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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 hydrolized s^{-1} mol^{-1} with a K_m = 150 μM (cGMP) and K_m > 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 s^{-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.

A variety of recent studies have demonstrated that the endogenous levels of cGMP in photoreceptor cells are significantly reduced by light (1–5). The reduction is attributed to the high levels of phosphodiesterase activity which have been demonstrated to be present in photoreceptors of cattle (6, 7), rabbits (1), rats, mice (4, 8), and frogs (2, 3, 9). This functional assignment for cGMP phosphodiesterase is supported by studies which have shown that the enzyme in its native membrane-bound state is markedly activated by light in the presence of GTP or ATP (9, 10) and that the photoreceptor guanylate cyclase, hence, the production of cGMP, seems to be unaffected by light (11, 12). Woodruff and collaborators (5) have shown that light can stimulate the turnover of cGMP to as great a level as 1000 to 2000 molecules of cGMP/molecule of rhodopsin bleached in 100 to 300 ms. Yee and Liebman (13) have very recently reported activation of cGMP hydrolysis by 10^4- to 10^5-fold upon illumination of rod outer segments. Considering this dramatic and rapid modulatory effect of light upon the catalytic function of cGMP phosphodiesterase, it has been postulated that this enzyme plays an important regulatory role in the primary process of photoreception (14) or may even play a direct role in the "triggering" process of visual photoreception (5, 13). Further characterization of the enzyme should help elucidate the mechanism of light activation and the role of activators and inhibitors and ultimately help to determine the true physiological role of the protein.

Isolation of the cGMP phosphodiesterase from frog rod outer segments has been accomplished by Miki *et al.* (15). Stimulated by this study and those of cGMP turnover in intact systems, we undertook the isolation of the cGMP phosphodiesterase from bovine rod outer segments which are available in large quantities. We have found that this source yields reasonable quantities of purified enzyme for characterization. This paper thus describes a simple, rapid procedure for isolation of cGMP phosphodiesterase from bovine rod outer segments with good yield and characterizes the final purified enzyme physically and enzymatically. In the course of purification, we have also isolated a second rod outer segment membrane protein which modulates the phosphodiesterase activity *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—Cyclic nucleotides and nucleotide triphosphates were purchased from Boehringer Mannheim and Sigma, ^{14}C-labeled cGMP (55 mCi/mmol) and cAMP (287 mCi/mmol) from Amersham, PMSF,[1] 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 001K5) 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

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

serum albumin as standard or according to Sedmak and Grossberg (18).

Phosphodiesterase Assay—The standard assay mixture consisted of a total volume of 20 μl containing 40 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 2 to 4 mM cGMP (8-^{14}C-labeled, specific activity, 22 to 45 μCi/mol), and 1 to 10 μg/ml of protein. In several assays, cGMP was replaced by cAMP, and Ca^{2+} 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. (i) 5-μl aliquots were spotted on PEI cellulose sheets; the sheets were then washed in 0.012 M LiCl, as described by Zusman (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% and was monitored in each experiment with control spots of labeled GMP. This assay was used 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 5′-nucleotidases and kinases which use GMP as substrate. For determinations of K_m and K_i, the substrate concentrations ranged from 0.01 to 4 mM at 3 μg of enzyme/ml and the constants were determined from Lineweaver-Burk and/or Dixon plots (21). One enzyme unit corresponds to 1 μmol (of cGMP hydrolyzed or GMP formed)/min.

Trypsin Activation—Stock solutions of trypsin (1 mg/ml in water) were freshly prepared. Purified or membrane-bound enzyme was activated by adding up to 10-fold excess of trypsin (ratio in milligrams of protein) to the enzyme in assay mixture minus cGMP. After incubation at 37°C for 1 min, 10-fold (mg) excess of trypsin inhibitor was added and cGMP hydrolysis was started by addition of substrate at a final concentration of 4 mM. Control experiments were performed by adding trypsin inhibitor before adding phosphodiesterase to ensure complete inactivation of trypsin by inhibitor.

5′-Nucleotidase Assay—Assay conditions were identical with that used for phosphodiesterase assay except that cGMP was replaced by GMP. Neither phosphodiesterase nor 80K protein showed any 5′-nucleotidase activity. Activity in rod outer segment membranes was shown to be insignificant.

Isolation of Rod Outer Segments—Bovine eyes[2] were obtained within 10 min of animal slaughter, collected in a light-tight box, and transported on ice to the laboratory in absolute darkness. After 2- to 4-h dark adaptation at 0°C, retinas were removed under dim red light in a 4°C cold room. All subsequent operations through elution of membranes with hypotonic buffer were carried out at 4°C in the dark and under dim red light.

ROS membranes were isolated according to Papermaster and Dreyer (22) with the following modifications: homogenizations of the retinas were performed with a motor-driven Teflon-glass homogenizer in 30% sucrose (w/w); all buffers contained, in addition to the standard salts, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM PMSF, 25 μg/ml of streptomycin, and 40 μg/ml of penicillin; centrifugation after dilution of the 30% sucrose supernatant was carried out at 7,000 × *g* for 4 min; and sucrose step gradients were centrifuged at 55,000 × *g* for 45 min in an SW 27 rotor.

ROS membranes were collected from the 1.11- to 1.13-mg/ml sucrose gradient interface which contained 80 to 90% of all rhodopsin. The rhodopsin content was measured as $A_{500\ nm}$ in a 1:10 dilution of the membranes in a buffer containing 1% Ammonyx-LO using an extinction coefficient of $E^{1\%}_{500\ nm}$ = 11.1. (This value was calculated from $\bar{a}_M$ = 40,000 M^{-1} cm^{-1} (23) and a molecular weight of rhodopsin of 36,000.[3]) The yield of rhodopsin was routinely 60 to 80 mg/100 retina. The ratio of $A_{280:500\ nm}$ was ~3.0.

Selective Washing of ROS Membranes—ROS membranes (in approximately 100 ml of buffered sucrose) were diluted with a double volume of an isotonic buffer containing 10 mM Tris, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM PMSF, 125 μg/ml of penicillin, and 20 μg/ml of streptomycin. The membrane suspension was centrifuged at 25,000 × *g* for 15 min in a Beckman JA-20 rotor and the isotonic supernatant was collected (SN 1.1). The pellet was resuspended in 50 ml of the same buffer and gently homogenized by forcing the suspension two to three times through a blunt needle (gauge 14) with the tip resting on the bottom of the bucket. The homogenized suspension was centrifuged under the same conditions and the supernatant was saved (SN 1.2). This washing cycle was repeated five times or until the $A_{280\ nm}$ of the supernatant was less than 0.05. Before reading *A*, the isotonic supernatants were centrifuged again in one run for 15 min to remove all traces of rhodopsin-containing membranes in the supernatant. For the hypotonic wash, the ROS pellet was resuspended and homogenized as described above in the buffer minus $MgCl_2$ and 0.1 M NaCl. This cycle was repeated four times or until the $A_{280\ nm}$ was less than 0.05. The supernatants were centrifuged at 25,000 × *g* for 30 min. The first three hypotonic supernatants contained 90% of all phosphodiesterase activity.

DEAE-Cellulose Column Chromatography—Preswollen DEAE-cellulose was preequilibrated with 0.1 M Tris, pH 6.8, until the pH dropped below 7.5. Columns (1.6-cm diameter) with bed volumes of ~34 ml were poured and equilibrated with 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, and 1 mM dithiothreitol overnight. Combined hypotonic supernatants containing ~10 mg of protein were applied and the column was washed with 1 to 2 void volumes of starting buffer containing 0.1 M NaCl, then eluted with a 200-ml linear gradient of 0.1 to 0.5 M NaCl in equilibration buffer. Appropriate fractions containing phosphodiesterase activity were pooled and concentrated with Millipore molecular separators.

Gel Filtration—Sephadex G-100 (Pharmacia) was preswollen in 10 mM Tris, pH 7.5, 0.1 mM $MgCl_2$, 500 mM NaCl, and 0.05 mM dithiothreitol for 5 h at 95°C. The gel was cooled to 4°C, defined, and degassed, and the column (1.1-cm diameter) was poured to a final bed volume of 50 to 60 ml. The gel was equilibrated in the swelling buffer plus 1 mM dithiothreitol and the void volume was determined using dextran blue. The sample was applied to the equilibrated column in less than 0.5 ml and eluted with the same swelling buffer plus 1 mM dithiothreitol. Fractions containing phosphodiesterase activity were combined, concentrated with Millipore molecular separators, and for storage dialyzed against 50% glycerol containing 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, and 0.1 mM EDTA. The final purified enzyme was kept in this buffer at −20°C.

SDS-Gel Electrophoresis—Gel electrophoresis was performed in a slab gel apparatus as described by Studier (24) using the discontinuous buffer system of Laemmli (25). Gel width was 0.08 cm (0.13 cm for second dimension of isoelectric focusing gels). Acrylamide concentration was 15%; bisacrylamide was 0.08% (T = 15.06% and C = 0.415% according to the nomenclature of Hjertén (26)). For comparison, gels were also run with 10% acrylamide and 0.26% bisacrylamide (25). Samples were incubated for 15 min at room temperature in a sample buffer containing 0.1 M Tris, pH 6.8, 0.1 M dithiothreitol, 30% glycerol, 2% SDS, and a trace of bromphenol blue as tracking dye. Gel slots contained 1 to 10 μg of protein, unless otherwise stated. Lower limit for band visibility is approximately 100 ng. Gels were stained and destained according to Weber and Osborn (27).

Isoelectric Focusing—Gels (5 cm × 7 cm slab gels) were polymerized in a manner similar to that described by Scheele (28). The gel mixture consisted of 5% acrylamide, 0.08% bisacrylamide, 20% glycerol, 2% ampholine (pH 3 to 9), and 0.02% ammonium persulfate. The gel slab rested horizontally on a water-cooled (4°C) plate and was pre-electrophoresed at 1 to 2 mA (manually controlled) until the voltage reached 250 to 300 V and a pH gradient had been established. Samples (50 μg/slot) were loaded on filter paper squares of various thickness (depending on volume loaded and sample concentration) and placed on the gel surface near the cathode. Focusing was performed at 360 V for 6 to 12 h.

For a typical run, a sample was loaded in triplicate and, after focusing, the gel was cut into three identical strips. One strip was used for second dimension SDS-PAGE (see Fig. 6), one was stained according to Vesterberg and Hansen (29), and the third was cut into 5-mm pieces for determination of phosphodiesterase activity and pH. The gel pieces were stored in 200 μl of H_2O overnight at 4°C, the pH gradient was determined in the supernatant using a microelectrode, and the pieces were subsequently used for enzyme assays. For assaying enzyme activity of the gel pieces, a gel cube of approximately 5-mm length was incubated with 50 μl of assay mix and aliquots of 20 μl were withdrawn after 30 min. Hydrolysis of cGMP was determined as described above.

[2] We are indebted to Mr. J. Colalillo of Cross Brothers, Philadelphia, PA, for his continued assistance.

[3] W. Baehr, M. J. Devlin, and M. L. Applebury, unpublished observations determined from SDS-gel electrophoresis used routinely in this laboratory.

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 Ballatio *et al.* (31). Slab gels (0.8 mm) were polymerized from a solution of 5.3% acrylamide, 0.15% bisacrylamide, 0.375 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/H_3PO_4, 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 "Isoelectric 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 $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M 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 $S_{obs} = (2.303/60\omega^2)\cdot(d\log r/dt)$, where ω = angular velocity, r = distance of boundary to center of

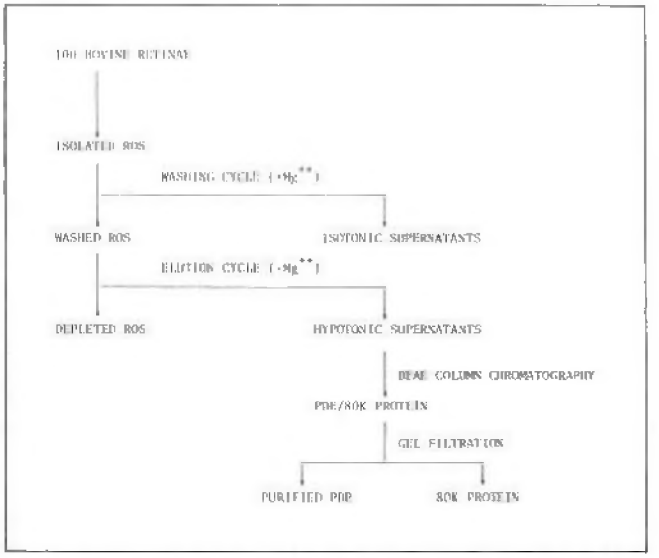

FIG. 1. **Schematic isolation procedure for cGMP phosphodiesterase and 80K protein from bovine ROS membranes.**

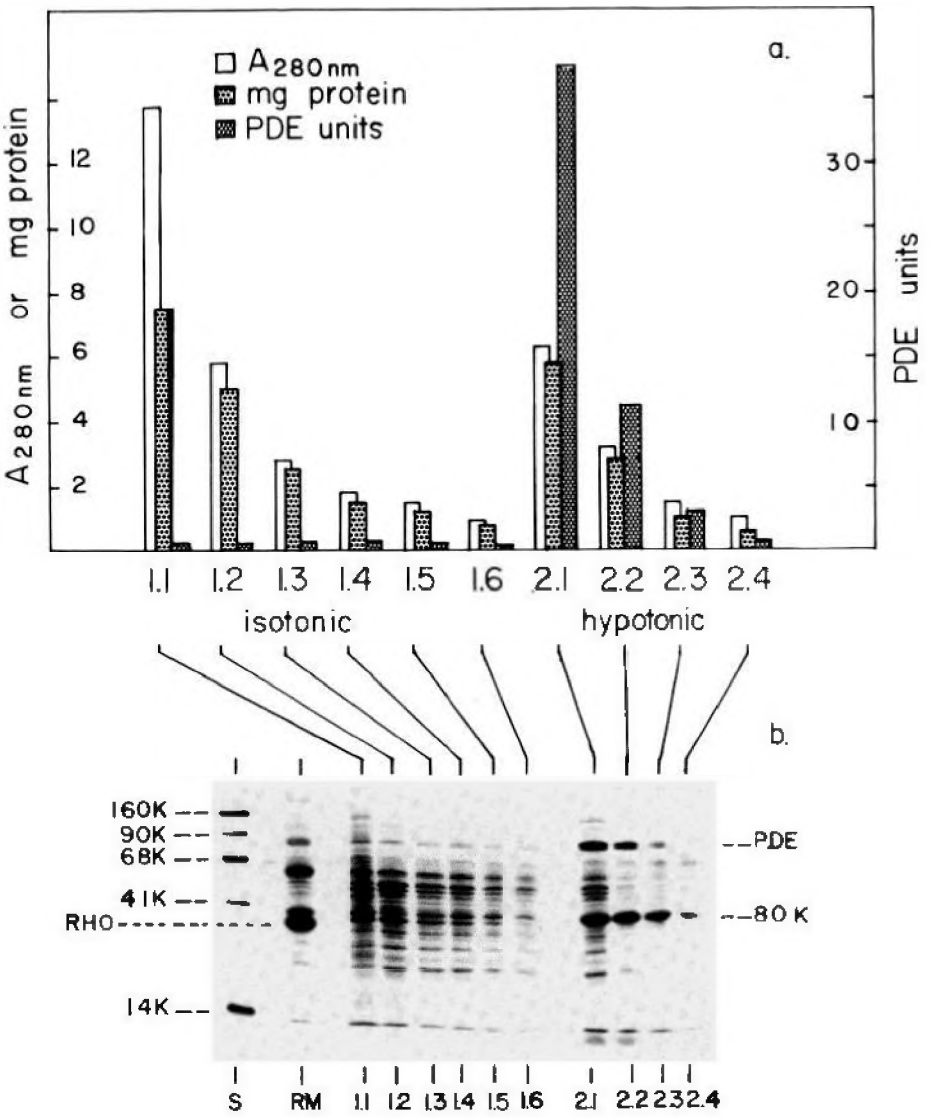

FIG. 2. **Elution of proteins from ROS membranes by selective washing (*a*) and comparison of proteins eluted into isotonic and hypotonic supernatants as detected by SDS-PAGE (*b*).** *a*, fractions 1.1 to 1.6 are successive supernatants from membranes washed with isotonic buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM PMSF containing 125 μg/ml of penicillin and 20 μg/ml of streptomycin). Fractions 2.1 to 2.4 are subsequent supernatants from these membranes washed with hypotonic buffer (as isotonic buffer but minus 5 mM $MgCl_2$ and 0.1 M NaCl). Protein released was measured as total $A_{280\ nm}$ or total milligrams using bovine serum albumin as a standard (18); phosphodiesterase (*PDE*) units are micromoles of GMP hydrolyzed/min for 100 retina. *b*, *S*, marker proteins (RNA polymerase from *E. coli* with β′, β, σ, and α of 165K, 155K, 90K, and 41K, respectively; bovine serum albumin, 68K; and lysozyme, 14K); *RM*, ROS membranes before washing; 1.1 to 1.6 successive isotonic supernatants; 2.1 to 2.4 subsequent hypotonic supernatants. *PDE*, phosphodiesterase; *80K* protein, protein isolated from ROS membranes having molecular weight of about 80,000; Rho, rhodopsin. The supernatants were concentrated by Millipore ultrafiltration before application; 15 μg of protein were loaded in each slot.

rotation, and t = time in minutes, and corrected to $s_{20,w}$ 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 $M_{app} = (2RT \cdot 2.303/(1 - \nu\rho)\omega^2) \cdot (d\log(A_{280\,nm})/dr^2)$, where R = gas constant, T = temperature, ν = partial specific volume, and ρ = 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. 2*b*) 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 α and β of the phosphodiesterase and the latter as the subunits δ and ϵ 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

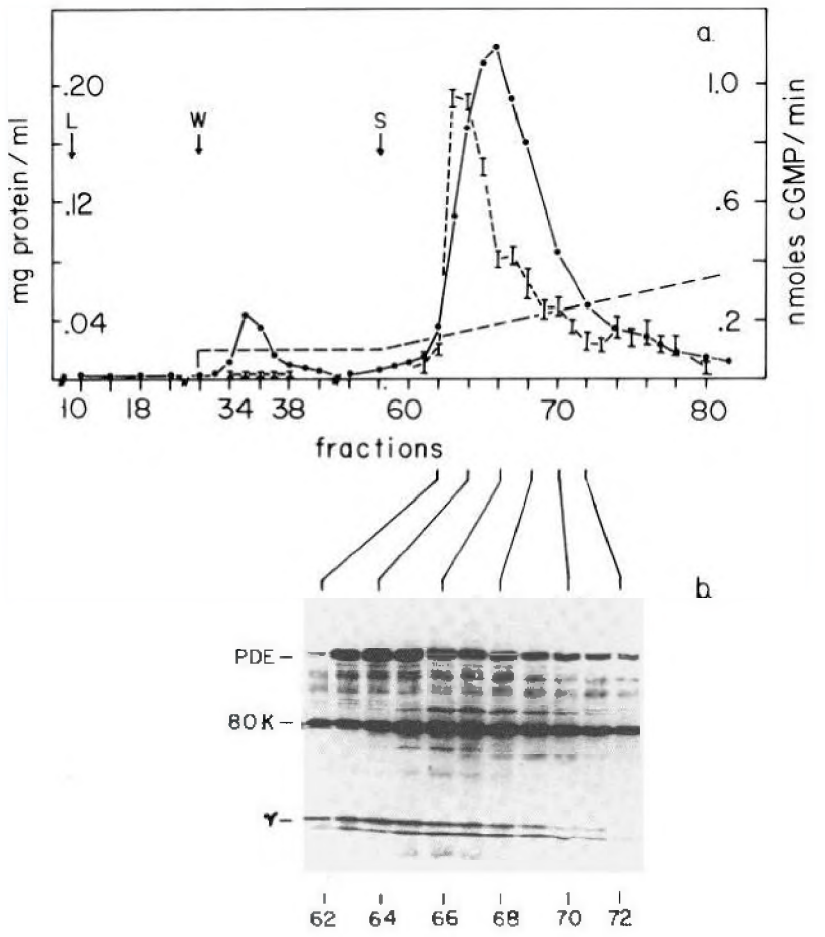

FIG. 3. **DEAE-column chromatography of combined hypotonic supernatants (*a*) and SDS-PAGE of protein in Fractions 62 to 72 from DEAE-chromatography (*b*).** *a*, the sample was loaded at *arrow* marked *L*; the column was then washed with equilibration buffer containing 0.1 M NaCl starting at *W* and eluted with a gradient from 0.1 to 0.5 M NaCl, starting at *S*. (●——●) μg protein/ml, (I- - -I) phosphodiesterase (*PDE*) activity, (- - -) NaCl gradient. *b*, 50 μl of each fraction was applied to the gel.

(*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. 4*b*), 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 $MgCl_2$, 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 phosphodiesterase at 4°C results in activation.

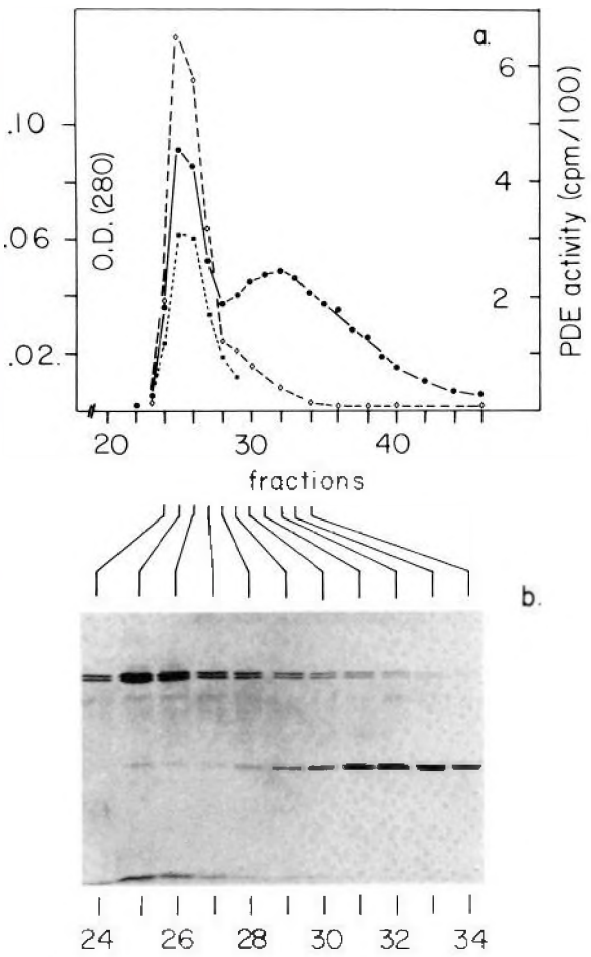

FIG. 4. **Gel filtration of concentrated DEAE-peak fractions on Sephadex G-100 (*a*) and SDS-PAGE of Fractions 24 to 34 (*b*).** *a*, the void volume was determined using dextran blue (■---■). Phosphodiesterase is excluded by Sephadex G-100, and 80K protein is retarded. (●——●) $A_{280\ nm}$; (◇---◇) phosphodiesterase (*PDE*) activity in cpm of ^{14}C-cGMP hydrolyzed. *b*, 50 μl of each fraction was applied to the gel.

TABLE I

Purification of cGMP phosphodiesterase from bovine retina

The values reported are averaged over at least five (and in some cases seven preparations) and are normalized to a starting material of 100 retina.

	Basal activity[a]	Total units	Total protein	Trypsin-activated activity[b]
	μmol GMP/min/mg	*μmol GMP/min*	*mg*	*μmol GMP/min/mg*
Homogenized retina	<0.1	~180	3600	
ROS membranes	0.5 ± 0.3[c]	50 ± 30	80[c]	100[c]
Hypotonic supernatant	7.5 ± 2.5	70 ± 10	10	500
DEAE cellulose	8.5 ± 2.5	55 ± 16	6.5	
G-100 Sephadex	15 ± 4	30 ± 5	2	750

[a] Assayed without GTP or light activation.

[b] Maximal activation obtained in 1-min digestion at 37°C with a 3-fold (mg) excess for ROS membranes and a 5-fold (mg) excess for the purified enzyme.

[c] Based on rhodopsin content of membranes taken from A_{500}. These retina contain an estimated 5 to 10% bleached rhodopsin.

TABLE II

Physical characterization of bovine ROS cGMP phosphodiesterase and 80K protein

	cGMP phosphodiesterase	80K
Molecular weight		
Sucrose gradient[a]	170,000 ± 10,000	
Sedimentation equilibrium	171,000 ± 5,000	75,000 ± 5,000
$S_{20,w}$		
Sucrose gradient[a]	8.8	
Sedimentation velocity	9.1 (0.5 M NaCl) 8.9 (0.04 M NaCl)	6.0 (0.5 M NaCl) 5.4 (0.05 M NaCl)
Subunits		
SDS-gel electrophoresis	84,000; 88,000 (13,000)	37,000; 39,000
Isoelectric pH[a]	5.0–5.5	4.5

[a] Measured for samples containing both phosphodiesterase and 80K protein.

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

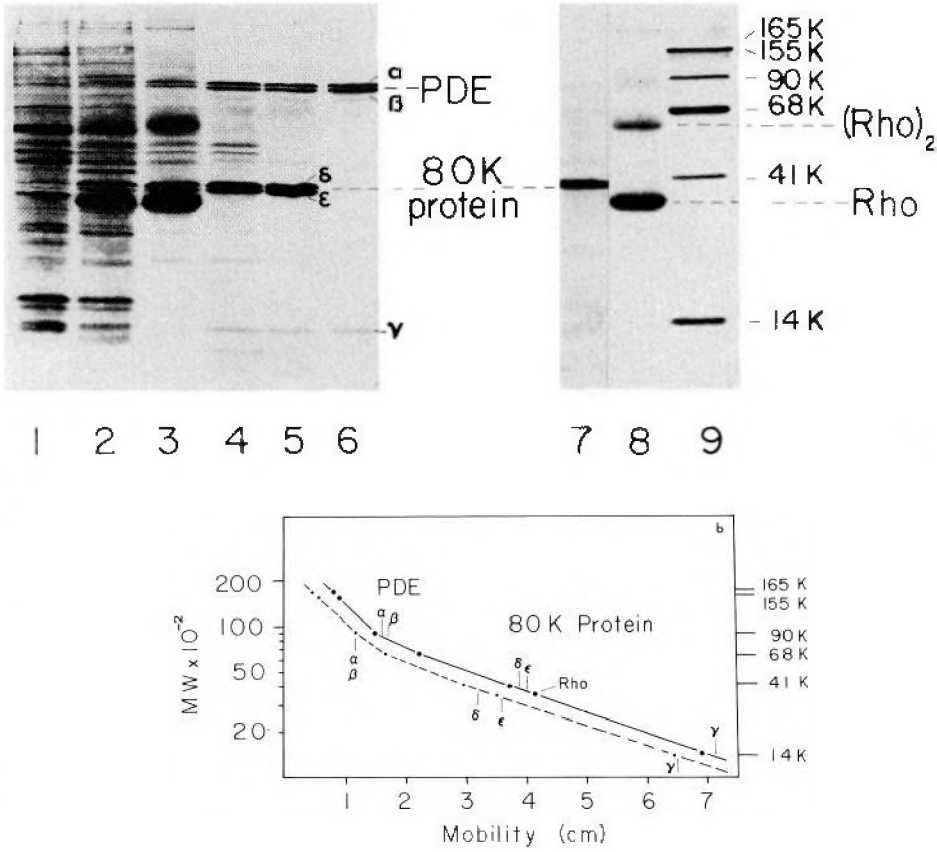

FIG. 5. **Purification of cGMP phosphodiesterase as followed by SDS-PAGE (*a*) and mobility of subunits of phosphodiesterase (*b*).** *a*, each slot contains 10 μg of protein. *1*, cattle retina homogenate; *2*, crude ROS membranes before sucrose step gradient; *3*, ROS membranes as harvested from interface 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. 2*b*. *Rho*, rhodopsin; (*Rho_2*), dimer of rhodopsin. *b*, mobility of subunits of phosphodiesterase (α, β), γ, and 80K protein (δ, ε) in comparison with marker proteins. (●——●) 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 Laemmli (25).

that this activity could be separated from the protein-containing subunits 40K (later shown to be 80K protein). Fig. 6 indicates that phosphodiesterase focuses as a rather broad band at pH 5.0 to 5.5, which does not sharpen after prolonged focusing. In contrast, 80K protein precipitates at pH 4.5 and is visible in the isoelectric focusing slab even without staining. Phosphodiesterase activity in the gel slab was identified by eluting protein from gel pieces and assaying for activity as described under "Experimental Procedures."

Sucrose Gradient Centrifugation—A second characterization of the phosphodiesterase was carried out by sucrose density gradient centrifugation. The conditions are similar to those used previously for estimation of molecular size of phosphodiesterases from other tissues (15, 37). Fig. 7 illustrates that the phosphodiesterase activity migrates faster than alcohol dehydrogenase (M_r = 140,000) but slower than catalase (M_r = 250,000). In comparison with the molecular weights of these marker enzymes, phosphodiesterase is estimated to have a molecular weight of 160,000 to 180,000 in the state first eluted from the membrane. A sedimentation coefficient of 8.8 may be calculated for the phosphodiesterase according to McEwen (34).

SDS-Gel Electrophoresis—Phosphodiesterase resolves into two major subunits α and β of 88,000 and 84,000 molecular weight upon electrophoresis in the presence of SDS. A third polypeptide, termed γ of molecular weight 13,000, is always associated with the phosphodiesterase enzyme throughout the purification (see Figs. 3–5 and 8). Integrated intensities of the Coomassie-stained bands suggest that the α and β subunits are always present in 1:1 ratio. The stoichiometry of the small subunit is less clear and would be dependent on amino acid composition but is estimated to be between 0.5 and 2. Resolution of α and β depends on the degree of cross-linking in a SDS-polyacrylamide gel. Fig. 5*b* demonstrates that phosphodiesterase moves as a single band in standard (25) 10% SDS

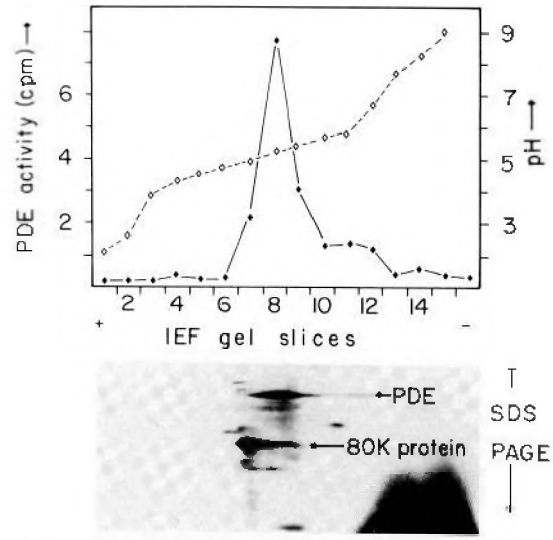

FIG. 6. **Two-dimensional electrophoresis of concentrated hypotonic supernatant (SN 2.1).** First dimension: isoelectric focusing (*IEF*) under nondenaturing conditions. The sample was applied on a filter square near the cathode. After 12 h of focusing, the gel slab was sliced into 16 pieces and the pH gradient (◇- - -◇) and phosphodiesterase activity (◆——◆) 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

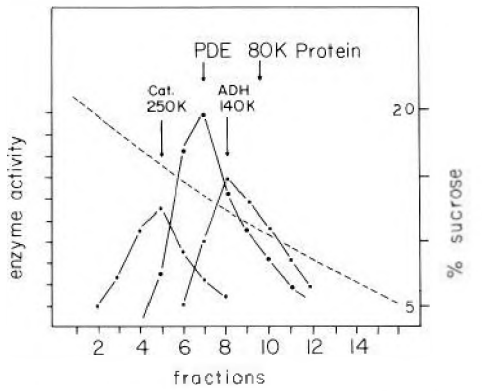

FIG. 7. **Sucrose density gradient of concentrated hypotonic supernatant in the presence of marker enzymes (*Cat* = bovine liver catalase, *ADH* = yeast alcohol dehydrogenase).** (– – –) sucrose gradient, determined refractometrically. (◆——◆) catalase activity, (■——■) ADH activity, (●——●) phosphodiesterase (*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.

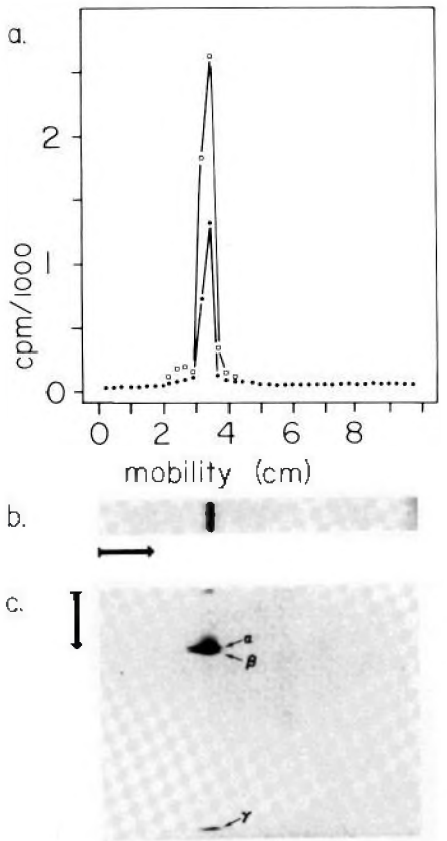

FIG. 8. **Two-dimensional gel electrophoresis of purified phosphodiesterase (*PDE*).** Fifteen micrograms of phosphodiesterase were loaded into three slots of a nondenaturing slab gel. After electrophoresis at pH 9.5, one gel strip was stained with Coomassie blue (*b*). The second strip was sliced and assayed for phosphodiesterase activity (*a*); (●——●) cpm of ^{14}C-GMP produced after 17-min and (□——□) after 34-min incubation time. The third strip was analyzed by SDS-PAGE (*c*) as described in the legend to Fig. 6.

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 conditions. 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 characterisitic 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 (δ) 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 (1.5×10^{-8} M) to 100 μg/ml (6×10^{-7} M) when initial velocities are measured at less than 2½ 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 2½ min. The activity of purified core enzyme is specific for cGMP,

TABLE III

Enzyme activity of bovine retina cGMP phosphodiesterase

Activity[a]	Specific activity	K_m	K_I
	μmol cGMP/min/mg		
Basal activity	15	150 μM cGMP (>4 mM cAMP)	
+GTP (0.16 mM)	15		
+$CaCl_2$ (0.5 mM)	15		
+Protamine (3 mg/ml)	120		
+Trypsin[b]	750		
+80K protein			
1:1 molar ratio	7.5		
1:10 molar ratio	1.5		
+IBMX			10 μM
+Papaverine			25 μM

[a] Assayed in 40 mM Hepes, 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 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 1:900 (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 retina.

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 α (M_r = 88,000) and β (M_r = 84,000) which are associated throughout the purification procedure by a third polypeptide γ (M_r = 13,000). We have tried to rule out the possibility that α and β might have been produced by proteolysis and that γ 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 α and β 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" γ 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 $A_{500\ nm}$ units of rhodopsin, $\bar{a}_M$ = 40,000 M^{-1} cm^{-1}), we obtain a ratio of 1:170. 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 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 to 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 phosphodiesterases (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. Maximally activating 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 additional subunits might be part of the holoenzyme. We have not ruled out this possibility, but can state that the catalytic sites are associated only with the M_r = 88,000, 84,000, and 13,000 subunits. In addition, characterization of the phosphodiesterase and 80K protein in samples in which both are present indicates that these two species behave as separate proteins once eluted from the membrane and not as a large holoenzyme (Figs. 6 and 7). 80K protein seems to modulate, not fully inhibit, activity since a 1:1 molar ratio of 80K/phosphodiesterase inhibits by 50% and a 10:1 ratio inhibits only by 85%. Presumably, the 80K protein which we have isolated is equivalent to the inhibitor protein first described by Dumler and Etingof (44) and possibly similar to that isolated from bovine brain (45, 46). From the total quantities of protein isolated, we estimate that the phosphodiesterase/80K ratio is at least 1:1, but 80K was not quantitatively released from ROS membranes and may be present in greater quantities in the native membranes.

Mechanisms have been suggested in which cGMP phosphodiesterase functions in modulation of photoreceptor sensitivity (9, 10) and/or in mediation of visual excitation (5, 13). We hope that biochemical characterization of the enzyme and the moieties that modulate this enzyme's activity will contribute to defining its physiological role.

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