Guanylate Cyclase from Bovine Rod Outer Segments: Solubilization, Partial Purification, and Regulation by Inorganic Pyrophosphate†

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ABSTRACT: In spite of its pivotal role in visual transduction, very little is known about guanylate cyclase of retinal photoreceptor cells. The enzyme has not yet been purified principally because of the difficulty in solubilizing it. We report here a simple method for solubilization of 67% of the cyclase activity from the retinal rod disk membranes (RDM). With Nonidet P-40 as detergent, the solubilization of cyclase is favored by a high concentration of KCl and exclusion of manganese. The solubilized and the residual insoluble enzymes are both highly unstable but could be partially stabilized by dithiothreitol. They were both insensitive to calcium, calmodulin, and atrial natriuretic factor. They also responded similarly to varying the manganese concentration in the assay. For the activity in both fractions, the K_m for GTP was about 230 μM, Lineweaver–Burk plots showed that substrate binding was cooperative, and Hill plots suggested that there are two substrate binding sites. Cumulatively, these observations showed that while the entire activity could not be solubilized, the solubilized and the residual insoluble activities probably belonged to the same enzyme. Partial purification resolved the solubilized enzyme into two activities refered to as enzymes 1 and 2. Both had substrate saturation kinetics similar to the solubilized enzyme and were inhibited competitively by inorganic pyrophosphate, one of the products of the cyclase reaction. The K_i for PP_i for enzyme 1 was 70–100 μM and 150–200 μM for enzyme 2. cGMP at concentrations up to 800 μM had no influence on the activity of either enzyme. Rod outer segments contain an inorganic pyrophosphatase activity, with a K_m for the substrate of 26 μM and a V_{max} of 175 μmol min^{-1} (μmol of rhodopsin)$^{-1}$. The enzyme is not regulated by light in the presence or absence of GTPγS, but is inhibited by calcium. The data suggest the possibility that calcium-mediated inhibition of inorganic pyrophosphatase could lead to a rise in intracellular pyrophosphate concentration which in turn inhibits cyclase. This could be one of the mechanisms by which calcium regulates cyclase activity in the rods.

During the last 2 decades, considerable progress has been made in demonstrating that cyclic GMP is the internal transmitter in vertebrate rod phototransduction [see reviews by Pugh and Cobbs (1986) and Stryer (1986)]. Light-initiated reactions lead to rapid hydrolysis of cGMP and consequent suppression of the inward dark current in rod outer segments. Full recovery of dark current was hypothesized to result from inactivation of light-activated enzymes (Liebman & Pugh, 1980) and increased synthesis and restoration of cGMP levels. While several reports showed that rhodopsin kinase, GTPase, and possibly S-antigen are involved in the inactivation of light-activated enzymes (Sitaramayya & Liebman, 1983; Miller et al., 1986; Wilden et al., 1986; Zuckerman & Cheasty, 1986; Bennett & Sitaramayya, 1988; Schleicher et al., 1989), studies on cGMP synthesis were hampered by the high cGMP phosphodiesterase activity in the crude system. Purification of the cyclase was hindered by the difficulty in solubilizing the enzyme from the rod disk membranes (RDM) (Krishnan et al., 1978; Fleischman, 1981). In view of the reports that cyclase is regulated by calcium and that it may play a significant role in the adaptation (Cohen et al., 1978; Lolley & Racz, 1982; Pepe et al., 1986; Koch & Stryer, 1988; Hodgkin & Nunn, 1988; Kondo & Miller, 1988), we have attempted to solubilize guanylate cyclase as a prelude to purification. In this report, we describe a simple procedure to solubilize a majority of this activity from RDM. The enzyme is partially purified and resolved into two components. Both components show regulation by inorganic pyrophosphate (PP_i). PP_i is hydrolyzed in the RDM by a pyrophosphatase activity which is sensitive to calcium, leading to the possibility that cyclase could be regulated by calcium via pyrophosphatase. A preliminary account of these results was presented to the Biophysical Society (Hakki & Sitaramayya, 1989).

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Materials and Methods

Fresh bovine eyes were purchased from MOPAC, Souderton, PA. [8-^{3}H]GTP and [α-^{32}P]GTP were from ICN, [^{32}P]PP_i was from NEN, Nonidet P-40 was from Fluka, and other chemicals were mostly from Sigma.

Buffers. (A) Isolation buffer: 20 mM MOPS, 2 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF, pH 8.0. (B) Extraction buffer: 10 mM Tris, 5 mM DTT, 0.5 mM PMSF, and 12.5 μg/mL each of aprotinin, benzamidine, and Leupeptin, pH 7.5. (C) Solubilization buffer: 25 mM Tris, 5% Nonidet P-40, 1 mM DTT, and 1 M KCl, pH 7.0. (D) Assay buffer: 25 mM Tris, 1 mM DTT, 1 mM IBMX, 1 mM cGMP, 1 mM GTP, 1 mM Mn^{2+}, and 1–2 μCi of [8-^{3}H]GTP or [α-^{32}P]GTP, pH 7.0.

Isolation and Washing of RDM. RDM isolated as described earlier (Sitaramayya, 1986) were washed 3 times in buffer A, twice in buffer B with 2 mM $MgCl_2$ and 50 μM GTP, once in buffer B with $MgCl_2$ alone, once in buffer C with 200 mM KCl, once in buffer C with 200 mM KCl and 2 mM EDTA, and finally in buffer C with 2 mM EDTA. This washing sequence is designed primarily to remove most of the soluble and peripheral proteins from the RDM. The final pellets were suspended in 100 mM Tris buffer, pH 7.0, and stored in approximately 4 mg of protein aliquots at −30 °C.

Solubilization. Frozen RDM were thawed and diluted to 1–4 mg of protein/mL in solubilization buffer. The suspension

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was vortexed for 1 min at maximum speed, an aliquot saved for activity and protein determination and electrophoresis, and the rest centrifuged for 1 h at 48000*g*. The pellet was small and opaque. An aliquot of the supernatant was saved again and the rest centrifuged at 109000*g* for 1 h (density of the medium is 1.04). The supernatant was decanted carefully. The pellet and the tube were rinsed twice carefully with buffer C and suspended in the same buffer.

Partial Purification of Cyclase. For these experiments, the membranes treated with detergent and KCl as described above were centrifuged directly at 109000*g* for 1 h. The supernatant was collected without disturbing the pellet, an aliquot was saved for protein and activity determinations, and 2.5 mL was applied to a 1.5 × 120 cm column of Biogel A 0.5m equilibrated in 25 mM Tris-HCl, 200 mM KCl, 0.1 mM PMSF, 1 mM DTT, 20 μg/mL benzamidine, 0.1% Nonidet P-40, and 0.02% asolectin, pH 7.0 (gel filtration buffer). The column flow rate was 7.8 mL/h. Two fractions were collected per hour, and the column was monitored at 280 nm. Guanylate cyclase activity and protein were determined in the fractions, and the protein composition of the fractions was checked by SDS–PAGE.

The fractions containing the cyclase activity were pooled and diluted so that the buffer composition did not change except that the KCl concentration was reduced to 50 mM. The diluted enzyme was loaded on a 1 × 3 cm column of DEAE-cellulose (DE52) equilibrated in ion-exchange buffer (same as gel filtration buffer except that the KCl concentration was 50 mM). The effluent was collected and column washed with 120 mL of equilibration buffer. The bound protein was eluted with a 50-mL linear gradient of 50–400 mM KCl in equilibration buffer. The elution was carried out at 20 mL/h, and 2-mL fractions were collected. The cyclase activity eluted as two distinct peaks, at 135 and 255 mM KCl referred to hereafter as enzymes 1 and 2. Fractions with higher activity in each peak were pooled separately and concentrated on a Centricon-30 to the desired volume. The cyclase activity of both enzymes decayed rapidly at 4 °C, and therefore all experiments on these preparations were carried out within 24 h after the DE52 chromatography.

Guanylate Cyclase Assay. All assays were done in room light in a final volume of 40 μL for 15 min at 37 °C in buffer D. When the influence of calcium was tested, manganese in the assay buffer was replaced by 5 mM $MgCl_2$, and the concentration of GTP was kept at 0.5 mM. Varied concentrations of free calcium were obtained by the addition of EGTA and $CaCl_2$ according to the computations kindly provided by Dr. Edwin Barkdoll using the stability constants from Tsien (1980). In all cases, the reaction was stopped with the addition of an equal volume of 40 mM EDTA and 2 mM each of cGMP, GTP, GDP, GMP, and guanosine followed by heating for 2 min at 80–90 °C. The tubes were then centrifuged at 2000*g* for 10 min, and 3-μL aliquots of each supernatant were applied on a PEI–cellulose plate. The plates were developed by using the lithium chloride system as described by Kavipurapu et al. (1982). The nucleot(s)ide spots were identified under UV light, cut out, extracted into 1 mL of 2 M KCl, and counted in 10 mL of ACS II scintillation fluid (Amersham). Under the conditions of assay, about 7% of the cGMP formed by RDM was converted to GMP, showing that a trace of phosphodiesterase activity still remained with the membranes in spite of extensive washing. The solubilized and insoluble fractions obtained from RDM as described above had no nucleotidase or cGMP phosphodiesterase activities (no counts in GMP or guanosine using [8-^{3}H]GTP as substrate). IBMX and cGMP were therefore deleted from assay buffer when these fractions were monitored for activity. While the insoluble fraction had no GTPase activity, the solubilized preparation had some, usually converting about 0.3% of added GTP to GDP. Under these conditions, it was not necessary to use a GTP-regenerating system for the cyclase assay, and none was used. Also under the assay conditions, the cyclase activity was linear with incubation time up to 20 min and with protein up to 0.5 mg/mL.

When frozen retinas were used in preparing RDM, the membranes washed by the same procedure as described above retained considerable amounts of GTPase and phosphodiesterase activities. RDM from fresh retinas were used for all the experiments reported here.

Inorganic Pyrophosphatase Assay. The intact rod outer segments used in these assays were isolated in the dark according to Schnetkamp and Kaupp (1985) from fresh dark-adapted bovine retinas. They were frozen in aliquots at −30 °C and thawed out before the assays. The assays were done according to Springs et al. (1981) at 37 °C in the dark or in room light in 100 mM Tris and 10 mM $MgCl_2$, pH 7.0, with or without 10 μM GTPγS. The pyrophosphate concentration in the assays was 4 mM (with about 10^6 cpm in [^{32}P]PP_i) except when it was varied between 50 μM and 1 mM for the determination of K_m. Under standard assay conditions, the activity was linear with time up to 10 min and with RDM in the assay up to 0.3 μM rhodopsin.

Other Methods. Electrophoresis was according to Baehr et al. (1979), and the gels were stained with Coomassie Brilliant Blue or silver stained by the method of Merril et al. (1981). Protein determinations were according to Markwell et al. (1981) and Read and Northcote (1981) using serum albumin as standard.

Results

Solubilization

Rod disk membranes formed 3.7 ± 0.9 (n = 9) nmol of cGMP min^{-1} (mg of protein)$^{-1}$ at 37 °C. The activity increased by about 15% in the presence of 5% Nonidet P-40. When membranes vortexed for 1 min at maximum speed in 5% Nonidet P-40 were centrifuged at 48000*g* for 1 h, the supernatant contained 16 ± 5% (n = 4) of cyclase activity. Vortexing intermittently for an hour before centrifugation did not increase the activity in the supernatant. The activity in the solubilized fraction (48000*g* supernatant) was not significantly different at concentrations of Nonidet P-40 between 0.2 and 5%. In the absence of the detergent, vortexing alone did not release measurable activity into solution.

Of various attempts made to increase the fraction of solubilized activity, treatment with KCl was the most successful. Figure 1 shows the influence of KCl up to 2 M on the activity and solubilization of guanylate cyclase. The activity was about 40% higher in KCl between 400 and 1000 mM but declines thereafter to below control level at 2 M KCl. In the absence of detergent, however, KCl had no significant effect on the activity or solubilization at 200–2000 mM concentration (data not shown). In the presence of the detergent, the fraction of solubilized enzyme increased with KCl concentration up to about 1–1.2 M, and thereafter higher concentrations did not significantly influence solubilization. Since the activity was inhibited at higher concentrations and solubilization was not better, further studies were all done at 1 M KCl and 5% Nonidet P-40. When the membranes were treated in the presence of manganese (5 mM), the solubilization was about 40%, but varied between 65 and 95% (82 ± 8% in seven

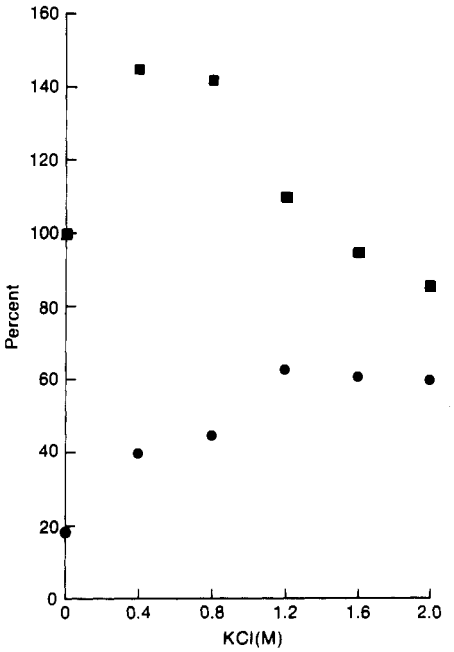

FIGURE 1: Influence of [KCl] on the activity and solubilization of guanylate cyclase. RDM, washed extensively to remove soluble and peripheral proteins, were divided into several portions, and each one was diluted to 1 mg of protein/mL in 25 mM Tris, pH 7.0, 1 mM DTT, 5% Nonidet P-40, and varying KCl concentrations between 0 and 2000 mM. The tubes were vortexed for 1 min, and an aliquot was removed from each tube to measure cyclase activity (■). The tubes were spun for 1 h at 48000*g*, and the supernatants were again assayed for activity (●). The experiment was repeated 4 times with similar results except that when DTT was deleted or 5 mM manganese was included, the solubilization was lower. At 1 M KCl and 5% Nonidet P-40 tested in the presence of DTT and without manganese, the solubilization in seven experiments was 82 ± 8%.

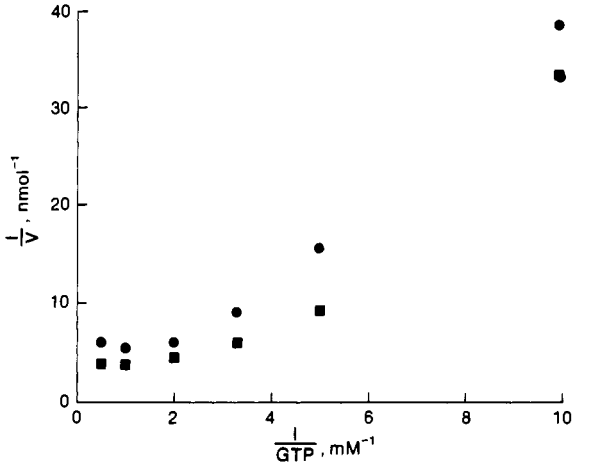

FIGURE 2: Substrate dependence of the cyclase activity. The solubilized and the residual insoluble (109000*g* pellet) fractions were assayed for cyclase activity as described under Materials and Methods except that the GTP concentration was varied between 0.1 and 2.0 mM. Manganese was equimolar with GTP at all concentrations of GTP. Lineweaver–Burk plot is shown. (●) Solubilized fraction; (■) insoluble fraction.

experiments) in its absence. Centrifugation of the 48000*g* supernatant for 1 h at 109000*g* and careful decanting of the supernatant revealed a very small and transparent pellet. Of the activity centrifuged, 66 ± 15% appeared in the 109000*g* supernatant, and 19 ± 15% appeared in the pellet (n = 5). In 20 experiments, where detergent- and KCl-treated RDM were spun directly at 109000*g* for 1 h, 67 ± 12% of the activity was found in the supernatant.

Solubilized and Residual Insoluble Cyclase Have Similar Properties

In order to verify if the solubilized and the residual insoluble (109000*g* pellet) enzymes are one and the same or different enzymes, their properties were investigated.

Stability. Both enzymes were almost totally inactive 2 days after preparation: only about 4% of activity remained. However, if DTT was included at 1 mM in the solubilization buffer, both enzymes were relatively stable: 59% of insoluble and 51% of solubilized activities were measured 2 days after preparation in DTT.

Influence of Manganese. Manganese supported the activity of both enzymes better than magnesium. Solubilized enzyme was 5-fold more active in manganese, and the insoluble fraction was 6-fold higher. As reported earlier by Krishnan et al. (1978), cyclase activity depended upon the GTP:Mn^{2+} ratio in the assays. The maximal activity was obtained at a GTP:Mn^{2+} ratio of 1:1. Compared to this activity, the activity at 1:5 was 92% and 86% for soluble and insoluble fractions, respectively, and at 1:10, it was 51% and 53%.

Atrial Natriuretic Factor. Both insoluble and solubilized fractions were tested for the influence of ANF. At 0.25 μM, sufficient to fully activate cyclase from bovine adrenal cortex (Tremblay et al., 1986), ANF affected neither enzyme activity significantly.

Kinetic Properties. Both enzymes were assayed at 0.1–2.0 mM GTP with manganese kept equimolar to GTP. The activities are shown in a Lineweaver–Burk plot (Figure 2). Both the enzymes showed positive cooperativity. Hill plots of the data gave n values of 2.5 for solubilized and 2.3 for insoluble enzyme. From plots of $1/v$ vs $1/[S^2]$, which were linear, the K_m measured for insoluble and solubilized enzymes was the same at 0.23 mM.

Influence of Calcium. Cyclase activity in either fraction was not influenced by 10^{-9}–10^{-6} M calcium. Also, addition of calmodulin to a final concentration of 6 μM did not elicit sensitivity to calcium.

Partial Purification Resolves Two Enzyme Activities

Solubilized guanylate cyclase appeared as a sharp peak in the excluded volume when chromatographed on Biogel A 0.5m or Sephadex G-200. On Biogel A 1.5m, the activity eluted behind the excluded volume but was spread over serveral fractions much like a smear rather than as a peak. These preliminary experiments suggested that the solubilized cyclase was present in more than one molecular size between 500 000 and 1 500 000 daltons (exclusion limits of Biogels A 0.5m and A 1.5m, respectively). Biogel A 0.5m chromatography was chosen as the first step in purification since the activity eluted from this column as a sharp peak in three to four fractions (Figure 3) and was reasonably well separated from the bulk of rhodopsin which appeared after the cyclase activity, though the fractions with cyclase activity also contained some rhodopsin. About 115 ± 66% (n = 18) of the activity loaded on a A 0.5m column was recovered in the fractions. The recoveries were lower when the KCl concentration in the column buffer was reduced to 50 mM.

After several repetitions of the chromatography on this column, it was no longer necessary to assay the fractions for cyclase activity before the next step. Routinely, the first two fractions in the exclusion peak were pooled, and they contained 89.7 ± 13.4% (n = 16) of the eluted activity and 12.5 ± 2.9% (n = 4) of the protein loaded on the column. The pooled fractions were diluted to 50 mM KCl and fractionated on DEAE-cellulose (DE52).

Most of the rhodopsin in the enzyme preparation loaded on the DEAE-cellulose column appeared in the effluent, and in

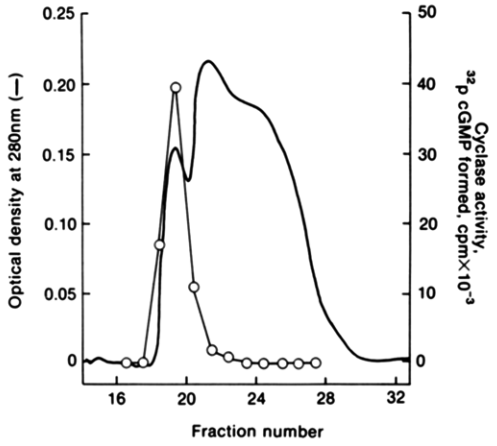

FIGURE 3: Gel filtration of cyclase. 2.5 mL of solubilized cyclase preparation was filtered on a 1.5 × 120 cm column of Biogel A 0.5m. The column was monitored at 280 nm, and the fractions were assayed for cyclase activity.

Table I: Partial Purification of Guanylate Cyclase from RDM

treatment	total protein (mg)	total act. (nmol/min)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]
solubilized preparation	10.25	12.73	1.2
Biogel A 0.5m column	2.73	14.52	5.3
DE52 column			
enzyme 1	0.19	2.47	13.0
enzyme 2	0.25	2.82	11.3

spite of extensive washing (up to 150 mL used for the 1 × 3 cm column), some remained bound to the column. Cyclase activity remained in the column during the loading and washing. In preliminary experiments, batch elution of the bound activity showed that cyclase eluted with 100, 150, 200, 250, and 300 mM KCl. Raising the KCl concentration up to 1000 mM did not result in elution of additional activity. Slower elution in 100 mM KCl (at 8 mL/h) did not prevent additional activity eluting at higher KCl. Of the activity loaded on the DE52 column, 34 ± 15% (n = 12) could be recovered in the eluted fractions.

Gradient elution of the DEAE-cellulose column with 50–600 mM KCl again revealed that cyclase activity continued to elute up to about 350 mM KCl. We have routinely employed a 50-mL linear gradient of 50–400 mM KCl to elute the activity. As shown in Figure 4, cyclase activity eluted as two distinct peaks at approximately 135 and 255 mM KCl. Fractions under the bars were pooled separately and were referred to as enzymes 1 and 2.

Table I shows the results of purification in a typical experiment. The specific activities of enzymes 1 and 2 are only about 10-fold higher than that of the starting material. When the starting material had higher specific activity, enzymes 1 and 2 had correspondingly higher specific activity, but the net increase after the two columns was generally only about 10-fold.

Stability of DE52-Purified Enzymes. As described above, the cyclase activity in RDM was stable but decayed rapidly upon solubilization. The partially purified enzymes after the DEAE-cellulose column were more unstable than the crude solubilized preparation, losing about 50–75% of the activity in about 24 h. Asolectin, PMSF, and benzamidine did not influence stability.

Identification of Cyclase Activity with Specific Proteins. The proteins that appear to be enriched in the enzyme fractions after DEAE-cellulose chromatography are the high molecular weight proteins of 220K (enzyme 1) and 240K–260K (enzyme

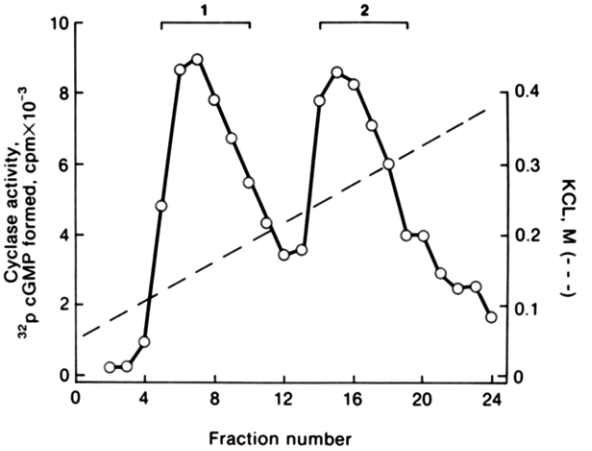

FIGURE 4: Ion-exchange chromatography of cyclase. The first two active fractions from the Biogel column were pooled and diluted to 50 mM KCl as described in the text and loaded on a 1 × 3 cm DEAE-cellulose (DE52) column. After being washed, the column was eluted with 50 mL of a liner gradient of 50–400 mM KCl. Fractions were assayed for cyclase activity, and selected fractions from the two peaks, as shown under the bars, were pooled separately and referred to as enzymes 1 and 2.

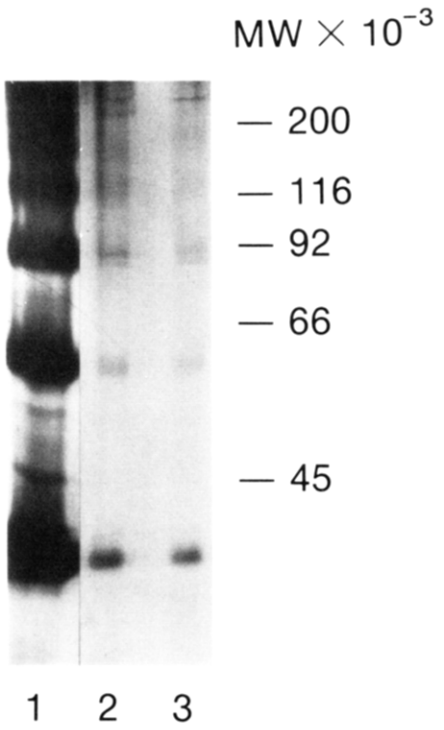

FIGURE 5: Protein composition of partially purified cyclase. Solubilized preparation (lane 1) and enzymes 1 (lane 2) and 2 (lane 3) were electrophoresed, and the gel was silver stained. Molecular weight calibration is shown on the right side.

2) (Figure 5). Whether the 220-kDa protein is a proteolytic product of the 240–260-kDa protein and whether either one of them is the cyclase are not certain. In spite of extensive washing of the DEAE-cellulose column after the enzyme was loaded, a fraction of rhodopsin remained attached to DE52 and eluted with the cyclase activity, suggesting that some rhodopsin may be tightly associated with cyclase.

Regulation of Cyclase by Inorganic Pyrophosphate

Cyclase catalyzes the conversion of GTP to cGMP and PP_i. It is a common practice to include millimolar cGMP in cyclase assays to protect the radiolabeled cyclase product, [^{32}P]cGMP, from hydrolysis by phosphodiesterase. In our experiments, up to 800 μM cGMP in the assays did not inhibit the cyclase

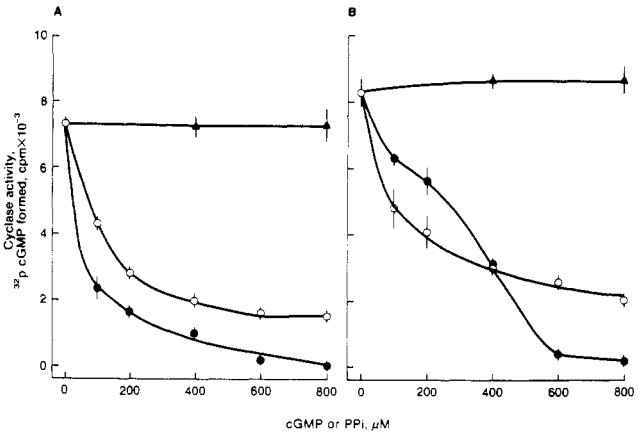

FIGURE 6: Influence of [PP_i] on cyclase. Enzymes 1 (A) and 2 (B) obtained as shown in Figure 4 were assayed in the presence of varying concentrations of PP_i (○, ●) or cGMP (▲). The assays contained either 0.2 mM each of manganese and GTP (○, ▲) or 1.2 mM manganese and 0.2 mM GTP (●). The data shown are the mean ± SD of triplicate assays. Similar results were obtained on four different batches of enzymes 1 and 2.

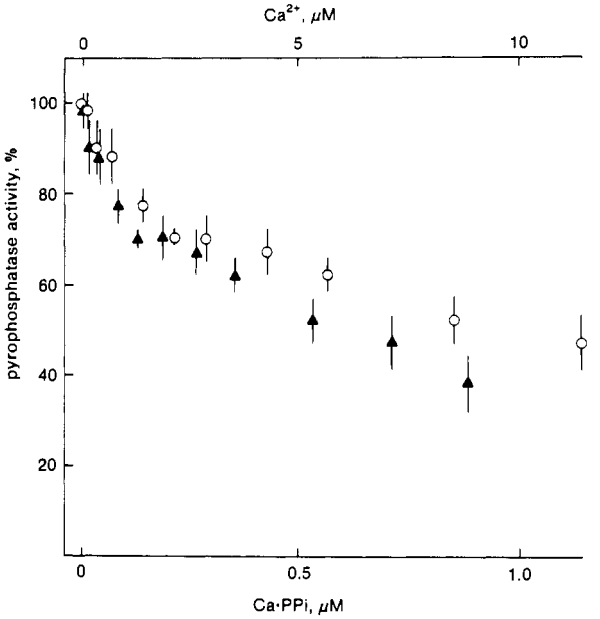

FIGURE 7: Influence of [calcium] and [Ca·PP_i] on inorganic pyrophosphatase activity of RDM. Pyrophosphatase was assayed as described under Materials and Methods except that the Mg^{2+} and PP_i concentrations were 1 and 0.5 mM, respectively. Different amounts of calcium were added to give the calculated free calcium (▲) and Ca·PP_i (○) concentrations. The data shown are mean ± SD of triplicate assays. Similar results were obtained in two experiments.

activity at 200 μM GTP as substrate (Figure 6). This suggested that cGMP has a very poor affinity to cyclase. However, the other product of the cyclase reaction, PP_i, inhibited the enzyme very strongly (Figure 6). When assays were done with equimolar GTP and Mn at 200 μM, PP_i inhibited both the partially purified enzymes to a maximum of 70–80%. At this concentration of Mn and GTP, about 177 μM GTP is present as GTP·Mn (stability constant of 353000 M^{-1}; Bartfai, 1979). GTP·Mn, not GTP, is the substrate for cyclase. Since PP_i also binds Mn, as PP_i concentration is increased, the GTP·Mn concentration should decrease, thereby decreasing (apparent inhibition) the activity of cyclase. Should that be the reason for the effect of PP_i, the presence of excess Mn should prevent the effect of PP_i. When 1 mM additional Mn was added to the assays, the inhibition by PP_i was even stronger. The activity was totally inhibited by 600 μM PP_i. The concentration of PP_i at which half the activity was inhibited was lower in the presence of additional Mn for enzyme 1 (Figure 6A). This showed that while PP_i could in part reduce cyclase activity by depleting Mn, it also serves as a strong inhibitor by itself. Between PP_i and Mn·PP_i, the latter appears to be a stronger inhibitor. With enzyme 2, the inhibition in the presence of added Mn is more complex (Figure 6B). At lower concentrations of PP_i (1 mM Mn and <400 μM PP_i), Mn alone might be binding to cyclase and reducing the interaction of PP_i with it, thereby decreasing the inhibition by PP_i alone. At higher concentrations of PP_i (1 mM Mn and >400 μM PP_i), Mn·PP_i is a better inhibitor than PP_i.

From experiments in which the cyclase activity was measured at [GTP·Mn] varied between 50 μM and 1 mM, with [PP_i] varied between 0 and 400 μM, the inhibition by PP_i was determined to be competitive with a K_i of 70–100 μM for enzyme 1 and 150–200 μM for enzyme 2.

Inhibition of Inorganic Pyrophosphatase by Calcium

Rod outer segments hydrolyze inorganic pyrophosphate to P_i. The activity was identical when assayed in infrared or room light. Also, the activity in room light was not influenced by the presence of 10 μM GTPγS, indicating lack of regulation by GTP binding proteins. The enzyme has a K_m of 26 μM and a V_{max} of 175 ± 31 μmol min^{-1} (μmol of rhodopsin)$^{-1}$ (n = 4). When the assays were done at 1 mM $MgCl_2$ and 0.5 mM PP_i, calcium had a strong inhibitory effect on the pyrophosphatase activity. Figure 7 shows the inhibition as a function of the free calcium and Ca·PP_i concentration. In the calculation of the free calcium concentration, stability constants of 501 200 M^{-1} for Mg·PP_i and 100000 M^{-1} for Ca·PP_i were used (O'Sullivan, 1969).

Discussion

Solubilization of Cyclase. Guanylate cyclase activity in rod outer segments was reported to be in the range of 1–10 nmol of cGMP formed min^{-1} (mg of protein)$^{-1}$ (Goridis et al., 1973; Pannbacker, 1973; Krishnan et al., 1978; Fleischman et al., 1980; Koch & Stryer, 1988). The activity in our preparations falls within this range. Previous reports indicated that Triton X-100, ammonyx LO, emulphogen, acetone, and 1-butanol (Goridis et al., 1973; Krishnan et al., 1978; Fleischman, 1981) have all failed to dissolve cyclase activity from RDM. We tested CHAPS and Nonidet P-40. At concentrations between 4 and 30 mM, CHAPS inhibited 80% of cyclase activity (data not shown) while in the presence of Nonidet P-40 the activity was about 15% stimulated. Treatment with Nonidet P-40 alone was not enough to release a significant portion of cyclase activity into solution. Of various attempts made to increase the solubilization by Nonidet P-40, a high concentration of KCl was found to be most effective, suggesting that cyclase is bound to other membrane components by strong electrostatic interactions. Conventionally, a protein appearing in the supernatant after 1 h of 100000g centrifugation (qualified by the density of the medium) is considered soluble (Hjelmeland & Chrambach, 1984), and by that definition, about 60–70% of RDM cyclase is solubilized by this procedure.

It is possible that either the solubilized or the insoluble enzyme is from membranes other than the RDM. Should that be the case, the properties of these enzymes are likely to be different. In our experiments, the instability of the two enzyme activities, the protection offered by DTT, the kinetic properties, and the lack of influence of ANF and calcium are very similar, suggesting that the two enzymes are identical.

Partial Purification. The high instability of the enzyme, especially after the DE52 column chromatography, made

purification difficult. Also due in part to that, the specific activity of the DE52-resolved enzymes is only about 10-fold higher than the solubilized preparation. Whether one of these enzymes is an artifact or a product of proteolysis is not known. In spite of their low enrichment, the two enzyme preparations are valuable in that they are free of contaminating GTPase and phosphodiesterase activities and can serve as screening tools for the calcium-dependent modulatory protein observed by Koch and Stryer (1988). The cyclase may, however, be tightly associated with rhodopsin and or other proteins and may exist in polymerized high molecular weight forms as suggested by its chromatographic behavior on Biogel A 0.5m and A 1.5m. Recent reports on some guanylate cyclases from other tissues suggest either that the enzyme is tightly associated with receptor proteins or that it has a receptor binding domain on itself (Paul et al., 1987; Bentley et al., 1988).

Cyclase has a complex dependence on KCl concentration. Chromatography of the enzyme in low [KCl] (50 or 100 mM) resulted in lower recovery of activity. Also, prolonged contact with high [KCl] also reduced the activity. Due to this, the enzyme recovered from chromatography on Biogel A 0.5m in 200 mM KCl has higher activity than the corresponding enzyme left in 1 M KCl for the same time (Table I).

Inhibition by PP_i. While this is the first report of inhibition of photoreceptor guanylate cyclase in a partially purified preparation, Detwiler and Rispoli (1989) observed that such inhibition can also occur in relatively intact preparations. From electrophysiological studies on detached and voltage-clamped Gecko rod outer segments, they noted that imidophosphate, an analogue of PP_i, reduced the dark current, which can be attributed to reduction in cyclase activity. Inhibition of cyclase by PP_i could be physiologically significant if the PP_i concentration in the rod outer segment is in a range that can exert a significant effect. Its concentration in the outer segment is currently unknown. Second, if the PP_i concentration remains invariant, it is unlikely to have a role in regulating visual transduction or adaptation. The inhibition of pyrophosphatase by calcium is therefore significant in that a change in free calcium concentration would lead to a corresponding change in the pyrophosphate level in the outer segment which in turn would influence the cyclase activity. Here again, the effect of calcium is meaningful only if it occurs at physiological calcium concentrations. As shown in Figure 7, pyrophosphatase is inhibited at submicromolar calcium and $Ca{\cdot}PP_i$ concentrations. Free calcium concentration in outer segments is reported to be around 0.2–0.5 μM (McNaughton et al., 1986; Ratto et al., 1988). On the basis of these findings, we propose the following sequence of events. (1) In dark-adapted rod outer segment, $[Ca]_i$ is higher, pyrophosphatase is inhibited, $[PP_i]$ is higher, and cyclase is inhibited. (2) Following a light flash, dark current is suppressed, calcium is extruded, pyrophosphatase activity increases, $[PP_i]$ is lowered, cyclase activity increases, and [cGMP] rises. (3) Dark current is restored, $[Ca]_i$ rises, etc. and the conditions of the dark-adapted state are restored.

Most of the reported studies on inorganic pyrophosphatase were done on the yeast enzyme, and very little is known about the enzyme from mammalian tissues. The yeast enzyme is known to be strongly inhibited by calcium. Ridlington and Butler (1972) carried out very elegant experiments with purified pyrophosphatase in which they measured the affinity of calcium and $Ca{\cdot}PP_i$ for the enzyme. The K_d for calcium was 800 μM and for $Ca{\cdot}PP_i$ 10^{-8}–10^{-7} M. As seen in Figure 7, photoreceptor pyrophosphatase is inhibited when calcium in the 10^{-7}–10^{-5} M range and $Ca.PP_i$ in the 10^{-8}–10^{-6} M range were present in the assays. Whether the inhibition was due to calcium or $Ca{\cdot}PP_i$ and what their affinities for the enzyme are can be answered unambiguously once the purified pyrophosphatase is available. We are currently attempting to isolate the enzyme.

Calcium was reported to influence cyclase activity through a soluble regulatory protein (Koch & Stryer, 1988). Our preparations did not show such regulation by calcium because we washed the RDM free of soluble proteins to remove interference by GTPase and phosphodiesterase. We suggest that the inhibition by calcium via pyrophosphatase, as shown in this report, could be an additional mechanism of regulating guanylate cyclase in rod outer segments.

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Registry No. 5'-GTP, 86-01-1; PP_i, 14000-31-8; Ca, 7440-70-2; guanylate cyclase, 9054-75-5; inorganic pyrophosphatase, 9024-82-2.

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N-Arylazido-β-alanyl-NAD$^+$, a New NAD$^+$ Photoaffinity Analogue. Synthesis and Labeling of Mitochondrial NADH Dehydrogenase†

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ABSTRACT: N-Arylazido-β-alanyl-NAD$^+$ [N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$] has been prepared by alkaline phosphatase treatment of arylazido-β-alanyl-NADP$^+$ [N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NADP$^+$]. This NAD$^+$ analogue was found to be a potent competitive inhibitor (K_i = 1.45 μM) with respect to NADH for the purified bovine heart mitochondrial NADH dehydrogenase (EC 1.6.99.3). The enzyme was irreversibly inhibited as well as covalently labeled by this analogue upon photoirradiation. A stoichiometry of 1.15 mol of N-arylazido-β-alanyl-NAD$^+$ bound/mol of enzyme, at 100% inactivation, was determined from incorporation studies using tritium-labeled analogue. Among the three subunits, 0.85 mol of the analogue was bound to the M_r = 51 000 subunit, and each of the two smaller subunits contained 0.15 mol of the analogue when the dehydrogenase was completely inhibited upon photolysis. Both the irreversible inactivation and the covalent incorporation could be prevented by the presence of NADH during photolysis. These results indicate that N-arylazido-β-alanyl-NAD$^+$ is an active-site-directed photoaffinity label for the mitochondrial NADH dehydrogenase, and are further evidence that the M_r = 51 000 subunit contains the NADH binding site. Previous studies using A-arylazido-β-alanyl-NAD$^+$ [A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$] demonstrated that the NADH binding site is on the M_r = 51 000 subunit [Chen, S., & Guillory, R. J. (1981) *J. Biol. Chem. 256*, 8318-8323]. Results are also presented to show that N-arylazido-β-alanyl-NAD$^+$ binds the dehydrogenase in a more effective manner than A-arylazido-β-alanyl-NAD$^+$.

The mitochondrial NADH dehydrogenase (EC 1.6.99.3) is the first enzyme of the respiratory chain, which conveys the electrons derived from NADH to ubiquinone via FMN and eight or nine iron–sulfur clusters as redox components (Ohnishi et al., 1985; Hatefi, 1985; Ragan, 1987). Upon treatment of the membrane-bound form of the enzyme, complex I, with chaotropic reagents (Davis & Hatefi, 1969; Galante & Hatefi, 1979), a water-soluble iron–sulfur flavoprotein preparation of NADH dehydrogenase can be isolated. This soluble enzyme preparation contains three subunits with molecular masses of 51 000, 24 000, and 9000 daltons and is capable of oxidizing NADH in the presence of quinones or ferric complexes as electron acceptors (Galante & Hatefi, 1979). Chen and Guillory (1981) showed that the M_r = 51 00 subunit of this enzyme could be labeled with tritiated A-arylazido-β-alanyl-NAD$^+$[1] (it was previously named arylazido-β-alanyl-NAD$^+$, further details under Results and Discussion), suggesting that the NADH binding site is on this subunit.

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[1] Abbreviations: N-arylazido-β-alanyl-NAD$^+$, N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$; A-arylazido-β-alanyl-NAD$^+$, A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$; arylazido-β-alanyl-NADP$^+$, N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}NADP$^+$; SDS, sodium dodecyl sulfate.

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