Isolation and Characterization of cGMP Phosphodiesterase from Bovine Rod Outer Segments

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A simple purification method has been developed for isolation of bovine cGMP phosphodiesterase from photoreceptor rod outer segments. The enzyme is peripherally membrane bound in its native state and is present in relatively high concentrations. In the bovine photoreceptors its molar ratio to rhodopsin can be estimated to be as great as 1:40 and not lower than 1:170. The isolated enzyme is purified to homogeneity as demonstrated by gel electrophoresis under native and denaturing conditions and analytical ultracentrifugation. The core enzyme has a molecular weight of approximately 170,000, as demonstrated by sucrose gradient centrifugation and analytical ultracentrifugation, and is composed of two major subunits, 88,000 (α) and 84,000 (β), augmented by a small subunit of 13,000 (γ). In its purified state, the enzyme is activated neither by light nor GTP in contrast to the native membrane-bound enzyme. As isolated, the molar activity of the enzyme is 45 mol of cGMP hydrolyzed s⁻¹ mol⁻¹ with a Kₘ = 150 μM (cGMP) and Kₘ > 4 mM (cAMP). Protamine activates the enzyme to 360 mol of cGMP sec⁻¹ mol⁻¹ (8-fold) and limited digestion by trypsin activates it to as great as 2100 mol of cGMP sec⁻¹ mol⁻¹ (50-fold).

In the course of isolation, a second rod outer segment disc protein is purified. This protein is also membrane-bound and extracted with the phosphodiesterase under hypotonic conditions in approximately equal molar amounts. It is composed of two subunits of 39,000 and 37,000 contributing to a molecular weight of approximately 80,000; hence, we have termed it 80K protein. It is itself devoid of cGMP hydrolytic activity but decreases phosphodiesterase activity by 50% when re-added to the purified enzyme in a 1:1 molar ratio.

EXPERIMENTAL PROCEDURES

Materials—Cyclic nucleotides and nucleotide triphosphates were purchased from Boehringer Mannheim and Sigma, ³H-labeled cGMP (55 mCi/mmol) and cAMP (287 mCi/mmol) from Amersham, PMSF, penicillin, streptomycin, papaverine, IBMX, and trypsin inhibitor from Sigma. All reagents for SDS-PAGE were electrophoresis grade from Bio-Rad. Ampholines, pH 3 to 9, were purchased from LKB. Molecular separators (Pellicon-type membranes, No. PTGC OOlK5) were from Millipore Corp. Ammonyx-LO was a gift from Onyx Chemical Co., Hoboken, NJ. DE-52 was purchased from Whatman and Sephadex resins from Pharmacia. PEI cellulose sheets were obtained from Brinkmann. All other chemicals were reagent grade.

Catalase (beef liver), alcohol dehydrogenase (yeast), and bovine serum albumin were obtained from Sigma; trypsin was from Miles Laboratories. RNA polymerase (Escherichia coli) was isolated according to Arndt-Jovin et al. (16). Protein concentrations were determined throughout this work according to Lowry (17) with bovine serum albumin.

The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; IBMX, 3-isobutyl-1-methylxantine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, rod outer segment; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing gel electrophoresis.

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Phosphodiesterase Assay—The standard assay mixture consisted of a total volume of 30 μl containing 40 mM Hepes, pH 7.5, 5 mM MgCl₂, 0.1 mM dithiothreitol, 2 to 4 mM cGMP (8-¹⁴C-labeled, specific activity, 22 to 45 μCi/mmol), and 1 to 10 μg/ml of protein. In several assays, CaM, Ca₂⁺, and GTP were added as indicated. The assay mixture was preincubated for 1 min at 37°C and the reaction was started by addition of membranes or enzyme. After time intervals of 30 s to 4 min, the reaction was stopped by immersing the capped tube in a 95°C bath for 1 min. Aliquots of these reaction mixtures were manipulated in the following two ways. (1) 5-μl aliquots were spotted on PEI cellulose sheets; the sheets were then washed in 0.012 M LiCl, as described by Zisman (19), and cut into individual strips. The amount of GMP formed was counted in toluene-based scintillation mixture. Retention of GMP was usually 60 to 80%. cGMP spots (and, for control, GMP spots) were cut out and counted.

Phosphodiesterase activity was determined as the change in radioactivity at 37°C. The reaction was started by addition of membranes or enzyme. The assay mixture was preincubated for 1 min at 37°C as indicated. The PEI plates were developed in 0.15 M LiCl and cGMP spots (and, for control, GMP spots) were cut out and counted. This method of measuring cGMP hydrolyzed was used for impure membrane preparations to avoid erroneous results due to the presence of cGMP phosphodiesterase activity in the membranes. For determinations of Kᵣ and Kₐ, the substrate concentrations ranged from 0.01 to 4 mM at 3 pg of enzyme/ml and the constants were calculated as described above.

This method of measuring cGMP hydrolyzed was used for impure phosphodiesterase assay. (ii) Alternatively, 5-μl aliquots were spotted on the origin of a PEI-cellulose TLC plate in a manner similar to that described by Keirns et al. (20). The PEI plates were developed in 0.15 M LiCl and cGMP spots (and, for control, GMP spots) were cut out and counted. This method of measuring cGMP hydrolyzed was used for impure membrane preparations to avoid erroneous results due to the presence of cGMP phosphodiesterase activity in the membranes. For determinations of Kᵣ and Kₐ, the substrate concentrations ranged from 0.01 to 4 mM at 3 pg of enzyme/ml and the constants were determined as described above.

Phosphodiesterase for analysis of purified phosphodiesterase samples. (ii) Alternatively, 5-μl aliquots were spotted on the origin of a PEI-cellulose TLC plate in a manner similar to that described by Keirns et al. (20). The PEI plates were developed in 0.15 M LiCl and cGMP spots (and, for control, GMP spots) were cut out and counted. This method of measuring cGMP hydrolyzed was used for impure membrane preparations to avoid erroneous results due to the presence of cGMP phosphodiesterase activity in the membranes. For determinations of Kᵣ and Kₐ, the substrate concentrations ranged from 0.01 to 4 mM at 3 pg of enzyme/ml and the constants were determined as described above.

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Native Slab Gel Electrophoresis—Electrophoresis of the enzyme under native conditions to illustrate protein homogeneity and correspondence of purified protein species to enzymatic activity was performed according to Davis (30) as modified by Ballato et al. (31). Slab gels (0.8 mm) were polymerized from a solution of 5.3% acrylamide, 0.15% bisacrylamide, 0.076 M Tris HCl, pH 9.5, 0.008% ammonium persulfate, and 0.08% N,N,N',N'-tetramethylethylenediamine (TemED). The stacking gel was polymerized from 3.75% acrylamide, 0.1% bisacrylamide, 0.4 M Tris/HCl, pH 9.0, 0.06% ammonium persulfate, and 0.04% TemED. Samples of enzymes were diluted 1:1 with 0.05 M Tris/glycine, pH 9.5, 20% glycerol, 0.1 M dithiothreitol, and a trace of bromphenol blue; 15 μg of protein were loaded into three adjacent slots. The gels were run at 100 V for about 3 h with 0.05 M Tris/glycine, pH 9.5, as electrode buffer. Following electrophoresis, the gel was cut into three parallel strips. One strip was stained and destained for protein, one strip was used for second dimension SDS-PAGE, and the third strip was cut into 2.5-mm pieces and assayed for enzymatic activity as described under “Analytical Focusing.”

Sucrose Density Gradient Centrifugation—Three-tenths milliliter of concentrated hypotonic SN 2.1 (2 mg/ml containing 200 μg of alcohol dehydrogenase and 250 μg of catalase) was layered on top of a 4.5-ml continuous 5 to 20% sucrose gradient in 10 mM Tris, pH 7.4, 1 mM MgCl₂, 0.1 mM dithiothreitol, and 0.1 mM NaCl. The gradient was centrifuged for 18 h at 4°C in a SW 65 rotor at 38,000 rpm and subsequently fractionated into 16 fractions. Alcohol dehydrogenase was assayed according to Valley and Hoch (32), catalase was assayed according to Beers and Sizer (33), and phosphodiesterase was assayed as described above. Sedimentation coefficients were estimated according to the method of McEwen (34).

Analytical Ultracentrifugation—Analytical ultracentrifugation was carried out with a model E ultracentrifuge (Beckman Instruments) equipped with a photoelectric scanner. Sedimentation coefficients were computed from the equation $S_{obs} = (2.303/60ω^2) \cdot (d \log r/dt)$, where $ω$ = angular velocity, $r$ = distance of boundary to center of rotation. 

![Fig. 1. Schematic isolation procedure for cGMP phosphodiesterase and 80K protein from bovine ROS membranes.](http://www.jbc.org/)
rotation, and \( t = \) time in minutes, and corrected to \( s_{w} \), according to Chervenka (35). Sample concentrations were 0.5 mg/ml in 0.1 M NaCl, 10 mM Tris, pH 7.5, 0.1 mM MgCl\(_2\), and 0.1 mM dithiothreitol. Four hundred-microliter samples were centrifuged at 31,410 rpm at 6°C and scans were taken in 16-min intervals.

Molecular weights were calculated according to Chervenka (35) from the equation 
\[
M_{w} = \left( \frac{2RT}{d\log (A_{w} / \rho)} \right)^{1/3},
\]
where \( R \) = gas constant, \( T \) = temperature, \( d \) = partial specific volume, and \( \rho \) = density of solution. One hundred-microliter samples were centrifuged at 6,995 rpm for phosphodiesterase and 9,945 rpm for 80K for 18 h at 4°C. Partial specific volumes of 0.74 ml/g were assumed.

**RESULTS**

**Isolation of Phosphodiesterase and 80K Protein from ROS Membranes**

The procedure developed here for purification of phosphodiesterase from bovine retina involves selective washing of ROS membranes to elute the cGMP specific enzyme. Subsequent column chromatography separates and purifies the ROS cGMP phosphodiesterase and a protein of undetermined function having a molecular weight of approximately 80,000 (Fig. 1). We have named the latter protein 80K. In order to achieve an initial step in purification, isolated ROS membranes are first washed with isotonic buffer, as defined under “Experimental Procedures.” As shown in Fig. 2, considerable loosely bound or contaminating protein is released into the supernatant. When the cycle of washing is repeated until the A\(_{280}\) is less than 0.05, as much as 20 mg of protein may be removed from ROS membranes isolated from 100 retina (Fig. 2). Only traces (about 0.5 total units) of phosphodiesterase are lost in this washing procedure.

Following the above extensive washing, Mg\(^{2+}\) and 0.1 M NaCl are omitted from the buffers and washing is continued in order to release effectively the phosphodiesterase activity (Fig. 2). SDS-gel electrophoresis of the hypotonic buffer supernatants (Fig. 2b) shows that the main protein constituents are characteristic doublet bands of M, 88,000/84,000 and 39,000/37,000 as determined in reference to standard proteins. The latter resolves clearly into two bands when electrophoresed in a 10% gel under Laemmli conditions (25), but the 88/84K doublet is then unresolved (see Fig. 5). As indicated below, the first doublet has been identified as the two subunits \( \alpha \) and \( \beta \) of the phosphodiesterase and the latter as the subunits \( \delta \) and \( \epsilon \) of a protein we call 80K protein. With three washes, the phosphodiesterase activity is completely removed from the remaining membranes. The latter 80K protein is not, however, quantitatively released in the absence of Mg\(^{2+}\) since we have observed residual protein to remain with the membranes (Fig. 5, Slot 8).

**Purification of Phosphodiesterase and 80K Protein**

**DEAE Column Chromatography—DEAE-cellulose chromatography** has been the major effective technique used to purify phosphodiesterase from several tissues (36). In our application, the chromatography only removes minor amounts of protein. As shown in Fig. 3, all proteins are quantitatively retained by the column and washing with 0.1 M NaCl releases only small amounts of material devoid of cGMP hydrolytic activity. Phosphodiesterase activity is released by a linear gradient of NaCl at about 0.18 M NaCl and the 80K protein at about 0.2 M NaCl. Separation of the two proteins is minimal, possibly due to the close isoelectric points of the two species (4.5 and 5.2; see below). For further purification the peak fractions of activity, containing 4.5 mg of protein, are pooled.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** DEAE-column chromatography of combined hypotonic supernatants (a) and SDS-PAGE of protein in Fractions 62 to 72 from DEAE-chromatography (b). a, the sample was loaded at arrow marked L; the column was then washed with equilibration buffer containing 0.1 M NaCl starting at W and eluted with a gradient from 0.1 to 0.5 M NaCl, starting at S. (●—●) \( \mu g \) protein/ml, (---) phosphodiesterase (PDE) activity, (---) NaCl gradient. b, 50 \( \mu l \) of each fraction was applied to the gel.
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Yield and Homogeneity—At this stage of isolation, the phosphodiesterase is judged essentially homogeneous as indicated by SDS-gel electrophoresis (Fig. 5, Slot 6), native gel electrophoresis (Fig. 8), and analytical ultracentrifugation (see below). About 2 mg of the phosphodiesterase enzyme, free from 80K, may be obtained starting from 100 bovine retina (Table I). There are significant losses of material from trailing edges of peak activities in both DEAE-chromatography and gel filtration. Approximately 1 mg of purified 80K from 100 bovine retina is obtained in this isolation procedure.

Characterization of ROS Phosphodiesterase

Early studies of hypotonic supernatants using isoelectric focusing and sucrose gradient centrifugation gave us indications of the ionic nature and molecular size even before the enzyme was purified using chromatography. When the cGMP phosphodiesterase was isolated to homogeneity by the procedure given above, the enzyme was physically characterized by native gel electrophoresis, SDS-PAGE, and analytical ultracentrifugation. These data are summarized in Table II.

**Isoelectric Focusing**—Two-dimensional electrophoretic chromatography of the hypotonic washes from ROS membranes first indicated to us that the enzymatic activity of the phosphodiesterase is associated with the 88/84K subunits and diesterase at 4°C results in activation.

**Table I**

<table>
<thead>
<tr>
<th>cGMP phosphodiesterase</th>
<th>80K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal activity*</td>
<td></td>
</tr>
<tr>
<td>µmol GMP/min</td>
<td></td>
</tr>
<tr>
<td>µmol GMP/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>Trypsin-activated activity*</td>
<td></td>
</tr>
<tr>
<td>µmol GMP/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>Homogenized retina</td>
<td></td>
</tr>
<tr>
<td>ROS membranes</td>
<td></td>
</tr>
<tr>
<td>Hypotonic supernatant</td>
<td></td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td></td>
</tr>
<tr>
<td>G-100 Sephadex</td>
<td></td>
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<tr>
<td>Isoelectric pH*</td>
<td></td>
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</table>

**Table II**

<table>
<thead>
<tr>
<th>Physical characterization of bovine ROS cGMP phosphodiesterase and 80K protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Sucrose gradient*</td>
</tr>
<tr>
<td>Sedimentation equilibri um</td>
</tr>
<tr>
<td>Sedimentation velocity</td>
</tr>
<tr>
<td>Subunits</td>
</tr>
<tr>
<td>SDS-gel electrophoresis</td>
</tr>
<tr>
<td>Isoelectric pH*</td>
</tr>
</tbody>
</table>

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(e.g. in Fig. 3, Fractions 62 to 70) and concentrated for gel filtration. The tailing fractions with low activity yield another 1 to 1.5 mg of protein.

**Gel Filtration**—Final separation of phosphodiesterase activity and 80K protein is achieved by molecular gel filtration. Fig. 4 illustrates that phosphodiesterase activity elutes in the void volume of a Sephadex G-100 column, while the 80K protein is retarded. Electrophoretic analysis, in the presence of SDS (Fig. 4b), indicates that phosphodiesterase activity is associated with a protein composed of three subunits, 88/84/13K in molecular weight; the 80K protein is associated with a protein of two subunits, 39/37K. For absolute separation of the two proteins, fractions of phosphodiesterase have been pooled, concentrated, and rechromatographed on the same column. Presence of 1 mM dithiothreitol in the buffers is necessary to effect separation. We have also found that, if solutions of phosphodiesterase and 80K are stored at 4°C for several days, the separation becomes more incomplete. Apparently 80K has a tendency to aggregate to 160K or larger species and makes good separation difficult. The final pools of phosphodiesterase activity and 80K protein are concentrated and dialyzed against 50% glycerol in buffer containing 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM MgCl₂, and 0.1 mM EDTA. Protein solutions are stored at -20°C and are stable for up to 6 months. We have observed that storing phospho-
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**Fig. 5. Purification of cGMP phosphodiesterase as followed by SDS-PAGE (a) and mobility of subunits of phosphodiesterase (b).** a, each slot contains 10 μg of protein. 1, cattle retina homogenate; 2, crude ROS membranes before sucrose step gradient; 3, ROS membranes as harvested from interface 1.11 to 1.13 mg/ml of sucrose; 4, concentrated hypotonic supernatant-containing phosphodiesterase activity; 5, pool of peak fractions of DEAE-cellulose column after concentration; 6, purified phosphodiesterase (PDE) after gel filtration; 7, purified 80K protein after gel filtration; 8, depleted ROS membranes; 9, marker proteins as listed in Fig. 2b. Rho, rhodopsin; (Rho*), dimer of rhodopsin. b, mobility of subunits of phosphodiesterase (α, β), γ, and 80K protein (δ, ε) in comparison with marker proteins. (M) mobility in low cross-linked gels as routinely used in this paper (see "Experimental Procedures"; (-----) for comparison, mobility of the same subunits are shown for 10% gels prepared according to Laemml (25).

**Fig. 6. Two-dimensional electrophoresis of concentrated hypotonic supernatant (SN 2.1).** First dimension: isoelectric focusing (IEF) under nondenaturing conditions. The sample was applied on a filter square near the cathode. After 12 h of focusing, the gel slab was sliced into 16 pieces and the pH gradient (0 - 0) and phosphodiesterase activity (M) were determined. For second dimension SDS-gel electrophoresis, an unsliced identical IEF slab was incubated in a buffer containing 0.1 Tris, pH 6.8, 1% SDS, and 5 mM dithiothreitol and polymerized horizontally into a stacking gel. After electrophoresis, the gel was stained with Coomassie blue. The broad stained zone (lower right corner) is Ampholine which stains permanently with Coomassie blue.

gels and resolves into α and β in low cross-linked gels. Since proteolysis is a possible cause of doublet band formation, attempts to inhibit any proteolysis with common proteolytic inhibitors and antibacterial agents were carried out. No
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Fig. 7. Sucrose density gradient of concentrated hypotonic supernatant in the presence of marker enzymes (Cat = bovine liver catalase, ADH = yeast alcohol dehydrogenase). (-- ---) sucrose gradient, determined refractometrically, (----) catalase activity, (---) ADH activity, (---------) phosphodiesterase (PDE) activity. The position of the peak of 80K protein was estimated from SDS-PAGE of the gradient fractions. The ordinate is given as arbitrary units for activity of all three enzymes.

change in the subunit patterns of phosphodiesterase or 80K has been observed upon using these agents or throughout the course of purification.

Native Slab Gel Electrophoresis—The purified enzyme, once separated from the 80K protein by G-100 molecular filtration, was subjected to electrophoresis under native con-

ditions. This demonstrates that the phosphodiesterase activity is associated with a single protein band; no other protein species can be detected under these conditions at pH 9.5. When the same material is subjected to two-dimensional SDS-PAGE, this single band resolves into three characteristic subunits of $M_r = 88,000, 84,000$, and $13,000$.

Analytical Ultracentrifugation—Once the enzyme was purified, traditional methods of sedimentation velocity and sedimentation equilibrium were applied to assess the molecular size and homogeneity. Symmetrical moving boundaries in sedimentation velocity and linear plots of log c versus $r^2$ in sedimentation equilibrium experiments indicate that the protein is a homogeneous species free of higher aggregation forms. The data, as shown in Table II, indicate a $S_{20,w}$ coefficient of 8.9 and molecular weight of 170,000 ± 10,000, and are in agreement with data obtained by sucrose gradient centrifugation. The errors in the values are sufficiently large that we are unable to confirm the presence of one additional small subunit of $M_r = 13,000$.

Characterization of ROS 80K Protein

In purifying the phosphodiesterase, we have inadvertently also purified a protein consisting of two subunits of $M_r = 39,000$ (β) and 37,000 (ε) (Fig. 5). Isoelectric focusing shows this protein to be slightly more acidic (pI 4.5) than the phosphodiesterase. Sucrose gradient centrifugation and analytical centrifugation confirm a molecular weight of 80,000 for the protein. Sedimentation velocity gives an $s_{20,w}$ of 5.4, which is roughly typical for a spherical macromolecule of $M_r = 80,000$. As observed for purified phosphodiesterase, sedimentation velocity and sedimentation equilibrium experiments with the isolated 80K protein indicated the purified protein is a homogeneous species free of higher aggregating forms.

Enzyme Activity and Modulation

Preliminary studies of phosphodiesterase activity and its modulation are given in Tables I and III. The specific activity of the enzyme in purified state shows no concentration dependence over the range of 2.5 μg/ml ($1.5 \times 10^{-5}$ M) to 100 μg/ml ($6 \times 10^{-7}$ M) when initial velocities are measured at less than 2% min assay time. We have noted that, with longer times of assay, the hydrolysis becomes nonlinear showing activation. All measures of activity (Table I and Table III) are derived from initial velocities measured over less than 2% min. The activity of purified core enzyme is specific for cGMP.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Specific activity</th>
<th>$K_a$</th>
<th>$K_i$</th>
</tr>
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<tbody>
<tr>
<td>Basal activity</td>
<td>15</td>
<td>150 μM cGMP</td>
<td></td>
</tr>
<tr>
<td>+GTP (0.16 mM)</td>
<td>15</td>
<td>(&gt;4 mM cAMP)</td>
<td></td>
</tr>
<tr>
<td>+CaCl(_2) (0.5 mM)</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Protamine (3 mg/ml)</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Trypsin(^b)</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+80K protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1 molar ratio</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10 molar ratio</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+IBMX</td>
<td></td>
<td>10 μM</td>
<td></td>
</tr>
<tr>
<td>+Papaverine</td>
<td></td>
<td>25 μM</td>
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</table>

* Assayed in 40 mM Hapes, pH 7.1, 5 mM MgCl\(_2\), 0.1 mm dithiothreitol, and 3 mM cGMP with additions indicated. The purified phosphodiesterase shows no light activation.

\(^b\) Maximal activation is achieved with 1-min digest at 37°C, with a 5-fold (mg) excess of trypsin.
but cAMP may be hydrolyzed at high concentrations which is consistent with the high $K_m$ for cAMP. The enzyme has a basal activity of about 15 units/mg and its activity is unaffected by light or the presence of GTP in contrast to the membrane-bound enzyme activity. CaCl$_2$ at 0.5 mM neither activates nor inhibits the basal activity.

Both protamine and trypsin are known activators of mammalian phosphodiesterases (38) as well as the frog ROS cGMP phosphodiesterase (15). Protamine maximally activates the purified bovine ROS enzyme about 5-fold. Maximal activation is achieved with a 1-min digestion at 37°C with a 5- to 10-fold (mg) excess of trypsin while higher concentrations of trypsin or longer periods of incubation lead to decreased activity. Under these conditions as much as a 50-fold activation may be achieved (Table I and III).

Several factors modulate the enzymatic activity among which is the 80K protein which was purified along with the phosphodiesterase. The 80K protein, purified to homogeneity following Sephadex gel filtration, is itself free of catalytic activity. Incubation periods of 8-fold. Trypsin is a more powerful activator. Maximal activation is achieved with a 1-min digestion at 37°C with a 5- to 10-fold (mg) excess of trypsin while higher concentrations of trypsin or longer periods of incubation lead to decreased activity. Under these conditions as much as a 50-fold activation may be achieved (Table I and III).

Several factors modulate the enzymatic activity among which is the 80K protein which was purified along with the phosphodiesterase. The 80K protein, purified to homogeneity following Sephadex gel filtration, is itself free of catalytic activity as indicated by assay for cGMP hydrolysis as a control (see also Fig. 6). When the purified 80K protein is added back to the purified phosphodiesterase, the enzymatic activity is inhibited. A molar ratio of 80K/phosphodiesterase of 1:1 effects 50% inhibition; a molar ratio 10:1 effects 85% inhibition. Both IBMX and papaverine are excellent inhibitors of activity (Table III). As for phosphodiesterase from other tissues (36), IBMX is more effective than papaverine.

**DISCUSSION**

Various works in the last 10 years have ascribed relatively high cyclic nucleotide phosphodiesterase activity to retinal rod outer segments (6, 9, 39). Robb (40) histochemically demonstrated the presence of a phosphodiesterase in the retinal rod outer segment, and the work of Zimmerman et al. (41) biochemically demonstrated that the activity is associated with rod outer segments since it co-migrated with photoreceptor membranes in sucrose gradients. Miki et al. (15) first isolated the rod outer segment phosphodiesterase from frogs and suggested that the enzyme is a peripheral membrane protein. In the frog, the enzyme was estimated to be present in very small amounts with a molar ratio of phosphodiesterase to rhodopsin approximately 1:300 (15). The importance of this enzyme to regulatory or triggering phenomena in the photoreceptor cell has been emphasized and the fascinating association of activation by rhodopsin has been demonstrated; thus, further characterization of this enzyme is of interest. Since bovine retina provides a larger source of starting material, we have turned to this tissue as a source of purification of the rod outer segment phosphodiesterase. A partial isolation of phosphodiesterase from bovine retina has been reported by Coquil et al. (42).

The purification procedure presented here (Fig. 1) takes advantage of the observation that phosphodiesterase co-sediments with membranes in buffers containing Mg$^{2+}$, but can be eluted from the membrane surface by depleting Mg$^{2+}$. In this manner, phosphodiesterase and one other major protein are selectively eluted from the washed ROS membranes. Subsequent DEAE column chromatography of the hypotonic supernatants achieves only minimal separation of the two protein constituents (Fig. 3) and serves mainly as an effective and fast concentration step of the rather large supernatant volume. Subsequent gel filtration quantitatively separates the phosphodiesterase and a 80K protein. The overall purification of phosphodiesterase from ROS membranes is approximately 30-fold. Using this simple and rapid isolation procedure, we were able to isolate 1 to 2 mg of phosphodiesterase and an equal amount of 80K protein from 100 bovine retinas.

The purified phosphodiesterase appears to be homogeneous and greater than 95% pure as judged by gel electrophoresis (Figs. 5 and 8) and analytical ultracentrifugation (see "Results" and Table II). The enzymatic activity is assigned to a core protein of two subunits $\alpha (M_r = 88,000)$ and $\beta (M_r = 84,000)$ which are associated throughout the purification procedure by a third polypeptide $\gamma (M_r = 13,000).$ We have tried to rule out the possibility that $\alpha$ and $\beta$ might have been produced by proteolysis and that $\gamma$ might be a proteolytic fragment by adding proteolytic inhibitors and bacteriostatic agents to the homogenization and flotation buffers. Under all conditions, we observe that the two large subunits $\alpha$ and $\beta$ are present in a 1:1 ratio and no further change in this ratio takes place even upon prolonged storage of ROS membranes. Further work will be necessary to confirm the stoichiometry and the nature of association of the small "subunit" $\gamma$ with the holoenzyme. Analytical ultracentrifugation and sucrose gradient centrifugation indicate that the enzyme, released from the membrane surface, is a roughly globular molecule of 170,000 molecular weight. This indicates that the bovine ROS phosphodiesterase is somewhat smaller than the analogous frog enzyme which has a $M_r = 240,000$ with subunits of $M_r = 110,000$ and 120,000 (15). Both of these enzymes differ from the bovine brain phosphodiesterase which has recently been purified to homogeneity (43). The latter has been shown to be a Ca$^{2+}$-dependent enzyme and to consist of a catalytic subunit of 59,000 and two other subunits of 61,000 and 15,000 constituting a holoenzyme of $M_r = 135,000.$

Since rhodopsin mediates the activation of the ROS phosphodiesterase (20) and since it has very recently been postulated that one rhodopsin may activate as many as 500 phosphodiesterases (5, 13), it is of interest to know the ratio of phosphodiesterase to rhodopsin in the rod outer segment discs. On the basis of quantity of phosphodiesterase isolated from 100 retina (with 2 mg of phosphodiesterase, $M_r = 170,000$; 80 $A_{500}$ units of rhodopsin, $A_{480} = 40,000$ $m^{-1} cm^{-1}$), we obtain a ratio of 1:1.0. We think that this is a lower limit. Losses of phosphodiesterase on both DEAE-chromatography and G-100 chromatography could be as great as 50% of the enzyme yield.

The more conventional way of estimating the total amount of catalytic subunit of cellular protein from comparison of the units of crude catalytic activity with the specific activity of purified protein is difficult to apply for this enzyme. The enzyme is very sensitive to light activation in the membrane-bound state in the presence of GTP (10, 13, 20), but not in the purified state, and, as discussed below, is inhibited by an accompanying $M_r = 80,000$ protein when isolated. We have also observed that the enzyme in the purified state, but not the crude state, shows an unusual activation when assayed over long periods of time at 37°C and is activated over a period of a few weeks upon storage at 4°C. Thus, the total units of activity reported at any stage of purification are not readily comparable.

Treatment of the protein with trypsin activates the enzyme in a manner analogous to that seen for phosphodiesterases in other tissues (15, 38; see Table I). If this treatment can be considered to achieve a completely activated state which is functionally similar in the native membrane and in the isolated form, a comparison of units of activity may be made to determine the starting amount of material. For this purpose trypsin activation was maximized in a 1-min digestion at 37°C by treatment with varying concentrations of trypsin for both native membranes and purified protein. For the purified enzyme the specific activity is enhanced to 750 units/mg (50-fold) and for membrane-bound enzyme to 100 units/mg of...
rhodopsin (200-fold). Using the respective molecular weights for phosphodiesterase and rhodopsin (170,000 and 36,000), this result suggests that the ratio of phosphodiesterase to rhodopsin could be as much as 1:40.

Like the brain and heart cyclic nucleotide phosphodiesterase (36, 42-44), many factors appear to modulate the activity of retinal ROS phosphodiesterase. This makes it difficult to obtain a true molar activity (turnover number) for this enzyme. In the membrane-bound state, ROS phosphodiesterase is activated by light in the presence of GTP and the activation has an action spectrum related to the absorption spectrum of rhodopsin (20). In the purified state, activity is not affected by light or GTP; it is decreased by addition of a purified 80K protein and increased by several exogenous activators such as trypsin and protamine (Table III). For the final purified enzyme, a turnover number of 45 mol s⁻¹ mol⁻¹ is observed. Maximal activation of the enzyme by trypsin suggests that the turnover number could be as high as 2130 mol s⁻¹ mol⁻¹. Whether the latter value is realistic remains to be confirmed when better understanding of the various factors that activate the phosphodiesterase has been achieved.

The role of 80K protein in modulating activity is less clearly understood. We initially encountered some difficulties in separating 80K protein from phosphodiesterase, especially in low salt and low dithiothreitol concentrations, and thought that the phosphodiesterase had been achieved. Maximally activating the enzyme by trypsin suggests that the phosphodiesterase has been isolated. Whether the latter value is realistic remains to be confirmed.

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