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ENZYMATIC FORMATION OF INOSINE 3′,5′-MONOPHOSPHATE AND OF 2′-DEOXYGUANOSINE 3′,5′-MONOPHOSPHATE

INOSINATE AND DEOXYGUANYLATE CYCLASE ACTIVITY

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Summary

Enzymes in particulate fractions from sea urchin sperm and in soluble fractions from rat lung were shown to catalyze the formation of inosine 3′,5′-monophosphate (cyclic IMP) and of 2′-deoxyguanosine 3′,5′-monophosphate (cyclic dGMP) from ITP and dGTP, respectively.

With sea urchin sperm particulate fractions, Mn^{2+} was an essential metal cofactor for inosinate, deoxyguanylate, guanylate and adenylate cyclase activities. Heat-inactivation studies differentiated inosinate and deoxyguanylate cyclase activities from adenylate cyclase, but indicated an association of these activities with guanylate cyclase. Preincubation of sea urchin sperm particulate fractions with trypsin altered in a very similar manner guanylate, inosinate, and deoxyguanylate cyclase activities, and various metals and metal—nucleotide combinations protected the three cyclase activities to comparable degrees against trypsin. The relative guanylate, deoxyguanylate and inosinate cyclase activities at 0.1 mM nucleoside triphosphate were 1.0, 0.5 and 0.08, respectively. With these three cyclase activities, plots of reciprocal velocities against reciprocal Mn^{2+}—nucleoside triphosphate concentrations were concave upward, suggesting positive homotropic effects.

With rat lung soluble preparations, relative guanylate, deoxyguanylate, inosinate and adenylate cyclase activities at 0.09 mM nucleoside triphosphate were 1.0, 1.7, 0.1 and 0, respectively. MnGTP was a competitive inhibitor of deoxyguanylate cyclase activity (K_i = 12.2 μM) and MndGTP was a competitive inhibitor of guanylate cyclase activity (K_i = 16.2 μM). Inhibition studies using ITP were not conducted. When soluble fractions from rat lung were applied to Bio-Gel A 1.5 m columns, elution profiles of guanylate, deoxyguanylate and inosinate cyclase activities were similar.

These results suggest that deoxyguanylate, guanylate and inosinate cyclase activities reside within the same protein molecule.

Introduction

The ubiquitous presence of cyclic AMP* and of cyclic GMP has been well established [1], but the natural occurrence of other nucleoside 3′,5′-monophosphates is unreported. Partially purified adenylate cyclases (ATP pyrophosphate-lyase, EC 4.6.1.1) from bacteria are known to catalyze formation of cyclic dAMP from dATP [2—4], and GTP** reportedly can be used as a substrate by adenylate cyclase from *Escherichia coli* [4], but ITP, GTP, dGTP, UTP, CTP, TTP and dUTP cannot be used as substrates by adenylate cyclase from *Brevibacterium liquefaciens* [2]. Partially purified adenylate cyclase from frog erythrocytes also seems to use dATP as an alternate substrate [5], and it has been suggested that cyclic dAMP formation by sheep thyroid homogenates and by fat cell ghosts is due to adenylate cyclase activity [6,7].

Ferguson and Price [8] have recently observed formation of cyclic IMP after the addition of either ATP or ITP to toad bladder homogenates. Because either cyclic AMP or cyclic IMP formation was stimulated by oxytocin, they suggested that cyclic IMP formation was due to adenylate cyclase activity. The formation of cyclic IMP from cyclic AMP by enzymatic deamination has also been reported [9,10].

Various enzymes that use GTP as a substrate can also use dGTP or ITP as substrates [11]; it therefore seemed possible that guanylate cyclase (EC 4.6.1.2) would have the capacity to use dGTP or ITP as a substrate. Adenylate and guanylate cyclase activities are both found in particulate fractions of sea urchin sperm homogenates [12,13], but only guanylate cyclase activity is found in soluble fractions from rat lung [14]. In this study, enzyme activities in sea urchin sperm particulate fractions and in rat lung soluble fractions have been shown to catalyze the formation of cyclic dGMP and of cyclic IMP from dGTP and ITP, respectively. These activities parallel that of guanylate cyclase under a number of experimental conditions.

Methods

Enzyme Preparation

Live specimens of the sea urchin *Strongylocentrotus purpuratus* were purchased during peak fertility (November—June) from Pacific Bio-Marine Supply Co., Venice, Califonia, and were maintained at 15°C to 17°C in artifical sea water.

Male sea urchins were placed on watchglasses, and ejaculation was induced by injecting 0.5 ml of 0.5 M KCl into the body cavity [15]. The samples were combined, strained through silk bolting cloth (35 μm × 35 μm pore size), and centrifuged in a clinal centrifuge at low speed to remove contaminating pigmented particles. The supernatant fluid containing sperm was decanted and

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* Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; cyclic IMP, inosine 3′,5′-monophosphate; cyclic dAMP, 2′-deoxyadenosine 3′,5′-monophosphate; cyclic dGMP, 2′-deoxyguanosine 3′,5′-monophosphate.

** For convenience, the charge on the nucleoside triphosphates has not been designated, but at pH 7.8 the charge is primarily NTP^{2-} in the presence of excess Mn^{2+}.

centrifuged at 2000 × *g* for 10 min. This supernatant fluid was discarded, and the sedimented sperm were resuspended in a solution containing 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 27.1 mM $MgSO_4$ and 4.4 mM $NaHCO_3$ at pH 7.8—8.0, and centrifuged at 10 000 × *g* for 15 min. The final sperm pellet was resuspended in 40 vols (wet weight/volume) of a solution containing 50 mM triethanolamine at pH 7.0, 1.0 mM dithiothreitol and 5 mM EDTA. The suspension was sonicated for 15 s at the amperage setting of 8 on a Branson LS-75 Sonifier and centrifuged at 39 000 × *g* for 20 min. The supernatant fluid was discarded, and the pellet resuspended, sonicated briefly (15 s) and centrifuged again. The pellet was then washed twice with 40 vols of a solution containing 50 mM triethanolamine (pH 7.0) and 10 mM dithiothreitol without EDTA. Finally, 1 g of sperm particles were resuspended with a Ten-Broeck homogenizer and sonicated in 50 ml of the triethanolamine—dithiothreitol buffer without EDTA. The resulting suspension, containing 2 to 3 mg protein · ml^{-1} was stored in 4 ml aliquots at —70° C, and each aliquot was used only once. Guanylate cyclase activity did not change detectably during at least 3 months of storage.

Partially purified soluble guanylate cyclase from rat lung was prepared by and obtained from T.D. Chrisman. This preparation is purified some 20-fold (compared to tissue extract) by isoelectric precipitation and is described in another paper [16].

Enzyme assays

Guanylate, adenylate, deoxyguanylate, and inosinate cyclase activities were determined by the same basic procedure. For measurements of all enzyme activities, the respective [^{3}H]nucleoside triphosphate was used as substrate, and the amount of cyclic [^{3}H]nucleotide formed at the end of an incubation was determined. Assay mixtures generally contained 32 mM triethanolamine buffer at pH 7.8, 0.8 mM dithiothreitol, 8 mM theophylline, 8 mM sodium azide, various concentrations of $MnCl_2$ and nucleoside triphosphate (0.006—0.6 mM) and 1—3 · 10^6 dpm of [^{3}H]nucleoside triphosphate in a volume of 0.5 to 0.6 ml. Sodium azide was added to inhibit nucleoside triphosphatases. Guanylate, deoxyguanylate and inosinate cyclase activities of sea urchin sperm particles were determined at 30° C with a constant rate of product formation to at least 10 min at 0.006 mM nucleoside triphosphate and to at least 30 min at 0.6 mM nucleoside triphosphate. Adenylate cyclase activity of sea urchin sperm decreased with time at 30° C, but maintained constant activity at 23° C for at least 10 min. Enzyme activities of rat lung soluble fractions were carried out at 37° C with constant rates of product formation for at least 30 min.

Reactions were stopped with 0.25 ml of 0.2 M zinc acetate containing about 1 mM of the respective unlabelled cyclic nucleotide. 0.25 ml of 0.2 M Na_2CO_3 was then added to induce coprecipitation of nucleoside tri-, di-, and monophosphates with $ZnCO_3$ and the samples were then centrifuged. When activities of guanylate, deoxyguanylate or inosinate cyclases were being estimated, the supernatant fluids were applied to small polyethyleneimine—cellulose columns (0.75 cm × 5.0 cm), previously equilibrated with 50 mM acetic acid [17]. The samples were allowed to run into the column, 1 ml

followed by 16 ml of 50 mM acetic acid and 16 ml of H_2O were used to wash the columns, and the cyclic nucleotides were then eluted with 20 mM LiCl. Part of each collected fraction was counted in a scintillation counter using "TTX-127" as the scintillator fluid [18], while another part of each fraction was read on a spectrophotometer for calculation of cyclic nucleotide recoveries.

When adenylate cyclase activity was measured, both polyethyleneimine—cellulose columns and small QAE-Sephadex columns (4 cm × 0.6 cm) were used. Polyethyleneimine—cellulose columns were used as described for cyclic GMP, except that they were equilibrated with 5 mM acetic acid, and cyclic AMP was eluted with 50 mM acetic acid [17]. QAE-Sephadex A-25 columns were prepared and used as described elsewhere [19].

Materials

Polyethyleneimine—cellulose was obtained from Serva Feinbiochemica (Heidelberg, Germany), and Dowex-50 (AG50W-X8, 100—200 mesh) from BioRad Laboratories. Precoated polyethyleneimine thin-layer plates were obtained from Carl Schleicher and Schull, and Bio-Gel A 1.5 m from BioRad Laboratories. Unlabeled nucleotides were from Schwartz/Mann, Nutritional Biochemicals Corp., or from Boehringer-Mannheim. [^{3}H]ITP, 2.7 Ci/mmole, [^{3}H]GTP, 5.62 Ci/mmole, ^{3}H-labeled dGTP, 6.51 Ci/mmole, and [^{3}H]ATP, 21 Ci/mmole, were obtained from Amersham-Searle or from New England Nuclear. Metals (chloride salts) were obtained from Fisher and theophylline from Merck & Co. Trypsin was purchased from Nutritional Biochemicals Corp. and soybean trypsin inhibitor from Sigma.

Results

Product confirmation

The incubation of sperm particulate material with [^{3}H]ITP yielded a radioactive product that migrated on polyethyleneimine—cellulose columns with authentic cyclic IMP (Fig. 1A). To show that cyclic [^{3}H]IMP was the product being measured, the following experiments were conducted: Samples that eluted from polyethyleneimine—cellulose columns in the same area as authentic cyclic IMP were acidified with 0.1 M HCl, applied to Dowex-50 (H^+ form) columns (0.62 cm × 30 cm) that had been equilibrated with 0.1 M HCl, and eluted with 0.1 M HCl. The radioactive material that migrated as cyclic IMP on polyethyleneimine—cellulose columns also migrated as cyclic IMP on Dowex-50 columns (Fig. 1B), and the specific activity (eluant CPM/eluant absorbance at 249 nm) remained constant. The elution patterns from polyethyleneimine—cellulose and Dowex-50 columns clearly differentiated cyclic IMP from cyclic AMP and cyclic GMP. To verify that the peak was actually a cyclic nucleotide, fractions from polyethyleneimine—cellulose were incubated with purified cyclic nucleotide phosphodiesterase [20]. Samples were then acidified, applied to Dowex-50 columns and eluted with 0.1 M HCl. The radioactive material now migrated with authentic IMP (Fig. 1C), and again specific activity did not change.

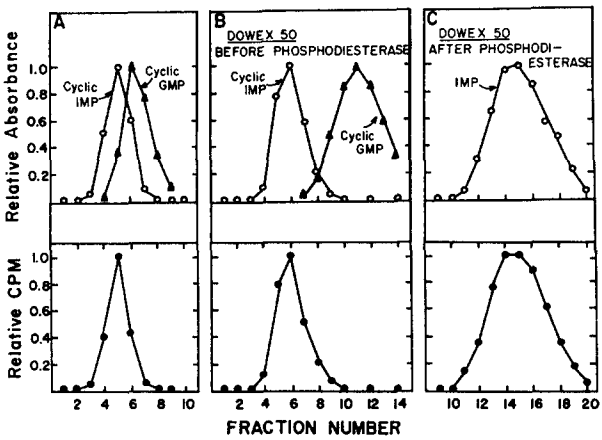

Fig. 1. Formation of cyclic [^{3}H]IMP from [^{3}H]ITP in the presence of sea urchin sperm particulate fractions. The ordinate in all cases represents the radioactivity or absorbance relative to the peak fraction. (A) Sea urchin sperm particulate fractions were incubated with 3.1 mM Mn^{2+} and 0.3 mM [^{3}H]ITP for 20 min as described in the Methods. After incubation, 0.25 μmoles of unlabeled cyclic IMP was added to some of the samples and unlabeled cyclic GMP to the other samples. These samples were then applied to polyethyleneimine—cellulose columns and after a wash with 16 ml of 50 mM acetic acid followed by a wash with 16 ml of H_2O, 20 mM LiCl was applied to the columns and 1 ml fractions collected. The absorbance was determined at 249 nm (cyclic IMP) or at 252 nm (cyclic GMP) as shown in the top panel. The radioactivity formed during the incubation is shown in the bottom panel. The radioactivity (bottom panel) is recovered in the same fractions as the authentic unlabeled cyclic IMP (top panel). (B) Fractions 3 through 8 from the polyethyleneimine—cellulose columns were pooled and acidified to 0.1 M HCl, and a 2-ml aliquot of the pooled polyethyleneimine—cellulose column effluent was applied to a Dowex-50 column (0.62 cm × 30 cm). 2-ml Fractions were collected, and the absorbance or radioactivity determined. The total recovery of authentic unlabeled cyclic IMP was 90% (top panel). The recovery of the radioactive material formed during the incubation was 87% (internal standards were used to correct for quenching). Thus, the ratio of the radioactive material to authentic cyclic IMP from the polyethyleneimine—cellulose columns did not change after chromatography on Dowex-50. (C) To 2 ml of the pooled fractions 3 through 8 from the polyethyleneimine—cellulose columns, the following was added: 0.1 ml of 1.0 M triethanolamine buffer at pH 7.8, 0.1 ml of 0.1 M $MnCl_2$, and 0.1 ml of purified cyclic nucleotide phosphodiesterase [20]. After incubation for 60 min at 30°C, samples were acidified and applied to Dowex-50 columns as in B. Both the previously unlabeled cyclic IMP and the radioactive product now migrated as IMP; authentic IMP (unlabeled) was shown in parallel columns to run in the exact same area. The recovery of IMP was 87% of the amount applied; the recovery of radioactive material was 83%.

The incubation of sperm particulate material with ^{3}H-labeled dGTP yielded a radioactive product that chromatographed on polyethyleneimine—cellulose near but not precisely with authentic cyclic GMP (Fig. 2). The chromatographic relationship between authentic cyclic GMP and the radioactive product is similar to the relationship observed between cyclic AMP and cyclic dAMP [17]. Authentic cyclic dGMP was not available for these studies.

^{3}H-labeled cyclic dGMP was shown to be the product of the reaction in the following manner: The radioactive substance migrating on polyethyleneimine—cellulose columns near cyclic GMP (see Fig. 2) was collected and treated with purified cyclic nucleotide phosphodiesterase as described for cyclic IMP. After incubation for 2 h at 30°C, part of the sample was applied to polyethyleneimine—cellulose thin-layer plates. Separation of GMP and dGMP on thin-layer plates was accomplished by using a solvent containing 100 mM borate and 0.5 M ammonium formate at pH 6.2. R_f values for various nucleotides in this

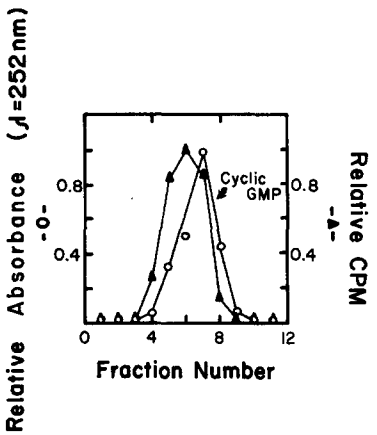

Fig. 2. Elution of a radioactive product of [^{3}H]dGTP from a polyethyleneimine—cellulose column. Assay mixtures contained 3.1 mM Mn^{2+} and 0.3 mM dGTP and were incubated for 30 min at 30°C. Other conditions were as described in the legend to Fig. 1A, with 0.25 μmoles of authentic cyclic GMP being added to the sample before application to the column.

system were as follows: GMP, 0.07; dGMP, 0.21; dGTP, 0.01; GTP, 0.01; cyclic AMP, 0.45; cyclic GMP, 0.38; cyclic dAMP, 0.45; cyclic IMP, 0.54; dATP, 0.01; ITP, 0.01 and GDP, 0.01. The authentic GMP and dGMP spots were observed by ultraviolet light, and corresponding sample areas were cut out and the radioactivity in them determined in a scintillation counter. No radioactivity was detected in the GMP area, but more than 80% of the ^{3}H applied was recovered in the dGMP area. Similar experiments were done using polyethyleneimine—cellulose columns, instead of thin-layer plates and comparable results were obtained. These same experiments were repeated using the rat lung soluble preparation, and the results were as reported for sea urchin sperm particulate fractions.

Cation requirements

When the respective total nucleoside triphosphate concentration was 0.26 mM and total metal (Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}) concentration was 1.3 mM, formation of cyclic IMP, cyclic dGMP and cyclic GMP by sea urchin sperm particulate fractions was detected only with Mn^{2+}. The lower limit of detection was around 1% of the rate with Mn^{2+}. When cyclic AMP formation was estimated with 0.67 mM ATP and 1.3 mM metal, again detectable formation was observed only with Mn^{2+}. The limit of detection in this case was about 6% of the rate with Mn^{2+}. That sea urchin sperm adenylate and guanylate cyclase activities require Mn^{2+} had been established previously [12,13].

Mn^{2+} has also been reported to be the preferred cation of partially purified guanylate cyclase from soluble fractions of rat lung [14], but cation effects on rat lung deoxyguanylate or inosinate cyclase activities were not studied in this report.

Heat inactivation

Although constant enzyme reaction rates were maintained for at least 10 min at 30°C with guanylate, deoxyguanylate and inosinate cyclase activities

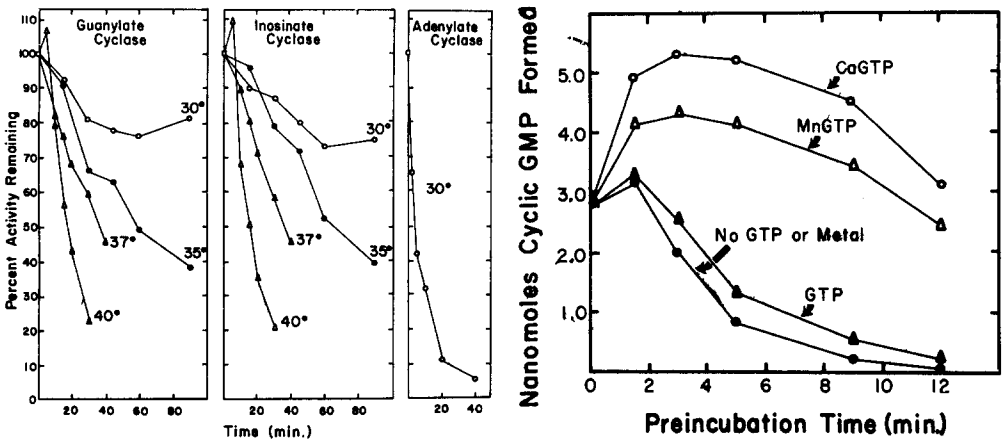

Fig. 3. Heat inactivation of sea urchin sperm adenylate, guanylate and inosinate cyclase activities by preincubation at various temperatures. The preincubation system had 2 mg protein · ml^{-1} in a buffer containing 50 mM triethanolamine and 10 mM dithiothreitol at pH 7.0. At the indicated times, 40 µl aliquots were transferred to assay mixtures for measurement of adenylate, guanylate or inosinate cyclase activities as described in the Methods. Assay mixtures contained 0.67 mM MnATP or 0.25 mM MnITP or 0.25 mM MnGTP and 1.2 mM free Mn^{2+} in addition to the standard ingredients.

Fig. 4. Sea urchin sperm guanylate cyclase activity after preincubation with trypsin at 30°C. The preincubation volume of 0.46 ml contained 30 mM triethanolamine buffer at pH 7.8, 13 mM sodium azide, 80 µl of sea urchin sperm protein, 40 µg of trypsin and 0.5 mM GTP and 1.7 mM total metal when added. Reactions were stopped at the times indicated by the addition of 200 µg of soybean trypsin inhibitor. 40 µg aliquots were assayed at 30°C for guanylate cyclase activity as described in the Methods with 0.28 mM GTP and 1.7 mM total Mn^{2+}, and a 15 min incubation time.

from sea urchin sperm, adenylate cyclase reaction rates at 30° C decreased with time. Measurements of residual ATP by luciferase assay [21] indicated that ATP depletion was not responsible for the non-linearity. However, non-linear behavior could be accounted for in part, if not entirely, by the relatively great heat lability of adenylate cyclase (Fig. 3). Adenylate cyclase denatured rapidly at 30° C, whereas guanylate cyclase activity declined only slightly (15—20%) after 40 min of preincubation at 30° C. Some cyclic AMP production was still observed after 40 min of preincubation at 30° C, but it was less than 0.1% of the rate of cyclic GMP formation. Inactivation of inosinate cyclase by heat paralleled the inactivation of guanylate cyclase (Fig. 3). Deoxyguanylate cyclase inactivation patterns at 30° C and 35° C also corresponded to those seen with guanylate cyclase (data not shown). These experiments clearly differentiated deoxyguanylate and inosinate cyclase activities of sea urchin sperm from adenylate cyclase.

Effects of trypsin

The possibility that guanylate, inosinate and deoxyguanylate cyclase activities represented the activity of one enzyme was tested further using trypsin. When sea urchin sperm particulate fractions were preincubated in the presence of trypsin, the apparent activity of guanylate cyclase increased initially and then declined with increased preincubation time unless CaGTP, MnGTP or other metal—GTP complexes were added (Fig. 4). In the presence of the metal—

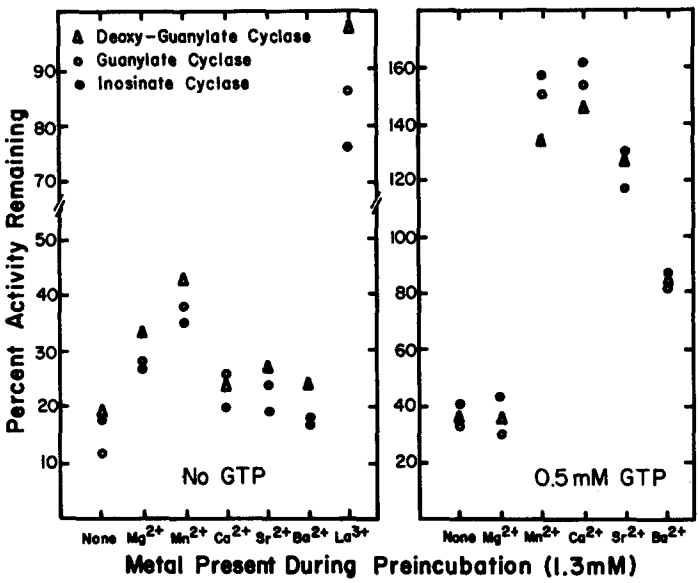

Fig. 5. Preincubation of sea urchin sperm particulate fractions with trypsin: apparent activities of inosinate, guanylate and deoxyguanylate cyclases after different preincubation conditions. The preincubation conditions were as described in the legend to Fig. 3, except that all preincubations were for 5 min and metal concentrations were 1.3 mM. 40 μl aliquots of the preincubation mixtures were assayed as described in the Methods. All assay mixtures contained 0.26 mM of the respective nucleoside triphosphate and 1.4 mM total Mn^{2+}. Relative activities of guanylate, inosinate and deoxyguanylate cyclase were similar to those in Fig. 7.

nucleotides, there was a substantial increase in guanylate cyclase activity after incubation with trypsin for 8 min or less. Trypsin induced solubilization of guanylate cyclase, as indicated by the recovery of virtually all of the enzyme in supernatant fractions after centrifugation at 100 000 × *g* for 1 h. The metal or metal—GTP combination used in the preincubation mixture did not alter the rate of enzyme solubilization by trypsin. The rate of total protein solubilization due to trypsin was estimated by following the loss of turbidity (absorbance at 560 nm). These rates were unaltered by metals or metal—GTP combinations used in the preincubation mixture, thus demonstrating that the preincubation condition did not appreciably affect trypsin activity in general.

Particulate fractions from sea urchin sperm were preincubated for 5 min with trypsin in the presence of various metals or metal—GTP combinations, and inosinate, deoxyguanylate and guanylate cyclase activities were subsequently measured (Fig. 5). Of the free metals tested, La^{3+} most effectively protected all three enzyme activities against trypsin. In the presence of GTP, Mn^{2+} and Ca^{2+} fully protected all three enzyme activities. With all metals and metal—nucleotides, the protection provided was virtually the same for all three cyclase activities.

When guanylate cyclase activity remaining after heat or trypsin treatment was plotted against inosinate cyclase activity remaining after such treatment, straight lines were obtained (Fig. 6), as evidenced by the correlation coefficients of 0.97 and 0.99. Similar comparisons between guanylate and deoxyguanylate cyclases were also made, and correlation coefficients of 0.99 were

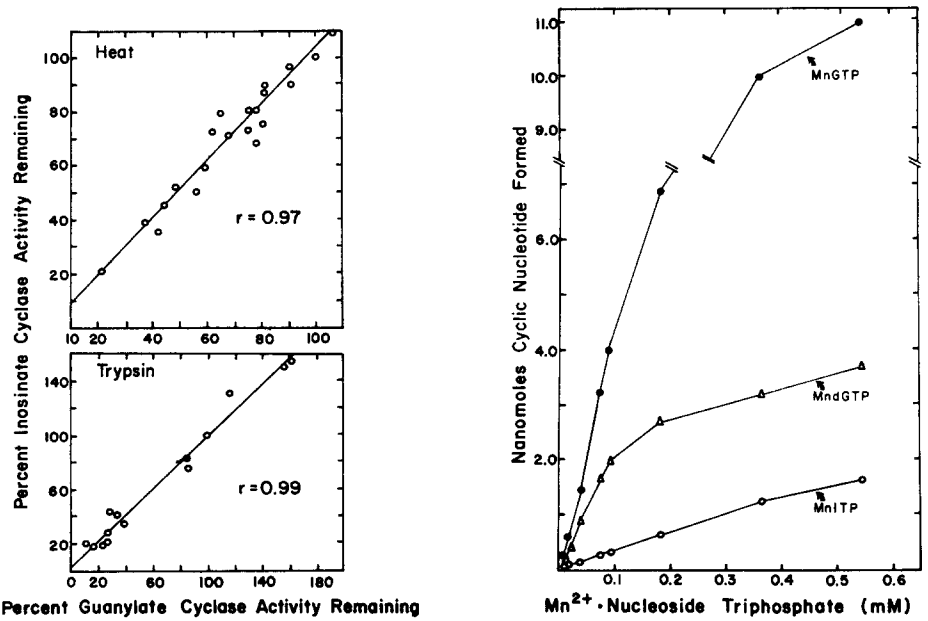

Fig. 6. Evidence that inosinate and guanylate cyclase activities reside within the same enzyme molecule. Data from Figs 3 and 5 were replotted as percent of enzyme activity remaining after all of the preincubation conditions. Lines were drawn by least squares analysis. r = correlation coefficient.

Fig. 7. Formation of cyclic nucleoside 3′,5′-monophosphate by sea urchin sperm particulate fractions. Assays were performed as described in Methods. All reaction mixtures contained a fixed free Mn^{2+} concentration of 1.6 mM and variable concentrations of the respective Mn^{2+}—nucleoside triphosphate as indicated in the figure. Velocities are expressed as the nanomoles of the corresponding cyclic nucleotide formed · 8 min^{-1} · 80 μg $protein^{-1}$.

observed for both heat and trypsin inactivation data. These results suggest that inosinate, deoxyguanylate and guanylate cyclase activities reside within the same protein molecule.

Relative activities of the cyclases

The relative activities of guanylate, inosinate and deoxyguanylate cyclases of sea urchin sperm particulate fractions are shown in Fig. 7. At low MnGTP and MndGTP concentrations, deoxyguanylate cyclase activity approached guanylate cyclase activity. Reciprocal plots were concave upward (not shown). The approximate substrate concentrations giving velocities equal to one-half V were GTP, 0.16 mM, dGTP, 0.07 mM, and ITP, 0.3 mM, for guanylate, deoxyguanylate, and inosinate cyclase activities, respectively.

The relative activities of guanylate, deoxyguanylate and inosinate cyclases of soluble fractions from rat lung were 1.0, 1.7 and 0.1 when estimated in the presence of 90 μM nucleoside triphosphate and 0.9 mM Mn^{2+}. At a substrate concentration of 90 μM V conditions are approximated with the rat lung enzyme. Adenylate cyclase activity was not detectable in the presence of Mn^{2+} or Mg^{2+}. In other experiments, sea urchin sperm particulate and rat lung soluble fractions were assayed for guanylate and deoxyguanylate cyclase activities using 90 μM GTP or dGTP at total Mn^{2+} concentrations ranging from 0.18 to

1.8 mM. In all instances, deoxyguanylate cyclase activity of sea urchin sperm was less than guanylate cyclase activity, while deoxyguanylate cyclase activity always exceeded guanylate cyclase activity when using soluble fractions from rat lung.

Inhibition studies

Inosinate cyclase activity of rat lung soluble fractions was too low to do meaningful inhibition studies, and because of this, ITP was not tested as an inhibitor of guanylate or deoxyguanylate cyclase activities. Previous work, however, has demonstrated that ITP inhibits rat lung guanylate cyclase activity [14].

Guanylate cyclase was inhibited in a competitive manner by MndGTP (Fig. 8), and the calculated K_i from the slopes replot was 12.2 μM. The apparent K_m for MnGTP in the absence of MndGTP was 4.4 μM. Deoxyguanylate cyclase activity was also competitively inhibited by MnGTP (Fig. 9). The apparent K_m for MndGTP (no MnGTP line) was 16 μM and the K_i for MnGTP was 16 μM.

Gel filtration profiles

When a homogenate of rat lung was centrifuged at 27 000 × g for 30 min and 4 ml of the resulting supernatant fluid applied to a Bio-Gel A 1.5 m

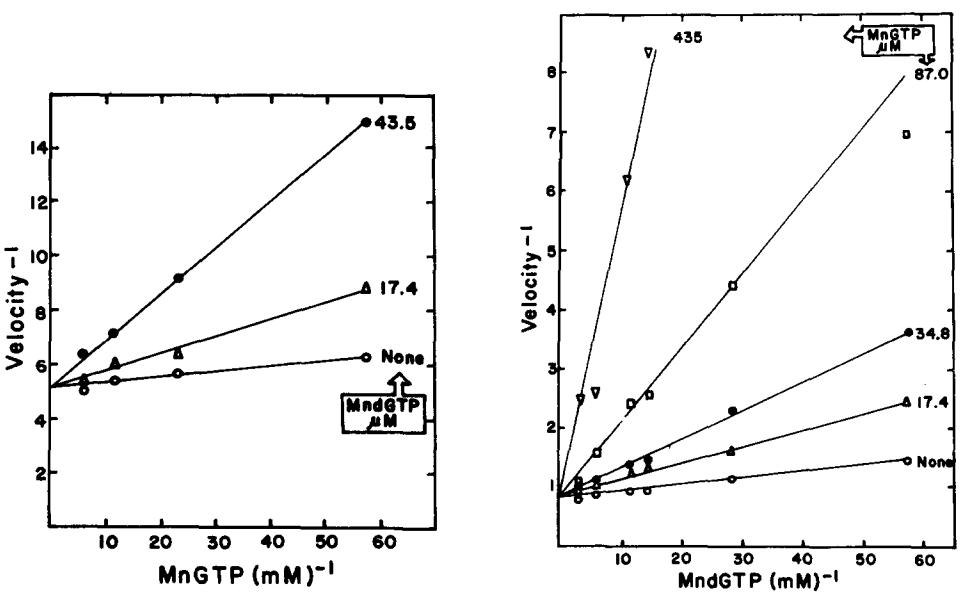

Fig. 8. Competitive inhibition of rat lung guanylate cyclase by MndGTP. Reaction mixtures contained a fixed free Mn^{2+} concentration of 0.9 mM and variable concentrations of MnGTP and MndGTP as indicated in the figure. Velocities are expressed as the nanomoles of cyclic GMP formed · 12 min^{-1} · 60 μg $protein^{-1}$.

Fig. 9. Competitive inhibition of rat lung deoxyguanylate cyclase by MnGTP. Reaction mixtures contained a fixed free Mn^{2+} concentration of 0.9 mM and variable concentrations of MnGTP and MndGTP as indicated in the figure. Velocities are expressed as the nanomoles of cyclic dGMP formed · 15 min^{-1} · 60 μg $protein^{-1}$.

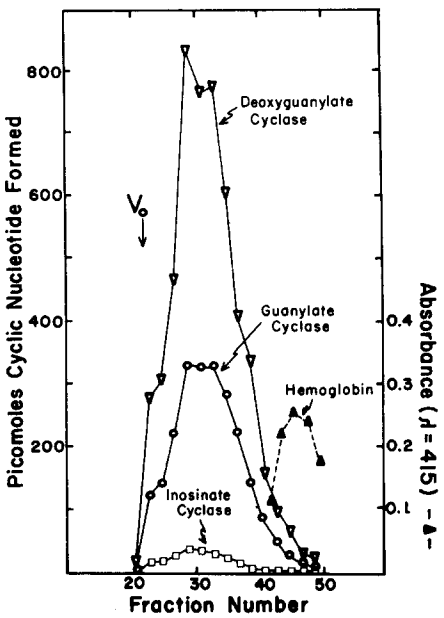

Fig. 10. Bio-Gel A 1.5 m elution profile of guanylate, inosinate and deoxyguanylate cyclase activities. A crude rat lung soluble fraction was made by centrifuging a 10% (wet weight/volume) homogenate of rat lung at 27 000 × *g* for 30 min. Four milliliters of the supernatant fluid was applied to a Bio-Gel A 1.5 m column (35 cm × 2.6 cm) equilibrated in 50 mM triethanolamine (pH 7.0), 1 mM dithiothreitol and 0.1% Triton X-100. Assay mixtures contained 2.2 mM total Mn^{2+}, 11 μM GTP and 0.2 ml of each 3 ml fraction. The ordinate equals the picomoles of cyclic GMP formed · 0.2 ml fraction^{-1} · 30 min^{-1}.

column as shown in Fig. 10, guanylate, deoxyguanylate and inosinate cyclase activity profiles were similar.

Discussion

Although cyclic dGMP and cyclic IMP, like cyclic dAMP, have not been reported to occur in nature, this report demonstrates enzymatic capability for formation of these two cyclic nucleotides. The similar responses of sea urchin sperm particulate inosinate, deoxyguanylate and guanylate cyclase activities to metals, heat and trypsin, and the competitive inhibitions and similar gel filtration profiles of these enzyme activities from rat lung soluble fractions, suggest that all three enzyme activities reside within the same protein molecule.

If adenylate cyclase from sea urchin sperm is capable of using ITP as an alternate substrate, the rate of conversion of ITP to cyclic IMP by this enzyme must be insignificant compared to the inosinate cyclase activity measured in this report, since inosinate cyclase activity remained fairly constant at 30° C while adenylate cyclase activity markedly declined. The capability of rat lung adenylate cyclase to use ITP as an alternate substrate was not studied.

At low substrate concentrations (10 μM), the rate of cyclic dGMP formation approaches the rate of cyclic GMP formation with sea urchin sperm particulate fractions, while in rat lung soluble fractions, deoxyguanylate cyclase activity exceeded that of guanylate cyclase under *V* conditions.

Deoxyribonucleotides present in DNA seem to be present in only low amounts when DNA synthesis is not occurring, but levels increase in proliferating tissue [22]. In proliferating chick embryo fibroblasts, ratios of ATP /dATP, GTP/dGTP and of CTP/dCTP are about 160, 28 and 7, respectively [23]. The ribonucleotide/deoxyribonucleotide ratio may be much lower in the nucleus, of course, since the deoxyribonucleotides are concentrated within this organelle. If guanylate cyclase activity exists in nuclei, then formation of cyclic dGMP would be a real possibility.

Acknowledgments

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