-kDa protein is a form of opsin with high apparent molecular mass (31). This finding seems to exclude the possibility that the cGMP-binding protein we identified was retained in their preparations of ROS as a contaminant. The resolution of this

issue awaits further study, as does more complete definition of the function and cellular localization of these proteins.

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