Purification of Soluble Guanylate Cyclase from Rat Lung

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The soluble form of guanylate cyclase from rat lung has been purified approximately 23,000-fold to homogeneity by isoelectric precipitation, GTP-Sepharose chromatography, and preparative gel electrophoresis. A single protein-staining band is observed after analytical gel electrophoresis on either 4 or 7.5% polyacrylamide gels. The final purified enzyme has a specific activity of about 700 nmol of cyclic GMP formed/min/mg of protein at 37°C in the presence of 4.8 mM MnCl₂ and 100 mM GTP. Both serum albumin and amiloride to slightly increase guanylate cyclase activity, but mainly stabilizes the purified enzyme; in its presence, specific activities in excess of 1 μmol of cyclic GMP formed/min/mg of enzyme protein can be obtained. When Mg²⁺ or Ca²⁺ are substituted for Mn²⁺, specific activities decrease to approximately 21 and 40 nmol of cyclic GMP formed/min/mg of protein, respectively. The apparent Michaelis constant for MnGTP in the presence of 4.8 mM MnCl₂ is 10.2 μM. Kinetic patterns on double reciprocal plots as a function of free Mn²⁺ are concave downward. The native enzyme has a molecular weight of approximately 151,000 as determined on Sephacyr S-200; sodium dodecyl sulfate-polyacrylamide gel electrophoresis results in two protein-staining bands with approximate molecular weights of 79,400 and 74,000. Thus, it appears that the soluble form of guanylate cyclase from rat lung exists as a dimer.

Results and Discussion

Step I: Isoelectric Precipitation—Frozen rat lungs (1.4 kg) were added to 3500 ml of buffer containing 25 mM triethanolamine, pH 7.6, and 1 mM dithiothreitol and homogenized with an Ultraturrax homogenizer. The homogenate was centrifuged at 13,000 × g for 60 min. The resulting supernatant fluid was used to prepare the isoelectric precipitated enzyme as previously described (6). After precipitation of the enzyme at pH 5.2 and centrifugation, the resulting pellets which contain enzyme activity were resuspended in 800 ml of a solution containing 25 mM triethanolamine buffer at pH 7.6 and 10 mM dithiothreitol. The enzyme could be stored at −70°C at this state until required. After thawing and prior to Step II, MnCl₂ and sodium azide were added to the enzyme solution to final concentrations of 1 and 10 mM, respectively. MnCl₂ was added because of its requirement for guanylate cyclase to bind to the GTP column, and sodium azide was added to protect the GTP column from guanosine triphosphatases.

Step II: GTP-Sepharose Chromatography—In initial studies, various agents were tested for their efficiency in causing elution of guanylate cyclase activity from GTP-Sepharose columns. Various concentrations of EDTA and GTP were tested and the best method appeared to be the use of low concentrations (0.5 mM) of EDTA. Although the enzyme elutes as a broad peak with 0.5 mM EDTA, in contrast to the situation with high concentrations of EDTA or MgGTP, the specific activity is higher. For the actual preparation of enzyme, 7.0 g of protein in 800 ml were added to a GTP-Sepharose column (2.6 × 20 cm) which had been equilibrated with a solution containing 25 mM triethanolamine at pH 7.6, 10 mM dithiothreitol, 10 mM sodium azide, and 1 mM MnCl₂. After sample application, the column was washed with 4 liters of the equilibration buffer. Both cyclic AMP and cyclic GMP phosphodiesterase activities were removed from the column by these procedures. Guanylate cyclase activity was eluted from the column by the addition of a solution containing 25 mM triethanolamine buffer at pH 7.6, 10 mM dithiothreitol, 10 mM sodium azide, and 0.5 mM EDTA. The fractions containing guanylate cyclase activity were pooled and immediately concentrated using an Amicon ultrafiltration unit (PM-30 membrane). The concentrated enzyme was then dialyzed extensively against a buffer containing 25 mM triethanolamine at pH 7.6, 10 mM dithiothreitol, and 50% glycerol. The enzyme was very stable at this step and could be stored for at least 6 months without loss of activity.

Step III: Preparative Gel Electrophoresis—A 4.5-cm high gel slab of 6% polyacrylamide was prepared using the 7-mm preparative gel holder from ISCO. The polyacrylamide gel was pre-electrophoresed for 5 to 6 h at a constant power of 25 W and fixed with 250 ml of 10% trichloroacetic acid. The gel was then stained overnight with Coomassie brilliant blue G-250 and destained with 30% acetic acid. A single protein-staining band was observed after electrophoresis. The gel was sliced into 0.5-cm sections and enzyme activity was determined in each section. The peak section was pooled and concentrated as described above.

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1 The data are presented as a supplement in a miniprint format immediately following this paper. (Experimental Procedures, Results. Fig. 4 through 8, Table III, and References are found on p. 243.) Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 960 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1121, cite author, and include a check or money order for $1.20 per set of photocopies. The abbreviation used in the miniprint is: Na-dodecyl-SO₄, sodium dodecyl sulfate.
watts, prior to sample application. The sample containing 20 mg of protein, 50% glycerol, 10 mM dithiothreitol, 25 mM triethanolamine buffer at pH 7.8, and 0.0007% bromphenol blue in 3.5 ml was applied to the top of the gel. Electrophoresis was then resumed at 25 watts constant power. The elution buffer flow rate was approximately 30 ml/h and fractions of 11 ml were collected. The enzyme elution profile is shown in Fig. 1. Fractions (Nos. 31 to 36) containing the enzyme were immediately concentrated with an Amicon ultrafiltration unit (PM-30 membrane) to a volume of about 8 ml. The concentrated enzyme was then dialyzed extensively against a buffer containing 25 mM triethanolamine at pH 7.6, 10 mM dithiothreitol, and 50% glycerol. The final volume of enzyme was about 3 ml, and this was frozen at -70°C in aliquots of 50 to 100 µl.

**Purification and Recovery**—A summary of the purification method is shown in Table I. The final specific activity of 693 nmol of cyclic GMP formed/min/mg of protein represents a 22,355-fold purification compared to the homogenate. This specific activity has been higher (up to 800 nmol of cyclic GMP formed/min/mg of protein) in some other enzyme preparations and can be increased to over 1 µmol of cyclic GMP formed/min/mg of protein with the addition of bovine serum albumin to the assay. The final enzyme recovery of 2.2% is similar to that of the purified enzyme from sea urchin sperm (10).

**Homogeneity**—The enzyme obtained from Step III migrates as a single protein-staining band on either 4 or 7.5% polyacrylamide gels (Fig. 2). Variation in the percentage of polyacrylamide would differentiate between different molecular weight proteins. Protein added to each gel has been varied between 3 and 20 µg, and no trace of contaminating protein-staining bands have been observed. In parallel gels, enzyme activity has been shown to be completely associated with the protein-staining band (not shown).

When the purified enzyme was incubated with [3H]GTP in the absence of inhibitors of guanosine triphosphatase or phosphodiesterase, essentially 100% (93%) conversion of GTP to cyclic GMP was observed (Table II). Thus, no interfering enzymes are present in concentrations detectable by enzymological methods.

![Fig. 1. Preparative gel electrophoresis of rat lung soluble guanylate cyclase. GTP-Sepharose purified enzyme (20 mg) in 3.5 ml of a solution containing 10 mM dithiothreitol, 25 mM triethanolamine buffer at pH 7.8, and 0.0007% bromphenol blue were applied to the 6% polyacrylamide slab (7 mm × 4.6 cm) and electrophoresed at 25 watts. Elution buffer was collected at a rate of approximately 30 ml/h in fractions of 11 ml. Guanylate cyclase activity was measured in the fractions as described under “Experimental Procedures.” The bromphenol blue marker dye peak was at Fraction 22. Fractions 31 to 36 were pooled and concentrated to a final volume of about 3 ml.](http://www.jbc.org/)

![Fig. 2. Analytical polyacrylamide gel electrophoresis of the purified guanylate cyclase. Purified enzyme (12.5 µg) was applied to 90-mm gels containing 7.5% polyacrylamide. Electrophoresis was at 5 mA/tube. After the bromphenol blue tracker dye had run to the end of the gel, electrophoresis was discontinued and the gel stained with Coomassie blue R-250. After destaining, the gel was scanned at 560 nm using the gel scanner attachment for the Beckman model 35 spectrophotometer.](http://www.jbc.org/)
TABLE II
The conversion of GTP to cyclic GMP by the purified guanylate cyclase from rat lung

Incubation mixtures contained 1.82 mM MnCl₂, 50 mM triethanolamine buffer at pH 7.8, 2.65 nmol of GTP, 4 x 10⁶ dpm of [³H]GTP, 0.19 mg/ml of bovine serum albumin, and 0.092 mg of enzyme protein in a final volume of 0.275 ml. Incubations were terminated with 2 ml of 50 mM acetic acid, rather than zinc acetate to allow for the determination of [³H]GTP. The [³H]GTP was isolated by elution with 1 M LiCl from polyethyleneimine-cellulose columns. After 220 min, 0.14 nmol of [³H]GTP remained; thus, all of the [³H]GTP utilized was converted to cyclic [³H]GMP.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Nanomoles of cyclic GMP formed</th>
<th>Nanomoles of GTP utilized</th>
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<tbody>
<tr>
<td>5</td>
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<td>220</td>
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**Molecular Weight**—The purified enzyme migrated with an apparent molecular weight of approximately 151,000 on Sephacryl S-200 (not shown). Marker proteins used on this column (1.5 x 70 cm) were ovalbumin, bovine serum albumin, aldolase, and catalase. The column was equilibrated with a buffer containing 25 mM triethanolamine at pH 7.6, 10 mM dithiothreitol, and 0.1 M NaCl.

When the purified enzyme was denatured with sodium dodecyl sulfate and dithiothreitol, two protein-staining bands with molecular weights of approximately 79,400 and 74,000 were observed (Fig. 3).

**General Properties**—Various properties of the purified enzyme are described in the miniprint section immediately following the main paper.

These results indicate that rat lung guanylate cyclase has been purified to apparent homogeneity by isoelectric precipitation, GTP-Sepharose chromatography, and preparative gel electrophoresis. The method of purification is relatively simple and yields an enzyme that is stable at -70°C for at least 6 weeks.

The purified enzyme appears to exist in a "state" other than that reported for the sodium azide-activated guanylate cyclase (11–14), since the Mg²⁺-stimulated activity is only about 3% of the Mn²⁺-stimulated activity. After sodium azide activation, the Mg²⁺-stimulated activity of crude guanylate cyclase preparations can become equivalent to the Mn²⁺-stimulated activity (11–14).

The enzyme obtained from Step I has been previously characterized with respect to metal and metal-GTP kinetics (6); the purified enzyme has an apparent Michaelis constant for MnGTP (10 μM) similar to that reported for the Step I enzyme. The negative cooperative type kinetics with respect to Mn²⁺ observed with the Step I enzyme also appear to be present with the purified guanylate cyclase.

The apparent native molecular weight of the purified enzyme agrees with estimates obtained for the soluble guanylate cyclase from kidney (7). The large apparent molecular weights reported by Chrisman et al. (6) for the rat lung enzyme were probably due to aggregation in low ionic strength buffers. After denaturation in sodium dodecyl sulfate, two subunits of unequal molecular weight are observed on 7.5% polyacrylamide. Because of the unequal size, it seems possible that only one of the subunits is associated with catalytic activity.

Although guanylate cyclases from other mammalian tissues have not been reported to be purified as of this time, specific activities of 700 to 1000 nmol of cyclic GMP formed/min/mg of protein may indicate expected specific activities of homogeneous soluble guanylate cyclases that are not in an "activated" state. These specific activities are about 10% of those reported for the sea urchin sperm enzyme (10). However, comparisons must take into account the fact that the sea urchin sperm enzyme is initially associated with the particulate fractions of the cell. Although the catalytic center for the particulate and soluble forms of guanylate cyclase may be similar, the secondary structure and composition, which results in the differential cell distribution, may significantly affect the turnover rate. It is also possible that activation of the purified soluble guanylate cyclase by nitric oxide (15) or by other agents could result in specific activities approaching those reported for the sea urchin sperm enzyme.

REFERENCES

References are found on p. 243.

**Fig. 3**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified guanylate cyclase. The enzyme was denatured as described under "Methods," and 10 μg of the denatured enzyme was then added to 90-mm gels (7.5%) and electrophoresed at 4 mA/tube until the bromphenol blue tracker dye reached the end of the gel. The apparent molecular weight was determined by the simultaneous electrophoresis of phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin.
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Experimental Procedures

Materials: RGB, ATP, GTP, alpha-[32P]GTP, Tris, EDTA, cycloheximide, dithiothreitol, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). CF3-125I was obtained from Amersham. Unlabeled iodothyronines were from Iodogen and [1,2-3H]inosine (1 Ci/mmol) from Amersham.

Methods

Evaluation of the effects of myriocapsin on guanylate cyclase activity. The effect of myriocapsin on guanylate cyclase activity was evaluated by measuring the production of cyclic GMP in intact rat lung homogenates. Intact rat lung homogenates were prepared as described previously (22). The homogenate was incubated with the appropriate concentrations of VIP (0-1000 nM) and GTP (0-5 mM) in the presence or absence of myriocapsin (0-100 µM) for 30 min at 37°C. The reaction was terminated by the addition of dithiothreitol (5 mM) and the sample was immediately applied to a column of cation-exchange resin (DEAE-Cellulose). The eluate was collected and assayed for cyclic GMP content as described previously (22).

Results and Discussion

The results of this study indicate that myriocapsin has a potent effect on the cyclic GMP production in rat lung homogenates. The maximal stimulation of cyclic GMP production was observed at 100 µM myriocapsin, with a 2.5-fold increase in cyclic GMP production compared to the control. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 1 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 2 shows the effect of VIP on cyclic GMP production in rat lung homogenates. The cyclic GMP production increased with increasing concentrations of VIP, reaching a maximum of 2.5-fold at 1000 nM VIP. The effect of VIP was dose-dependent, with a 50% increase in cyclic GMP production at 100 nM VIP.

Figure 3 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 4 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 5 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 6 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 7 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 8 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 9 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 10 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 11 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 12 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.

Figure 13 shows the effect of myriocapsin on cyclic GMP production in rat lung homogenates in the presence of VIP. The cyclic GMP production increased with increasing concentrations of myriocapsin, reaching a maximum of 2.5-fold at 100 µM myriocapsin. The effect of myriocapsin was dose-dependent, with a 50% increase in cyclic GMP production at 25 µM myriocapsin.
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