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CHARACTERIZATION OF GUANYLATE CYCLASE OF ROD OUTER SEGMENTS OF THE BOVINE RETINA

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Summary

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) of bovine retinal rod outer segments is almost completely particulate, i.e. associated with rod outer segment membranes. In contrast to particulate guanylate cyclase in other tissues, treatment of rod outer segments with Triton X-100 does not solubilize the enzyme but inhibits it. Enzyme activity is dependent on the presence of divalent cation, especially Mn^{2+} with only poor activation by Mg^{2+} (10-fold lower) and no activation seen with other cations. Expression of maximal activity required Mn^{2+} and GTP in equimolar concentrations with an apparent K_m of $8 \cdot 10^{-4}$ M and V of 10 nmol/min per mg protein. Excess of Mn^{2+} over that required for the formation of the Mn · GTP complex was inhibitory. Ca^{2+}, Ba^{2+} and Co^{2+} inhibited enzyme activity when assayed with the Mn · GTP substrate complex. In the presence of a fixed concentration of 1 mM Mn^{2+}, the enzyme exhibited strong negative cooperative interactions with GTP, characterized by an intermediary plateau region in the substrate vs. enzyme activity curve, a curve of downward concavity in the double reciprocal plot and a Hill coefficient of 0.5. Nucleotides such as ITP, ATP and UTP at higher concentrations (1 mM) inhibit enzyme activity while ATP at lower concentrations (0.1 mM) stimulates activity by 40%. NaN_3 has no effect on the guanylate cyclase. It is thus possible that the guanylate cyclase may be regulated in vivo by both the metal : GTP substrate ratio and the free divalent cation concentration as well as by the ATP concentration and thus play an important but yet undefined role in the visual process.

Introduction

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), The enzyme that catalyzes the formation of cyclic GMP from GTP, is widely

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distributed in many tissues and occurs in high concentration in lung [1] and sea urchin sperm [2]. The highest guanylate cyclase activity reported so far however is found in rod outer segments, specialized organelles of photoreception of the retina consisting of 80—90% rhodopsin [3—7]. A small yet significant effect of light on guanylate cyclase activity has been observed [7]. In association with high cyclase activity, isolated bovine rod outer segments exhibit high phosphodiesterase activity [8] and up to 400—600 pmol cyclic GMP/mg protein [7] indicating the probability that cyclic GMP plays a special role in the visual process. Study of guanylate cyclase in isolated outer segments thus affords an excellent system for studying the enzyme's properties in a highly purified yet physiologically intact organelle.

Materials and Methods

Cyclic[8-^{3}H]GMP and [α-^{32}P]GTP were obtained from New England Nuclear Corp., Boston, Mass. 2-Deoxy[α-^{32}P]GTP (dGTP) was obtained from New England Nuclear Corp. Guanylylimidodi[α-^{32}P]phosphate (Gpp(NH)p) was obtained from ICN-Life Sciences Group, Irvine, Calif. All α-^{32}P-labeled nucleotides, unlabeled GTP cyclic[^{3}H]GMP were purified by chromatography on Dowex-50 H^+ [7]. Dowex-50 H^+ resin (Bio-Rad AG50W, X-4 H^+ form, 200—400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif. Neutral Alumina (activity grade 1) was obtained from ICN-Life Sciences Group. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. All unlabeled nucleotides were purchased from Boehringer-Mannheim Co., New York, N.Y. All other reagents were of analytical grade.

Preparation of rod outer segments. Rod outer segments from dark-adapted bovine eyes were prepared as previously described [8]. We have shown earlier by electron microscopy that this preparation consists almost exclusively of rod outer segment membranes with very little contamination by other retinal structures [7]. The final pellet of outer segments was suspended in 100 mM Tris · HCl buffer, (pH 7.4) to give a concentration of about 1 mg/ml protein. The suspension of rod outer segments was bleached by exposure to room light and was then used either immediately or was stored under liquid N_2. Protein was determined by the method of Lowry et al. [9] using bovine serum albumin as a standard.

Solubilization of guanylate cyclase. The outer segment suspension (1.5 mg protein) was mixed at 4°C with different batches of Triton X-100 or Ammonyx LO (Onyx Chem. Co., Jersey City, N.J.) at a final concentration of 0.2 or 0.4%. The suspension was vortexed for 15 s, centrifuged at 12 000 $\times g$ for 10 min and the pellet was resuspended in 1 ml of the 100 mM Tris · HCl buffer. When appropriate, the supernatant fluid was dialyzed against the same buffer. A 10 μl aliquot, (4—15 μg protein) was assayed either before or after dialysis for guanylate cyclase activity.

Assay of guanylate cyclase. Guanylate cyclase activity was assayed by the method of Krishna and Krishnan [10]. The final incubation mixture contained 80 mM Tris · HCl buffer (pH 7.4), 2.5 mM isobutyl methylxanthine, 1 mM cyclic GMP (containing 20 000 dpm of cyclic[8-^{3}H]GMP), 0.8—1.0 mM $MnCl_2$, 1 mM [α-^{32}P]GTP (10—50 cpm/pmol) and 10—20 μg of outer segment

protein in a final volume of 0.1 ml. The mixture was incubated at 37°C for 1 min after the addition of the enzyme; the reaction was terminated by the addition of 100 μl 100 mM ethylenediamine tetraacetate (EDTA), followed by 300 μl water. The samples were then applied to a 0.5 × 4.0 cm column of Dowex-50 H^+. GTP and GDP were eluted with 0.5 ml water and were discarded. Cyclic GMP was then eluted with 1.0 ml water and the eluate was allowed to pass directly through an 0.5 × 4.0 cm column dry neutral alumina. The alumina column was eluted with 2.0 ml of 0.1 M Tris · HCl (pH 7.4). The eluates were collected in scintillation vials and radioactivity determined after the addition of 18 ml Aquasol (New England Nuclear Corp.). Control mixtures, containing either heat-denatured enzyme or EDTA were routinely incubated with each set of enzyme assays. The blank values were 0.5—3 pmol. In order to estimate the degradation of cyclic GMP, cyclic[^{3}H]GMP was included in the incubation mixture. This measured the loss of cyclic GMP both during the incubation period due to phosphodiesterase activity, and also during the isolation procedure. A recovery of 70—80% of added cyclic[^{3}H]GMP was routinely obtained. Degradation of cyclic[^{3}H]GMP was kept within 10% by limiting the amount of enzyme (10—20 μg protein) and the time of incubation (1 min). Cyclic[^{32}P]GMP isolated by the above procedure was characterized by various methods and found to be more than 99% pure [10].

The amount of [α-^{32}P]GTP remaining at the end of the incubation period was determined by chromatographing the reaction mixture over Dowex-1-formate (BioRad AG1-X8 200—400 mesh; BioRad Laboratories, Richmond, Calif.) and eluting the GTP with increasing concentrations of formic acid [11]. Since at least 99% of the substrate remained at the end of the 1 min incubation under the above conditions, no regenerating system for GTP was employed.

Results

Assay conditions. Guanylate cyclase activity in isolated outer segments ranged from 8 to 10 nmol of cyclic GMP formed/min per mg protein. The enzyme was stable for at least 3 months when stored under liquid nitrogen. Under the present conditions, guanylate cyclase activity was linear with time up to 20 min and with various protein concentrations at least up to 25 μg (figure not shown). The enzyme activity measured at times greater than 20 min did not provide a reliable value for enzyme activity because 50% or more of the cyclic GMP formed was degraded by phosphodiesterase. Even after correction for this loss, the values obtained were variable. However, the degradation of cyclic GMP by phosphodiesterase durings the routine 1 min incubation was less than 10% when 10—20 μg protein was employed; inclusion of cyclic [^{3}H]GMP during the assay was used to correct for this loss.

Substrate specificity. Adenylate cyclase activity has previously been shown to be low and variable in isolated rod outer segments [12]. Analogs of GTP were also poor substrates for the enzyme. For example, activity with 1 mM dGTP at pH 7.4 was only 1% of that obtained with GTP (i.e. about 80 pmol/min per mg protein). Activity with 1 mM Gpp(NH)p as substrate was only 10% of that seen with GTP even when assayed in the pH range of 8.5—10.5.

Solubilization studies. Guanylate cyclase activity remained associated with

the outer segment membranes after disruption by vortexing, homogenization or sonication. Virtually no enzyme activity was subsequently found in the supernatant fraction after centrifugation at 12 000 × *g* for 10 min. Treatment with 0.2% Triton X-100 resulted in the loss of 30% of the enzyme activity while treatment at a concentration of 0.4% of Triton caused a loss of 50% of the enzyme activity. Ammonyx LO at a concentration of 0.2% released 10% of the guanylate cyclase activity into the soluble fraction but decreased specific activity by two-thirds. Treatment of rod outer segments with solvents like *n*-butanol and acetone was unsuccessful in solubilizing the enzyme activity. Moreover, mechanical agitation and sonication with or without detergent resulted in major losses of enzyme activity with no significant solubilization.

Cation requirement. Mn^{2+} served as a metal cofactor for guanylate cyclase; enzyme activity in the absence of added divalent cation was virtually undetectable. The enzyme activity was also tested in the presence of 0.5 mM GTP and 0.5 mM concentrations of Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, or Co^{2+}. Under these conditions, magnesium was only 10% as effective as Mn^{2+} in serving as a metal cofactor. The enzyme activity in the presence of the other divalent ions was below the limit of detection in the assay, i.e. less than about 0.5% of that obtained with Mn^{2+}. Similar results were obtained when these divalent cations were tested at 0.1, and 1 mM concentrations.

Mn · GTP complex. The guanylate cyclase from rod outer segments was absolutely dependent on Mn^{2+} for its activity (figure not shown). Maximal activity was also dependent on an approximate 1 : 1 ratio between Mn^{2+} and GTP, when GTP was used either at 1 or 0.1 mM. These results indicated that a Mn · GTP complex was the substrate in the reaction rather than GTP alone.

Fig. 1 shows the effect of increasing concentrations of GTP on guanylate cyclase activity. At each substrate (GTP) concentration, Mn^{2+} was present at equimolar concentrations as well as at twice and three times the GTP concentration. These data show that, at concentrations of GTP up to 0.15 mM, the presence of Mn^{2+} in excess of 1 or 2 molar equivalents of GTP, significantly stimulate the guanylate cyclase activity (Fig. 1, inset). However, at higher substrate concentrations, the presence of Mn^{2+} in excess of that necessary for the formation of the Mn · GTP complex inhibited the enzyme activity and the inhibition increased with increasing concentrations of free Mn^{2+} (Fig. 1). Mn^{2+}, when present in excess of 1 or 2 molar equivalents of GTP (i.e. GTP : Mn^{2+} 1 : 2 or 1 : 3), significantly increased the apparent affinity of the substrate (Mn · GTP) for the guanylate cyclase enzyme but reduced the *V* by one-half. Apparent K_m values were calculated to be $8 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ and $1.5 \cdot 10^{-4}$ M and the *V* values were 10, 7.1 and 5 nmol/min per mg protein for GTP : Mn^{2+} ratios of 1 : 1, 1 : 2 and 1 : 3, respectively.

Cation interactions. Previous reports [13,14] have shown that Ca^{2+} and Ba^{2+} but not Mg^{2+} increase the activity of rat lung soluble guanylate cyclase in the presence of Mn^{2+} when added in concentrations less than that of GTP. A number of divalent cations were thus tested as activators or inhibitors of outer segment guanylate cyclase. The concentration of GTP and Mn^{2+} in these experiments was 1 and 0.5 mM, respectively (GTP : Mn^{2+}, 2 : 1). Under these conditions Ba^{2+}, Ca^{2+}, Mg^{2+} and Co^{2+} inhibited guanylate cyclase activity when tested in the range of 0.1—0.5 mM (Table I). Whether the inhibition of

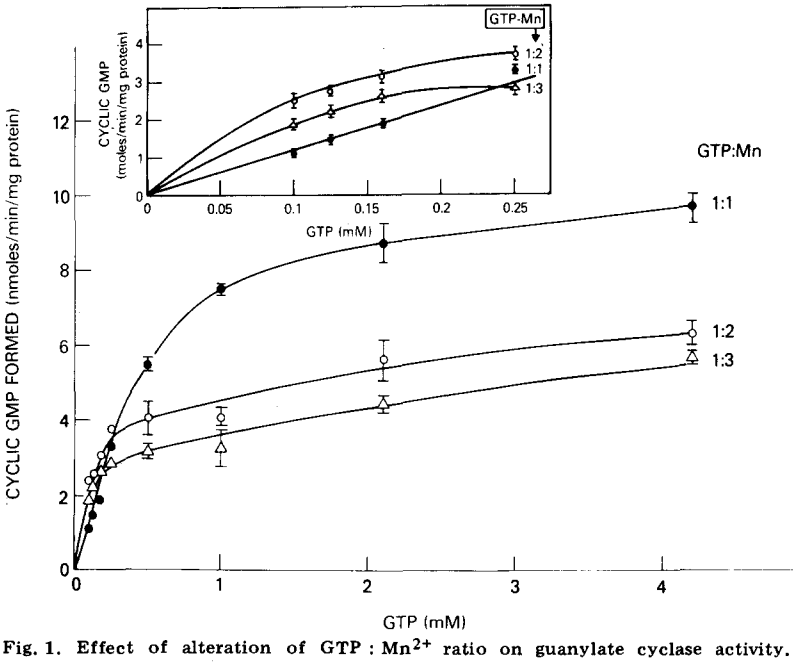

Fig. 1. Effect of alteration of GTP : Mn^{2+} ratio on guanylate cyclase activity. Rod outer segment membranes (12 μg protein) were incubated for 1 min with varying concentrations of [α-^{32}P]GTP (specific activity 21.5 cpm/pmol) and $MnCl_2$ at different concentration levels as shown in the figure; 1 mM cyclic[^{3}H]GMP (20 000 dpm), 2.5 mM isobutyl methylxanthine and 80 mM Tris · HCl (pH 7.4) buffer were present in a total volume of 0.1 ml and assays were performed as described in the text. The inset shows the data at low substrate concentrations replotted on an expanded scale.

TABLE I

EFFECT OF VARIOUS DIVALENT CATIONS ON GUANYLATE CYCLASE ACTIVITY

Guanylate cyclase was assayed in the presence of Mn^{2+} and other divalent ions as indicated. 1.0 mM [α-^{32}P]GTP (spec. act. 12 cpm/pmol was used. General conditions were as described in Fig. 1. The incubations were started by addition of the enzyme (20 μg protein). The values represent the mean of three determinations (±S.E.).

Primary cation	Concentration (mM)	Other cation	Concentration (mM)	Guanylate cyclase (nmol cyclic GMP formed/mg per min)
Mn^{2+}	0.5	None	—	9.35 ± 0.15
Mn^{2+}	0.5	Ba^{2+}	0.1	8.60 ± 0.50
			0.3	7.40 ± 0.40
			0.5	6.70 ± 0.40
Mn^{2+}	0.5	Ca^{2+}	0.1	7.90 ± 0.65
			0.3	7.20 ± 0.10
			0.5	6.30 ± 0.10
Mn^{2+}	0.5	Mg^{2+}	0.1	7.40 ± 0.75
			0.3	7.30 ± 0.20
			0.5	6.90 ± 0.10
Mn^{2+}	0.5	Co^{2+}	0.1	5.90 ± 0.30

guanylate cyclase was caused directly by the metal or indirectly by a metal · GTP complex is presently not clear.

Low concentrations of Ca^{2+} (10^{-6}—10^{-4} M) did not affect guanylate cyclase activity (data not presented). However, the activity of the enzyme was inhibited by 50% (from 9.5 to 4.7 nmol/mg per min) in the presence of 1 mM Ca^{2+}.

Negative cooperative interactions with substrate. The guanylate cyclase exhibited strong negative cooperative interactions with the substrate GTP, when the enzyme was assayed at a fixed concentration of 1 mM Mn^{2+}. As can be seen from Fig. 2, the curve of GTP concentration vs. velocity shows an intermediary plateau region which is indicative of negative cooperativity [15]. Insert A shows the double reciprocal plot which reveals a concave downward curve. Insert B shows the Hill plot which yields a Hill coefficient of 0.5. A curve of downward concavity in the double reciprocal plot and an '*n*' value of less than 1 are typical of an enzyme exhibiting negative cooperativity [16].

Effect of nucleoside triphosphates. ATP and other nucleotides inhibit guanylate cyclase in several tissues but the inhibitory effect is not due to metal-chelation by ATP [1,10,17]. Several nucleoside triphosphates were tested at various concentrations for their effect on outer segment guanylate cyclase (Table II). In these experiments, equimolar concentrations of GTP and Mn^{2+} were employed. ITP was the most potent inhibitor while 1 mM ATP and UTP inhibited the enzyme by 30%. In contrast, guanylate cyclase was activated by lower concentrations of ATP. This apparent stimulation could not be explained on the basis of prevention of GTP hydrolysis by ATP, since more than 99% of

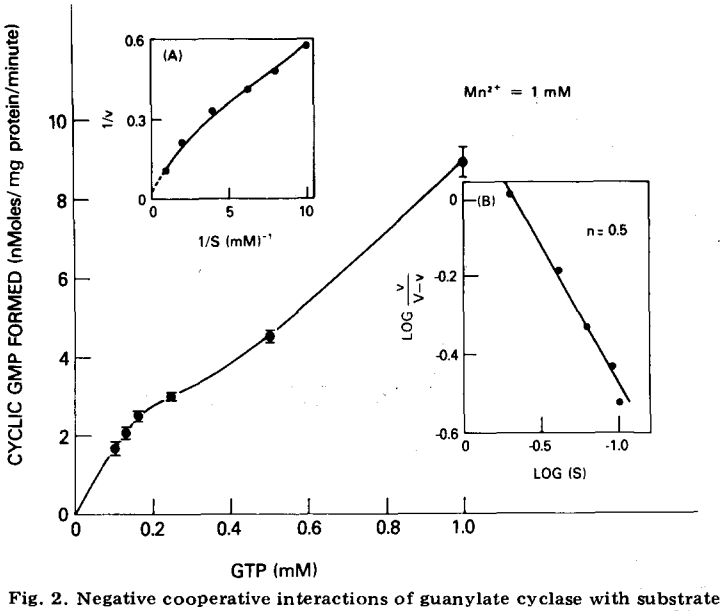

Fig. 2. Negative cooperative interactions of guanylate cyclase with substrate. $MnCl_2$ was present at a fixed concentration of 1 mM at each of the substrate concentrations of [α-^{32}P]GTP (specific activity 26 cpm/pmol) as indicated in the figure. The incubations were started by the addition of the enzyme (10 μg protein) and all assays were carried out in duplicate as described under Fig. 1. Inset A shows the double reciprocal plot; inset B shows the Hill plot. The Hill coefficient *n* was calculated to be 0.5.

TABLE II

EFFECT OF VARIOUS NUCLEOTIDES ON GUANYLATE CYCLASE ACTIVITY

Guanylate cyclase was assayed as described in Fig. 1 with 1 mM [α-^{32}P]GTP (specific activity 18 cpm/pmol) and 0.8 mM $MnCl_2$. Various nucleotides were present at concentrations indicated and the incubations were started by addition of 20 μg of protein. Assays were carried out in triplicate as described in the text. The values represent mean ± S.E. of three determinations.

Nucleotides	Guanylate cyclase (nmol cyclic GMP formed/mg per min)
No addition (1 mM [α-^{32}P]GTP alone)	9.50 ± 0.65
ITP 10^{-3} M	3.75 ± 0.21
10^{-4} M	9.65 ± 0.18
10^{-5} M	8.55 ± 0.18
ATP 10^{-3} M	6.50 ± 0.90
10^{-4} M	13.30 ± 0.50
10^{-5} M	10.35 ± 0.50
UTP 10^{-3} M	6.40 ± 0.40
10^{-4} M	9.00 ± 0.50
10^{-5} M	7.90 ± 0.18

the substrate GTP remained at the end of the incubation in the presence or absence of ATP. The mechanism of guanylate cyclase activation by ATP is thus presently not known.

Effect of temperature. Guanylate cyclase activity was measured at various temperatures (Fig. 3). The Arrhenius plot of $1/T$ against log v shows a break in the curve at 30°C [18]. This indicated that there was a change from one value

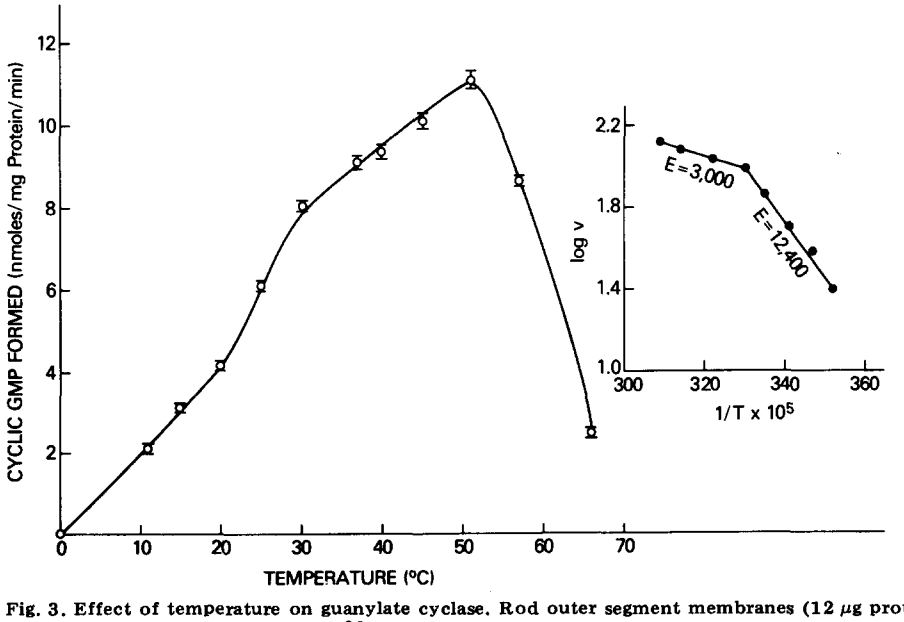

Fig. 3. Effect of temperature on guanylate cyclase. Rod outer segment membranes (12 μg protein) were incubated for 1 min with 1 mM [α-^{32}P]GTP (specific activity 24.5 cpm/pmol) and 0.8 mM $MnCl_2$ under conditions described in Fig. 1 at various temperatures as indicated. The inset shows the Arrhenius plot ($1/T$ vs. log v); the molar activation energy was calculated to be 12 400 and 3000 cal/mol.

of activation energy to another at the transition temperature of 30°C. The two values of molar activation energy for guanylate cyclase were 12 400 and 3000 cal/mol. The inflection at 30°C is probably caused by a phase transition occurring in the membrane.

Effect of azide on guanylate cyclase. It has been reported that NaN_3 stimulates both soluble and particulate guanylate cyclase from liver and kidney and the particulate enzymes from cerebral cortex and cerebellum [19]. No effect of azide on the guanylate cyclase from lung, heart, and the soluble enzymes from cerebral cortex and cerebellum has been observed [19]. The effect of NaN_3 on guanylate cyclase of rod outer segments was tested at several concentrations ranging from 10^{-5} to 10^{-3} M (data not shown). The rod outer segments were first preincubated with or without NaN_3 at room temperature for 10 min; other components of the incubation system were added after preincubation and assayed as outlined under Materials and Methods. The data show that NaN_3 does not affect outer segment guanylate cyclase nor does it prevent the partial inactivation of the enzyme that occurs during the preincubation period. The requirement of catalase in the activation process remains to be assessed.

Discussion

The single protein rhodopsin comprises more than 85% of the total protein of rod outer segments [20,21]. Of the enzyme activities that have now been reported in the photoreceptor units, the enzymes that synthesize [3—5] and degrade [8,22—24] cyclic GMP appear to be present in exceptionally high activity. Moreover, the actual concentration of cyclic GMP in outer segments has recently been shown to be regulated by light [25]. Study of guanylate cyclase in isolated outer segments thus affords a unique opportunity for examining various characteristics of the enzyme in an organelle of almost homogeneous composition and of well-delineated function.

Both soluble and particulate forms of guanylate cyclase have been found in homogenates of lung, heart, cerebral cortex, and cerebellum [26]. In contrast, in sea urchin sperm [27] and in rat small intestine [17], the enzyme appears to be almost entirely particulate. Similarly, rod outer segment guanylate cyclase appears to be entirely membrane-associated. Unlike the other particulate enzymes, however, rod outer segment guanylate cyclase is not solubilized by detergent treatment. In fact, treatment with even a low concentration of Triton or Ammonyx caused a loss of the enzyme activity.

Mg^{2+} was only 10% as effective as Mn^{2+} in activating guanylate cyclase. Other divalent cations tested were totally ineffective. Thus, Mn^{2+} was the best metal cofactor by far and the response to increasing its concentration was rather sharp; the maximal response was observed when the cation and GTP were at equimolar concentrations. The activation of guanylate cyclase by low concentrations of Mn^{2+} is of great interest because the amount of Mn^{2+} within the rod outer segments as in other tissues would probably be low. The concentration of the Mn · GTP complex would also be expected to be low because a recent finding in our laboratory revealed that GTP levels in isolated outer segment membranes are low i.e. less than 10 pmol/mg protein (unpub-

lished data). Thus, the control of Mn · GTP formation is one possible mode of regulating guanylate cyclase in outer segments. Under the conditions existing within the rod in vivo, the enzyme-metal specificity may be altered in subtle ways allowing divalent metal ions like Ca^{2+} and Mg^{2+} to influence enzyme activity. Both calcium and magnesium are present in high concentration in the neural retina and pigment epithelium [28,29] with isolated bovine outer segments containing 11 mol Ca^{2+} and 15 mol Mg^{2+} per mol of rhodopsin. Specific particulate and soluble compartments of the two cations appear to be present in rod outer segments with light causing a redistribution of calcium but not magnesium [28]. Since even 0.5 mM Ca^{2+} can inhibit guanylate cyclase by 32% (see Table I), it is tempting to postulate that the observed mobilization of calcium inhibits guanylate cyclase in vivo, resulting in decreased cyclic GMP levels [25] and other subsequent events possibly related to the visual process. Further experimental evidence is necessary to substantiate this hypothesis.

The kinetics of guanylate cyclase activity observed with Mn · GTP as substrate in the absence of free Mn^{2+} was essentially Michaelian. However, when the GTP : Mn^{2+} ratio was altered in favor of a high free Mn^{2+} concentration, the enzyme exhibited a negative cooperative interaction with GTP characterized by an intermediatry plateau region in the substrate vs. velocity curve. These data also show a concave downward curve in the double reciprocal plot and provide a Hill coefficient of less than 1. These criteria are typical of enzymes exhibiting negative cooperative interactions [15,16]. This feature of the enzyme could be especially important in a neural tissue such as the retina where rapid "on-off" mechanisms are necessary to facilitate messenger or impulse transfer. Somewhat similar results could also be obtained if more than one guanylate cyclase of differing substrate and metal affinities were present in the membranes. Even if this were the case, the multiplicity of enzymes would provide yet another mode of regulating cyclic GMP formation in the photoreceptor units.

The activation of guanylate cyclase by low concentrations of ATP is also significant from the point of view of regulation. Perhaps a concentration even lower than 0.1 mM ATP is needed to achieve activation of the cyclase in vivo. The level of ATP in isolated bovine rod outer segment membranes is only about 100 pmol/mg protein (unpublished data) although a value of 2—5 mM has been found in isolated frog outer segments [30]. Fluctuation of ATP content could thus be an important factor in cyclase regulation in vivo with activation and inhibition of activity by low and high ATP concentrations, respectively. The question of cell energetics in the photoreceptor unit is also intriguing, especially in light of the lack of mitochondria in outer segments. It has been shown that the 3′-bond of cyclic nucleotides is richer in energy than the terminal phosphate bond of ATP [31]. In view of the uniquely high guanylate cyclase activity and cyclic GMP content in rod outer segments [7] and the presence of light-activated cyclic GMP phosphodiesterase activity [23,24], it could very well be that hydrolysis of cyclic GMP furnishes the energy requirements necessary for neural events subsequent to the initial photic stimulation in retinal photoreceptors.

It thus seems likely that the guanylate cyclase system in bleached rod outer segments may be governed, at least in part, by (1) the interaction of the

enzyme with a divalent metal · GTP complex, (2) by free metal ligands and (3) by fluctuations in ATP concentration. If light-induced depolarization of the outer segments results in the release of Ca^{2+} from disks [32] and possibly other divalent metal ions, their mobilization may control guanylate cyclase activity and thus markedly affect the level of cyclic GMP in retinal photoreceptors.

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