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Extracellular cGMP Phosphodiesterase Related to the Rod Outer Segment Phosphodiesterase Isolated from Bovine and Monkey Retinas

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ABSTRACT: A phosphodiesterase (PDE) has been characterized in the interphotoreceptor matrix (IPM) of light-adapted fresh bovine retinas. It is obtained through a gentle rinsing of the retinal surface under conditions where the light-activated rod outer segment (ROS) enzyme remains attached. The enzyme has an apparent native molecular weight of 350000 by gel filtration and appears as a doublet at M_r 47000 and 45000 on sodium dodecyl sulfate–polyacrylamide gels. It has an apparent K_m value for cGMP of 33 μM and an apparent K_m value for cAMP of 2200 μM. It is activated 3–6-fold by protamine and over 40-fold by trypsin. Protamine has no effect on the K_m for cGMP while trypsin decreases the K_m for cGMP by a factor of 2. The enzyme occurs in at least two forms as evidenced by two distinct peaks of activity after gel electrophoresis under nondenaturing conditions. A heat-stable inhibitor is tightly bound to the enzyme. The inhibitor obtained from the IPM PDE inhibits 98% of the activity of the trypsin-activated ROS PDE: conversely, the inhibitor obtained by boiling the ROS PDE completely inhibits the trypsin-activated IPM enzyme. A high-affinity monoclonal antibody to the active site of the ROS PDE, ROS 1 [Hurwitz, R., Bunt-Milan, A. H., & Beavo, J. (1984) *J. Biol. Chem. 259*, 8612–8618], quantitatively absorbs the IPM PDE. These observations indicate a clear relationship between these two PDEs even though their location, sizes, and specific functions in the retina appear to be distinct.

The major cGMP phosphodiesterase (PDE)[1] activity in retina is present in the rod outer segment organelle of the photoreceptor cell (Miki et al., 1975; Baehr et al., 1979). The ROS PDE is activated by light (Miki et al., 1973; Chader et al., 1974) and is thought to play a role in phototransduction through regulation of cGMP levels (Bitensky et al., 1978; Miller, 1983). The enzyme is a peripheral protein tightly bound to the photoreceptor disk membranes when exposed to isotonic salt and Mg^{2+}, but it can be released by vigorous washing with low ionic strength buffer (Kuhn, 1982; Miki et al., 1975; Baehr et al., 1979). The purified ROS PDE has a molecular weight of 185000 and is composed of three subunits: M_r 88000 (α), M_r 84000 (β), and an inhibitory subunit, M_r 13000 (γ) (Baehr et al., 1979; Hurley & Stryer, 1982).

The photoreceptor cell outer segments and the processes of the pigment epithelial cells interdigitate. The extracellular material that surrounds these opposing surfaces is known as the interphotoreceptor matrix (IPM). There has been a recent upsurge of interest in the IPM as a separate, defined extracellular compartment important to visual function, specifically in connection with its role in retinol (vitamin A) transport and as a potentially excellent model for studying the components and functions of the extracellular matrix (Adler & Martin, 1982; Lai et al., 1982; Liou et al., 1982; Pfeffer et al., 1984).

When the IPM material is run on SDS–PAGE, about a dozen Coomassie blue staining protein bands can be visualized (Adler & Severin, 1981). However, prior to the present study, only one of these proteins had been purified and partially characterized. This protein is the interphotoreceptor retinoid binding protein (IRBP), a glycoprotein that could be involved in retinol transport between the pigment epithelium and retina (Lai et al., 1982; Liou et al., 1982; Adler & Martin, 1982; Pfeffer et al., 1984).

We have now identified a second protein in the IPM, a cGMP PDE (designated IPM PDE). It can be easily isolated through an extremely gentle, isotonic wash of whole, light-adapted retinas. This yields the constituents of the IPM and, at the same time, leaves the ROS PDE membrane bound. The

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[1] Abbreviations: PDE, phosphodiesterase; IPM, interphotoreceptor matrix; ROS, rod outer segments; IRBP, interphotoreceptor retinol binding protein; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Con A, concanavalin A; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; Cl_3CCOOH, trichloroacetic acid.

IPM PDE is freely soluble and has subunit sizes approximately half those of the ROS PDE. However, our present study demonstrates that it is probably related to the membrane-bound ROS enzyme. The relationship is indicated most strongly by the cross-reactivity of the IPM enzyme to a highly specific antibody to the catalytic site of the ROS PDE, ROS 1 (Hurwitz et al., 1984). In addition, both ROS PDE and IPM PDE contain a tightly bound, heat-stable inhibitor which is interchangeable: each inhibitor is capable of completely inhibiting the activity of both IPM and ROS PDE. Trypsin appears to activate both of these enzymes by removal of this inhibitor (Hurley & Stryer, 1982).

Experimental Procedures

Materials

Protamine sulfate (grade X), cAMP, cGMP, NADH, retinol, PMSF, dithiothreitol, TPCK-treated trypsin, soybean trypsin inhibitor, protamine–agarose, human α_2-macroglobulin, leupeptin, and rabbit anti-mouse IgG were obtained from Sigma Chemical Co., St. Louis, MO. [1-^{3}H]Retinol (14.3 Ci/mmol) and Protosol were from New England Nuclear Corp., Boston, MA. Protein A–agarose was from BRL, Gaithersburg, MD. Ultrogel AcA 22 was from LKB, Rockville, MD. Concanavalin A–Sepharose was from Pharmacia. Bio-Gel A 1.5 M, reagents, and molecular weight standards for native and SDS–PAGE were obtained from Bio-Rad, Rockville Center, NY. Hydrofluor and Betafluor were from National Diagnostics, Somerville, NJ. Guanosine-5′-monophosphate kinase, pyruvate kinase, lactic dehydrogenase, and adenylate kinase were from Boehringer Mannheim.

Buffers. Buffer A consisted of 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.8 mM PMSF. Buffer B consisted of 0.04 M sodium phosphate, pH 7.0, 0.16 M Na_2SO_4, 1.6 mM EDTA, and 20% glycerol. Buffer C contained 50 mM Tris-HCl, pH 7.6, and 140 mM NaCl.

Methods

Preparation of Crude ROS IPM Supernatant. All procedures were conducted at 4 °C. Frozen retinas from light-adapted bovine eyes were obtained from the Hormel Co., Austin, MN, and stored at −70 °C until use. Approximately 100 retinas were thawed at room temperature in a water bath and then divided into six portions in round-bottom centrifuge tubes. Three milliliters of buffer A was added to each tube. Each sample was vortexed for 15 s and then centrifuged at 800*g* for 5 min. The supernatant fractions were pooled and homogenized in a glass–glass homogenizer (20 strokes) followed by centrifugation at 110000*g* for 60 min. The 110000*g* supernatant containing soluble extracellular matrix components, soluble ROS components, and some soluble contaminants was used in subsequent gel filtration experiments. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Preparation of Interphotoreceptor Matrix Proteins. Fresh bovine eyes were obtained from a local slaughterhouse and kept packed in ice at all times. Retinas were carefully removed from the underlying pigment epithelium with fine forceps, cut at the optic nerve, and placed gently in Dulbecco's PBS containing proteolytic inhibitors at 0 °C: PMSF (0.8 mM) was used in the preparation from frozen retinas; a mixture of PMSF and leupeptin or α_2-macroglobulin was used in the fresh retinal preparations (see legend to Figure 4). After approximately 30 s, retinas were removed and discarded. The wash fluid was centrifuged at 110000*g* for 30 min and the tiny pellet discarded. The supernatant was designated the IPM wash. Monkey retinas were processed in the same way.

ROS PDE. Enzyme was purified by the method of Baehr et al. (1979) except that a final step of chromatography on Blue Sepharose was included.[2] The specific activity ranged between 5 and 10 units/mg (micromoles per minute per milligram).

Gel Filtration. All operations were carried out at 4 °C under dim red light. Portions (15 mL) of the high-speed supernatant, prepared as described above, were incubated in the presence of either 1 μM retinol or 1 μM [1-^{3}H]retinol. The entire sample (15 mL, approximately 400 mg of protein) was applied to a Bio-Gel A 1.5 M column (2.6 × 33 cm) which had been previously equilibrated with buffer A. Fractions of 3.3 mL were collected at a flow rate of 40 mL/h. The relative fluorescence intensity (340-nm excitation, 477-nm emission) was measured in each fraction by using the ratio mode of an SPF-500 spectrophotofluorometer (American Instrument). The fraction having the greatest relative fluorescence was reincubated with 1 μM retinol or 1 μM [1-^{3}H]retinol and applied to an Ultrogel AcA 22 column (1.6 × 35 cm). Fractions of 1.3 mL were collected at a flow rate of 10.4 mL/h.

Concanavalin A Chromatography. Eight milliliters of Con A–Sepharose was placed into a Bio-Rad polypropylene Econo column and washed with buffer C. The supernatant from 50 bovine retinas (approximately 150 mg of protein) was applied at a flow rate of 0.5 mL/min. The column was washed with buffer C until the A_{280} returned to the base line. Eight-milliliter fractions were collected. Fractions containing the bulk of the protein and activity (2–6; 40 mL) were pooled.

Protamine–Agarose Chromatography. A 15-mL, 5 cm × 1.8 cm protamine–agarose column was equilibrated with buffer C at a flow rate of 0.5 mL/min. The pooled peak from the Con A–Sepharose column was applied at the same flow rate and washed with buffer C until the A_{280} returned to the base line. The column was then eluted with buffer C containing 1 M NaCl. The PDE was eluted in three fractions of 8 mL each.

Native Disc Gel Electrophoresis. A 2.5% stacking gel and a 5.0% running gel were used. Electrophoresis was performed by using 1 mA per tube for 20 min followed by 2.5 mA per tube until the tracking dye reached the bottom of the gel (Davis, 1964). Following electrophoresis, the gels were cut at the dye front and either stained (0.05% Coomassie blue R-250 in 50% methanol and 9.3% acetic acid) or cut into 1-mm slices. The slices were placed in small plastic tubes and eluted with gentle shaking in 75 μL of a solution containing 0.04 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1 mM dithiothreitol, 1 mM $MgCl_2$, 10% glycerol, and 40 μg/mL bovine serum albumin. After removal of aliquots for PDE assay, either the remainder of the eluate was used for SDS gels or the gel slices were quantitatively transferred to scintillation vials, 0.3 mL of Protosol and 10 mL of Betafluor were added, and the radioactivity was determined.

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out according to the method of Laemmli (1970). Gels were fixed at room temperature with 15% Cl_3CCOOH for approximately 2 h, stained with 0.25% Coomassie blue R-250 in 45% methanol–9.2% acetic acid for 16 h at room temperature, and destained with glacial acetic acid–methanol–water (1:3:7). Standard proteins were the following (molecular weights in parentheses): myosin (200000), β-galactosidase (116000), phosphorylase *b* (92500);, BSA (66400),

[2] Unpublished observation.

ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (22 000), and lysozyme (14 400).

Sucrose Density Gradient Centrifugation. Samples (250 μL) were incubated with either 2×10^{-7} M [^{3}H]retinol alone or label plus a 500-fold molar excess of unlabeled retinol. Incubation was under dim red light at 0–4 °C for 2 h. The samples were layered on continuous sucrose gradients (4.6 mL, 5–20% sucrose in buffer A without PMSF) and centrifuged at 243000*g* for 16 h at 4 °C (SW 50.1 rotor of a Beckman LS-50 preparative ultracentrifuge). Approximately 35 fractions, 130 μL each, were collected from each tube, and the radioactivity was determined following the addition of 15 μL of Hydrofluor.

Trypsin Treatment of PDE. Trypsin was made up as a 1 mg/mL solution in 1 mM HCl–1 mM $CaCl_2$ and used at a final concentration of 20 μg/mL. The reaction was run at room temperature and stopped by adding aliquots of the incubation mixture to tubes in ice containing soybean trypsin inhibitor to yield a final inhibitor concentration of 100 μg/mL.

Enzymatic Assay of PDE. Fluorometric enzymatic analyses were performed by using the method of Carter et al. (1979) with the reactions combined into one step and run directly in the fluorometer in 1 mL of reagent. The reagent contained 50 mM imidazole hydrochloride pH 7.4, 75 mM KCl, 5 mM $MgCl_2$, 4 μM NADH, 16 μM phosphoenolpyruvate, 9.4 μM ATP, and 0.25–0.5 mg/mL protamine sulfate. The auxiliary enzymes were added together to the reagent in the fluorometer tube to give final concentrations of 9 μg/mL guanosine-5′-monophosphate kinase (or 2.5 μg/mL adenylate kinase where cAMP was substrate), 9 μg/mL pyruvate kinase, and 1 μg/mL lactic dehydrogenase. cGMP concentration was 1 mM unless otherwise indicated. There was approximately 0.05% GMP in the cGMP; once that had reacted, there was no further decrease in NADH fluorescence without PDE addition. Rates were the same with or without bovine serum albumin. EGTA (0.2 mM) had no effect on enzyme activity. The reaction was started with the addition of PDE. The lag time for the auxiliary enzymes was about 1.5 min. Assays were run at 23 °C. Addition of boiled PDE (inhibitor) had no effect on the activity of the auxiliary enzymes.

An advantage of the direct fluorometric assay is that one can monitor the time course of the reaction. Once the lag due to the auxiliary enzymes is over, another lag is observed due to dissociation of the inhibitor form the PDE. This lag phase is followed by a gradual acceleration which becomes more marked at high enzyme concentrations. Progress curves at both low (8 μM) and high (1 mM) cGMP concentrations showed similar lag plus acceleration phases. Because of the complex progress curves, and the lack of linearity between the amount of enzyme and rate (see Results), kinetic parameters are approximate. However, the same amount of enzyme was always used in determining K_m's so that differences due to dilution were eliminated. Furthermore, we always used the same section (maximum rate portions) of the progress curves to calculate our rates. The plots of $1/V$ vs. $1/S$ were linear and were used to determine K_m values. Trypsin treatment activated the enzyme (see Results) and completely abolished the second lag phase so that rates were linear once the initial lag due to auxiliary enzymes was over.

Immunoadsorption of cGMP PDE. The protein A–agarose suspension 600 μL) was centrifuged, washed 3 times, and resuspended in an equal volume of PBS. Rabbit anti-mouse IgG (200 μL) was added and the tube incubated 60 min at 37 °C. The protein A–IgG complex was collected by centrifugation, washed 4 times with PBS, and then resuspended

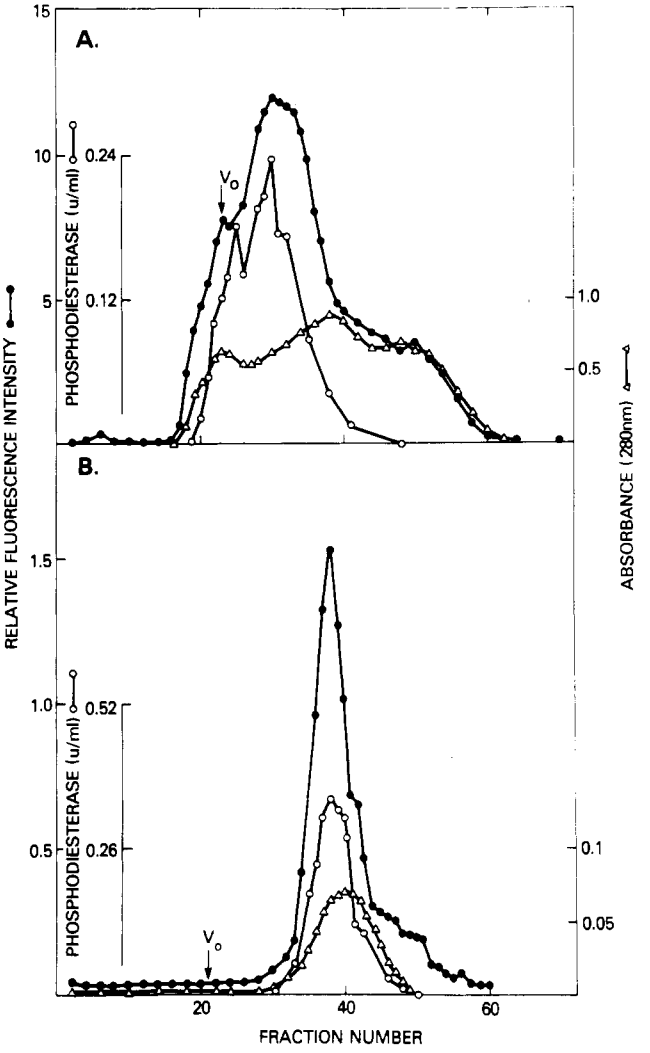

FIGURE 1: Gel chromatography of IRBP and IPM PDE. (A) The 1100000*g* supernatant from crude ROS of whole unwashed, frozen retinas was chromatographed on Bio-Gel A 1.5M (see Methods). (B) Fraction 30, the peak of both PDE and IRBP from the Bio-Gel column, was chromatographed on Ultrogel AcA 22. Fractions (3.3 mL) were assayed for relative fluorescence intensity (340-nm excitation, 477-nm emission, a measure of IRBP), absorption at 280 nm, and phosphodiesterase activity (units per milliliter) (see Methods).

in 200 μL of PBS. This was vortexed and divided into two equal volumes, and each was recentrifuged. One hundred microliters of PBS was added to one tube plus 25 μL of ROS 1 antibody. The control tube had 125 μL of PBS added. The tubes were incubated 60 min at 37 °C. The complexes were centrifuged, washed 4 times with 50 mM imidazole, pH 7.4, and resuspended in 100 μL of buffer. Bovine IPM wash (20 μL; 30 μg of protein) was added to each tube which was then incubated overnight at 4 °C. After centrifugation, the supernatants were assayed for PDE activity.

RESULTS

PDE from Intact, Frozen Retinas. During purification of the IRBP from the ROS IPM supernatant of frozen bovine retinas (see Methods), a cGMP PDE activity was detected. Although neither the IPM PDE nor the IRBP appears to be present in the retina per se, it was initially convenient to use the larger number of frozen retinas (which contained the associated IPM matrix) to begin the purification procedure. PDE activity was followed through Bio-Gel A 1.5 M and Ultrogel AcA 22 column chromatography (Figure 1A,B). The Bio-Gel column profile showed two peaks each of retinol binding and PDE activity with the major peak of both activities

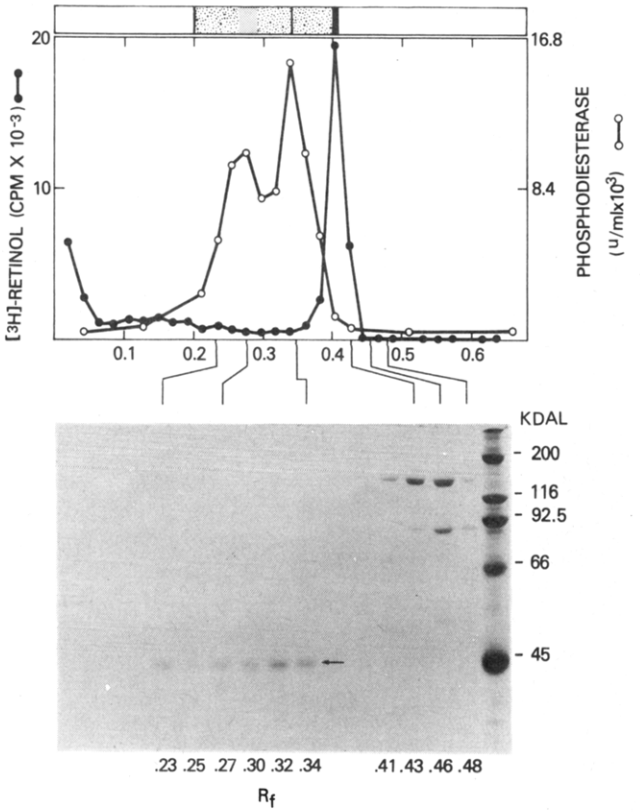

FIGURE 2: Native and SDS disc gel electrophoresis of material from the Ultrogel peak. The peak fraction from the Ultrogel column (fraction 30) was concentrated (Minicon macrosolute concentrator, Amicon Corp.) and reincubated with 1 μM [^{3}H]retinol under dim red light at 0 °C. Approximately 170 μg of protein was applied to three native 5% gels (see Methods). After electrophoresis, one gel was stained for protein with Coomassie blue R-250 (diagram, top of figure). A second gel was cut and eluted overnight, and aliquots were assayed for phosphodiesterase activity (upper panel). [^{3}H]Retinol was determined on the solubilized gel slices (see Methods). A third gel (lower panel) was sliced and eluted overnight with 0.063 M Tris, pH 6.8, and 5% mercaptoethanol. SDS was added to 2%; the samples were applied to a 3% stacking gel, electrophoresed on an 8% running gel at 50 V for 16 h, and stained for protein with Coomassie blue. The R_f's are shown at the bottom of the figure; molecular weight standards are shown in the lane at the far right.

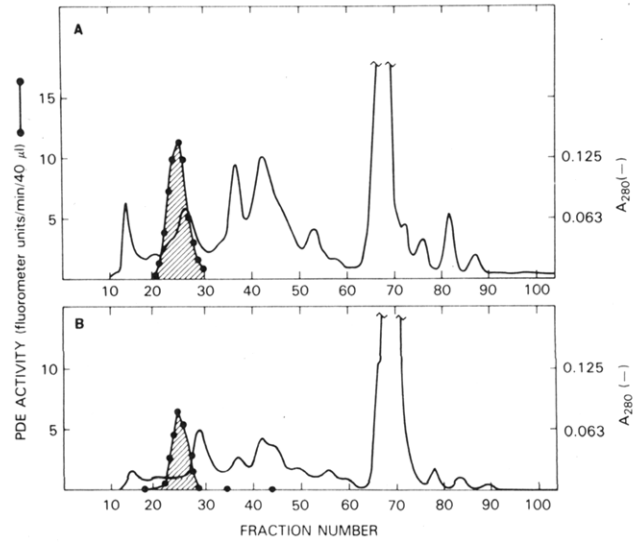

FIGURE 3: HPLC of interphotoreceptor matrix proteins. An IPM wash was obtained from bovine (A) and monkey (B) retinas at 0 °C by using PBS containing 0.8 mM PMSF. Samples (200 μL) of the 110000g supernatants were injected into an Altex Model 322 HPLC system equipped with two Beckman Spherogel TSK-3000SW (30 cm × 7.5 mm) size exclusion columns connected in series. Chromatography was carried out by using buffer B at a flow rate of 0.5 mL/min. The effluent was monitored continuously at 280 nm. Fractions (100 μL) were collected, and phosphodiesterase activity was measured on 40-μL aliquots.

coinciding at fraction 30. When fraction 30 was subsequently run on an Ultrogel column, the peaks of retinol binding and PDE activity showed an even more complete coincidence at fraction 38 (Figure 1B).

Although these proteins are closely associated on these columns, they can be separated on disc gel electrophoresis under nondenaturing conditions. The material from the peak of the Ultrogel column was incubated with [^{3}H]retinol and applied to three 5% gels. After electrophoresis, one gel was stained for protein; a second gel was sliced and eluted, the eluates were assayed for PDE activity, and the radioactivity was determined (see Methods). There were two distinct peaks of cGMP PDE activity in the eluted gel at R_f's of 0.26 and 0.34 (Figure 2, upper panel). These peaks corresponded to two Coomassie blue bands at these same R_f's on the stained gel (Figure 2, upper diagram). A darkly staining band at R_f 0.41 corresponded to the sharp peak of [^{3}H]retinol binding activity. A fourth band on the stained gel at R_f 0.20 was not identified.

The third gel was cut and eluted, and the eluates from the individual slices were run on an SDS slab gel (Figure 2, lower panel). The major protein seen in the region corresponding to the PDE on the native gel (R_f's of 0.23–0.34) was a band with a molecular weight of 45 000. A low cross-linking 15% acrylamide gel run later resolved this band into a doublet, M_r = 47K + 45K (see Figure 4, lanes 12 and 13). The IRBP subunit (apparent M_r 146 000) and an unknown protein (M_r 86 000) were also present at R_f's of approximately 0.41 (Pfeffer et al., 1983). This latter protein is more accurately sized in Figure 4 where it is seen as a band running between the 88K and 84K subunits of the ROS PDE.

PDE from IPM. Another more efficient way to isolate the IRBP and PDE was a simple washing procedure of the retinal surface. This process yielded the constituents of the IPM and gave higher yields of both IRBP and the IPM PDE with fewer manipulations (see Methods). The yield of PDE activity/bovine retina was approximately 2–3-fold higher in the IPM as compared to unwashed whole retinas. An exact quantitation of amount based on activity was not possible due to the presence of an endogenous inhibitor (see below). After centrifugation, the soluble proteins of the matrix material could be applied directly to a TSK-3000SW HPLC column.

Bovine IPM PDE elutes near the void volume at fraction 25 (Figure 3A). The IPM proteins isolated from fresh monkey retinas have also been analyzed on this column (Figure 3B); there was a PDE peak at the same position (fraction 25) as was seen for bovine IPM. The large peak (fractions 63–75) on the A_{280} profile was not protein as measured by the method of Lowry et al. (1951); it was most likely some component of PBS. A set of standard proteins run through this column yielded an apparent molecular weight of 350 000 for both bovine and monkey IPM PDE while the purified ROS PDE eluted at a position corresponding to M_r 190 000. Sucrose gradients (5–20%) of the bovine IPM also indicated that the native IPM PDE was of very high molecular weight; PDE activity sedimented almost to the bottom of the tube under conditions where the 7S IRBP was located near the middle of the gradient.[2]

Partial Purification of IPM PDE. Several chromatography steps were undertaken to obtain partial purification of the IPM PDE. In addition, two different types of proteolytic inhibitors were used in the initial rinse medium to see if that would affect

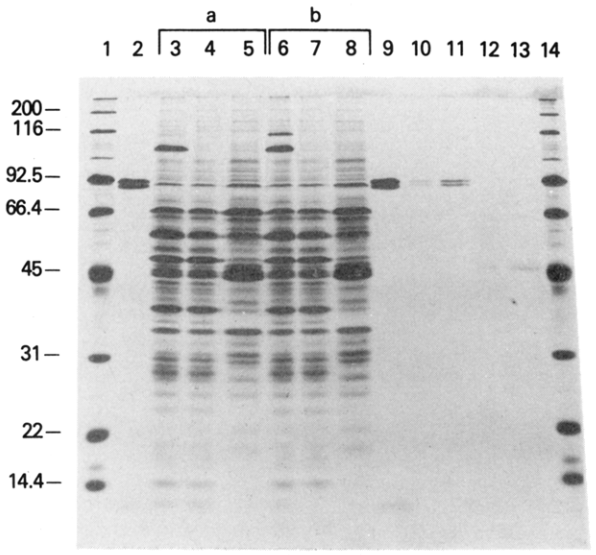

FIGURE 4: Partial purification of IPM PDE as followed by SDS–PAGE. Fifty fresh bovine retinas were rinsed for 30 s each at 0 °C in PBS containing 2 mM $MgCl_2$, 0.5 mM EGTA, and either 100 μM PMSF and 100 μM leupeptin (a) or 1 mg/mL α_2-macroglobulin (b). Thirty microliters of each fraction was applied to a 15% acrylamide–0.08% bis(acrylamide) gel (Baehr et al., 1979; Laemmeli, 1970) run at 60 V for 16 h. The gel was stained and destained as described under Methods. Lanes 1 and 14, marker proteins (see Methods); lanes 2 and 9–11, 6.6, 6.6, 0.22, and 1.1 μg of pure ROS PDE, respectively; lanes 3 and 6, the 110000*g* supernatants; lanes 4 and 7, the flow through from a concanavalin A–Sepharose column; lanes 5 and 8, the 1 M NaCl eluate from a protamine–agarose column; lane 12, the peak fraction from HPLC; lane 13, the pooled shoulders of the HPLC peak.

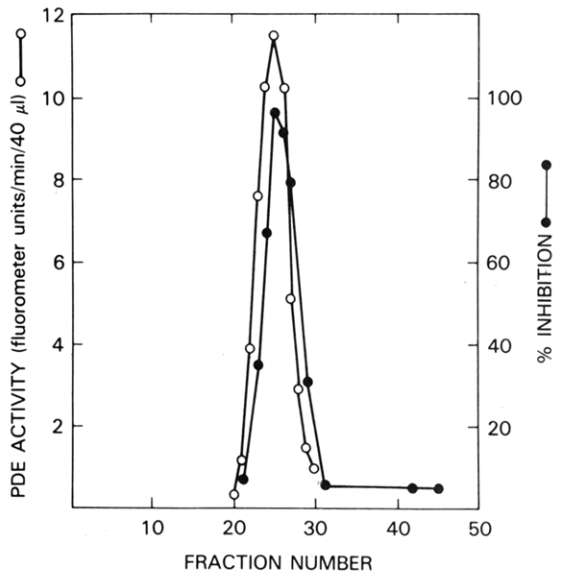

FIGURE 5: Location of PDE inhibitor on HPLC. Aliquots of fractions from the HPLC column run in Figure 3 were heated at 100 °C for 75 s and then cooled in ice. Ten-microliter aliquots of these boiled fractions were added to 1 mL of PDE reagent containing 1 μL of trypsin-activated 110000*g* supernatant (see Methods). The tubes were incubated at room temperature for 15 min before the addition of cGMP to start the reaction. Phosphodiesterase activity of the untreated column fractions shown in Figure 3 and also percent inhibition caused by these boiled fractions are shown.

the SDS pattern of proteins isolated. PMSF and leupeptin were added to the rinse medium of one group of retinas, and 1 mg/mL α_2-macroglobulin was added to the other. Ca^{2+} was omitted to avoid problems with Ca^{2+}-activated proteases, and for this reason also, EGTA was included. Both groups were processed identically through three steps: centrifugation at 110000*g* for 30 min, Con A–Sepharose chromatography, and protamine–agarose chromatography (see Methods). The α_2-macroglobulin preparation was processed further on HPLC (for method, see legend to Figure 3). Fractions were analyzed by SDS–PAGE (Figure 4). SDS–PAGE patterns in the two groups were identical at all stages (a vs. b, Figure 4) with the exception of the M_r 114K subunit of α_2–macroglobulin seen in lane 6. It is a glycoprotein and is removed during passage through Con A–Sepharose.

The Con A column serves to remove IRBP, the major glycoprotein of the IPM, and a protein with which the IPM PDE is closely associated on all types of columns tested (sizing, charge, and affinity). In the 8% SDS–PAGE (Figure 2, bottom panel), IRBP migrates with an apparent molecular weight of 146 000. However, on a low cross-linked 15% acrylamide gel (Figure 4, lanes 3 and 6), the apparent molecular weight of 100 000 is close to the value obtained by analytical ultracentrifugation.[2]

The protamine–agarose column yields approximately a 5-fold purification by tightly binding the IPM PDE and allowing approximately 80% of the protein to flow through: the doublet at 47K and 45K on SDS–PAGE is increased in amount relative to most of the other proteins present (lanes 5 and 8, Figure 4).

The protamine peak from set b was dialyzed overnight against buffer B, and 500-μL aliquots were applied to an HPLC TSK-3000SW column run in the same buffer. The peak fraction analyzed on SDS–PAGE shows that the predominant protein remaining is a doublet at 47K and 45K (lanes 12 and 13).

Pure ROS PDE run on the same gel shows the characteristic doublet at 88K and 84K as well as the 13K inhibitory subunit when 6.6 μg of protein was present (Figure 4, lanes 2 and 9–11). There is no evidence in the IPM supernatants for the 88K and 84K doublet. There is a band at 86K (migrating between the two ROS PDE subunits) which ran on native gels in an area with no PDE activity (Figure 2) and which by periodic Schiff stain was a glycoprotein.[2] Interestingly, there appears to be a band migrating close to that of the ROS PDE 13K subunit in both the IPM and Con A–Sepharose flow through (Figure 4a and lane 6 of Figure 4). However, it is greatly diminished after chromatography on protamine–agarose (Figure 4, lanes 5 and 8).

PDE Inhibitor. The activity of the PDE was not a linear function of the amount of protein present when tested in the IPM and Ultrogel fractions. Instead, the activity per unit amount of protein increased with increasing dilution. This indicated the presence of an inhibitor which might be dissociating as dilution increased. The presence of an inhibitor was tested directly by boiling aliquots of the fractions eluted from the HPLC column shown in Figure 3. This procedure completely destroyed PDE activity while leaving the inhibitory activity intact. These boiled aliquots were then added to reaction tubes containing trypsin-activated PDE (Figure 5). Maximum inhibition coincided with the maximum PDE activity at fraction 25. There was a slight shift apart at the trailing edge perhaps due to the dilution effect of the column, but, in general, there was close alignment of the two peaks. Under the conditions used, 96% of the PDE activity could be abolished.

The PDE from ROS also contains an inhibitor which has been isolated and identified as a subunit of this enzyme, M_r 13 000 (Hurley & Stryer, 1982). To test the ability of that inhibitor to inhibit the IPM PDE, purified ROS PDE was boiled for 1 min and added to trypsin-activated IPM PDE as well as to trypsin-activated ROS PDE. The converse exper-

Table I: Interchange of Inhibitors between IPM and ROS PDEs[a]

enzyme	addition	activity (μmol min^{-1} mg^{-1})	% activity remaining
IPM PDE[b]	none	0.30	100
(trypsin treated)	10 μL of boiled ROS PDE[c]	0.003	1
	10 μL of boiled IPM PDE[d]	0.016	5
ROS PDE[b]	none	100	100
(trypsin treated)	10 μL of boiled ROS PDE	1	1
	10 μL of boiled IPM PDE	2	2
IPM PDE	none	0.006	
(native)	10 μL of boiled ROS PDE	0.004	76
	10 μL of boiled IPM PDE	0.005	83
ROS PDE	none	4.1	
(native)	10 μL of boiled ROS PDE	2.8	68
	10 μL of boiled IPM PDE	2.8	68

[a] Enzyme activity was determined after incubating enzyme alone or enzyme plus inhibitor in 1 mL of reagent for 15 min at 23 °C. cGMP was added to start the reaction. The ROS PDE was a purified enzyme; the IPM PDE was a crude preparation obtained from washed retinas (see Methods). [b] Assayed without protamine present. [c] Two micrograms of protein. [d] Fourteen micrograms of total protein.

iment was also performed (Table I). Between 95% and 99% of the trypsin-activated activity of both enzymes could be blocked by the presence of their own as well as the other's inhibitor. Since native IPM PDE and ROS PDE already contained bound inhibitor, incubation of native enzyme with additional inhibitor caused little further inhibition (Table I).

Cross-Reaction with Monoclonal Antibody. IPM PDE was incubated with a protein A–agarose–IgG–antibody complex directed against the catalytic site of the ROS PDE (see Methods). After centrifugation to pellet the complex, no activity remained in the supernatant. A control incubation of IPM PDE with a protein A–IgG complex (no antibody) left all the activity in the supernatant.

Protamine and Trypsin Activation. The enzyme is activated by both protamine and trypsin. Protamine activation (with cGMP as substrate) occurs almost maximally (about 3-fold) at the lowest level tested, 0.05 mg/mL. Very little further stimulation was seen even when levels were increased a further 20-fold to 1 mg/mL. The maximum stimulation, however, depended to some extent on the enzyme concentrations; with protamine concentration held constant at 0.5 mg/mL, activation increased to 6-fold when protein concentration was decreased from 15 to 8 μg/mL.

Trypsin is a much more potent activator of the PDE (Figure 6). When the native enzyme, which contains bound inhibitor, is incubated with trypsin, a 16-fold activation occurs within 2 min, and greater than 40-fold is attained by the end of 12 min. Once activated by trypsin, activity assayed in the presence of protamine is about half of that assayed in its absence, a reversal of the case seen with native enzyme. However, because the basal activity of untreated PDE is low without protamine present, the percent increase in stimulation with trypsin treatment is dramatically greater when protamine is omitted from the assay (Figure 6). Even 1 min of trypsin treatment, while yielding only partial activation, gave increased rates which were linear, and did not respond to protamine; i.e., only slight activation produced an enzyme with the characteristics of the complete trypsin response.

PDE Kinetic Parameters. Because of the complexities of the assay mentioned under Methods, the kinetic parameters

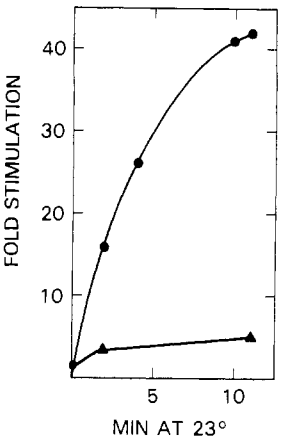

FIGURE 6: Trypsin activation of PDE. One hundred microliter portions of the IPM wash were incubated with 20 μg/mL trypsin for varying periods of time at room temperature before the reaction was stopped by the addition of soybean trypsin inhibitor (see Methods). Assayed without protamine (●); assayed with protamine present (▲).

do not obey strict Michaelis kinetics but are nevertheless useful approximations for comparison with the kinetics of the ROS PDE which is also subject to these same limitations. PDE activity was measured as a function of cGMP with and without protamine: the K_m for cGMP was 38 μM in the presence of protamine and 33 μM without. Thus, protamine did not alter the apparent K_m, although it increased the apparent V_m as seen above. The K_m for cAMP was not determined without protamine, but 0.5 mg/mL protamine did increase the rate at 1 mM cAMP. The K_m for cAMP, 2.2 mM, was 70-fold higher than that for cGMP determined under the same conditions. The effect of trypsin treatment was 2-fold: it not only caused a large increase (approximately 40-fold) in the V_m (Figure 6) but also caused a decrease in the apparent K_m by a factor of 2 (to 16 μM determined in the absence of protamine).

Discussion

It is clear from our preliminary work that the IPM PDE and ROS PDE are distinct enzymes. Isolation procedures for the IPM PDE have been exceedingly gentle (dipping whole retinas briefly in cold, isotonic buffer containing Mg^{2+}) and have employed PMSF and leupeptin or α_2-macroglobulin. Purification of the ROS PDE, on the other hand, requires quite different procedures. These include early steps of both extensive washing of the ROS membranes and homogenization in isotonic buffer accomplished by forcing the memrane suspension through a 14-gauge needle. These vigorous processes, although repeated 5 or more times, cause only trace losses of ROS PDE from the membranes (Baehr et al., 1979). Subsequent removal of the ROS PDE from the membrane requires both a hypotonic buffer and the absence of Mg^{2+}. Thus, these two PDEs are removed from different compartments of the retina under very different conditions. Even more compelling evidence comes from the molecular size data: SDS gels run at several stages of our purification show increasing amounts of 47K and 45K subunits accompanied by an increase in PDE specific activity. There is no evidence for the presence of the 88K and 84K doublet even when gels are analyzed with a very sensitive silver stain.[2]

However, several lines of evidence indicate a connection between this new PDE (located in the IPM) with the PDE in the ROS. Most significantly, an antibody specific for the catalytic site of the ROS PDE (Hurwitz et al., 1984) completely removes the activity from the supernatant of both the IPM and ROS enzymes, and the inhibitors obtained from each of the separate enzymes can completely inhibit their own

activity as well as the activity of the other PDE. In addition, both PDEs are activated by trypsin and protamine to a similar extent, and the apparent K_m's are similar in magnitude for both cAMP and cGMP. The effect of trypsin in both cases is to decrease the K_m by a factor of 2 (Hurley & Stryer, 1982), and once activated by trypsin, activity is greater in the absence of protamine.[2]

The high-affinity ROS 1 antibody (to bovine ROS PDE) is very specific: it has been shown to block the catalytic activity only of other ROS PDEs (human, monkey, bovine, rat, and goldfish) but not to affect any of the other types of PDEs tested including the cGMP-stimulated and the calmodulin-dependent types (Hurwitz et al., 1984). This is not too surprising since it has been shown by peptide mapping that bovine ROS PDE appeared to be unrelated to other PDEs tested (rat liver insulin sensitive and rat brain calmodulin sensitive) (Takemoto et al., 1982). Thus, the evidence to date indicates that the ROS PDE is a distinct entity unrelated to other PDE types, and this adds additional significance to the observed relationship with the IPM PDE. However, peptide mapping will need to be done on these two PDEs to determine the full extent of their similarities.

It seems possible that the 88K + 84K form of the ROS enzyme could give rise to the 47K + 45K form by a cleavage occurring either before or during secretion. Since both enzymes normally contain tightly bound inhibitor, it seems likely that it is the PDE–inhibitor complex (88–84–13K) which is cleaved such that the IPM PDE is secreted with inhibitor attached (47–45–13K), thereby providing a source of extracellular inhibitor as well as enzyme. This provides an explanation as to how a large activation can be achieved by tryspin digestion of the IPM PDE; we are presumably removing a 13K inhibitory subunit, as was shown to be the case with the ROS enzyme, although we have not as yet had the pure enzyme needed to answer this question directly. It is interesting to note that the eluate from the protamine column is missing a 13K band (lane 4 vs. lane 5, Figure 4). Whether this could be the inhibitory subunit is currently under investigation. Tight binding of the 47K + 45K subunits to protamine might loosen the 13K subunit and be the mechanism for activation.

The question of a product–precursor relationship with the IPM and ROS PDE enzymes has yet to be elucidated. Although it seems likely that a conversion of PDE forms may occur in vivo, it also appears that due to the extreme tightness of the binding of the ROS PDE to the disc membranes, it is highly unlikely that this specific and unusual cleavage occurs during our gentle isolation procedure with proteolytic inhibitors present. Either PMSF and leupeptin or α_2-macroglobulin was present during the crucial initial stages of isolation. α_2-Macroglobulin is especially useful as it has the capability of inhibiting the great majority of known proteinases (Barrett, 1981). Furthermore, data from other laboratories indicate that proteolysis is not a problem in the retinal system. Kohnken et al. (1981) isolated the ROS PDE from bovine retinas without PMSF and observed only the expected 88K and 84K subunits; Miki et al. (1975) isolated the ROS PDE from frog retinas also without PMSF present and obtained only two large subunit sizes. The fact that cleavage to the 47K + 45K subunits does not happen during in vitro trypsin digestion is additional evidence that the ROS PDE is not very susceptible to proteolytic degradation. The effect of trypsin treatment on bovine ROS PDE has been shown to be the removal of the 13K inhibitory (γ) subunit; a small amount of a 70K fragment is generated, but the 88K and 84K subunits are essentially unaltered (Hurley & Stryer, 1982). Furthermore, if the PDE were indeed formed as a result of proteolysis during isolation, more activity should have been present when whole cells were broken for more proteolysis could then occur, but this was not the case. In fact, we isolated more activity when we gently washed whole, fresh, unbroken retinas.

A somewhat analogous situation appears to exist in the slime mold *D. discoideum*, where an extracellular PDE along with its inhibitor is secreted into the medium (Kessin et al., 1979). Here also, a low molecular weight, heat-stable inhibitor can inhibit both intra- and extracellular forms of the PDE, and there is a decrease in subunit size upon secretion (Rutherford & Brown, 1983).

The fact that a cGMP PDE isolated from monkey IPM elutes at exactly the same position on HPLC as that from bovine IPM makes it seem highly probable that this IPM PDE is not confined to one species of animal but has a more general distribution as is true for the other identified IPM protein, IRBP (Bunt-Milam & Saari, 1983). Although the coelution of IRBP and IPM PDE on sizing columns (Bio-Gel and Ultrogel) could be fortuitous, the fact that these two proteins bound together on a cGMP affinity column makes it seem as if the association could be moderately tight and specific since IRBP, by itself, would not be expected to bind.[2]

The function of this new form of PDE is not known, but evidence from other tissues [pancreas (Kapoor & Krishna, 1977), pineal (O'Dea et al., 1978); pituitary (O'Dea et al., 1978), and liver (Tjornhammar et al., 1983)] shows that a cGMP efflux process exists in several cell types. Release of cGMP from the retina has not yet been investigated, but a light-induced Ca^{2+} flux from the ROS into the extracellular space has been described (Gold & Korenbrot, 1980; Yoshikami et al., 1980). cGMP levels in ROS are high (Farber & Lolley, 1974; Orr et al., 1976) and characterized by a high turnover rate (Goldberg et al., 1983) due, in part, to the light-activated ROS PDE. The regulation of cGMP concentration must be important in the ROS since elevated cGMP concentration has been shown to be associated with ROS degeneration in the mouse (Farber & Lolley, 1976), dog (Aguirre et al., 1978), and human (Ulshafer et al., 1980). Secretion, in addition to high ROS PDE activity, could provide another means for the control of cGMP levels. Another possibility is that secreted cGMP could serve as an intercellular signal, perhaps to the pigment epithelial cells which serve important functions in the phagocytosis of shed ROS as well as in the transport of vitamin A and other essential nutrients to the retina. In either case, an IPM PDE activity would be important in regulating the extracellular cGMP levels.

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Registry No. PDE, 9068-52-4; cGMP, 7665-99-8; cAMP, 60-92-4; trypsin, 9002-07-7.

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Mammalian Glycinamide Ribonucleotide Transformylase: Purification and Some Properties†

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ABSTRACT: Glycinamide ribonucleotide transformylase, the first of the two formyl group transferases of de novo purine biosynthesis requiring 10-formyltetrahydrofolate, has been purified 1500-fold, nearly to homogeneity, from the murine lymphoma cell line L5178Y. Purification of the enzyme was facilitated by the use of a gelatin protease "affinity" resin. This mammalian enzyme is a monomer of approximate M_r 110000. The kinetic studies are consistent with a sequential reaction mechanism and yield Michaelis constants of 0.4 mM for the substrate, glycinamide ribonucleotide, and 0.25 μM for the cofactor analogue 10-formyl-5,8-dideazafolate. A minimum V_{max} of 2 μmol/(min·mg) was obtained for the purified enzyme, from which a turnover number of 4 s^{-1} was calculated.

The pathway of de novo purine biosynthesis was first elucidated by Buchanan and Greenberg and their co-workers in the early 1950s (Buchanan, 1960). Their work led to the identification of the intermediates of this pathway and the enzymic activities responsible for their interconversion. Glycinamide ribonucleotide (GAR)[1] was identified as one of these intermediates and a preliminary characterization of the GAR TFase reaction was reported (Hartman & Buchanan, 1959). These experiments, as well as subsequent enzymological studies

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[1] Abbreviations: GAR, glycinamide ribonucleotide; GAR TFase, glycinamide ribonucleotide transformylase; 2-ME, 2-mercaptoethanol; Me_2SO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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