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Purification of soluble guanylate cyclase from rat liver

(cyclic GMP/cyclic AMP/nitroprusside/nitric oxide/fatty acids)

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ABSTRACT **Soluble guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] has been purified from rat liver and exhibited a single protein band on polyacrylamide gels coincident with activity and indicative of a molecular weight of 150,000. The apparent specific activity of the purified enzyme was 276 nmol of cyclic GMP formed per mg per min with Mn^{2+} as the cation cofactor and 23.8 nmol of cyclic GMP formed per mg per min with Mg^{2+}. This represented 9200-fold and 7400-fold purifications of Mn^{2+} and Mg^{2+} activities, respectively. The specific activity of soluble guanylate cyclase was not constant with protein concentration. At all stages of purification, increasing the enzyme concentration in the guanylate cyclase assay increased the apparent specific activity of the preparation. The purified enzyme could be activated by nitroprusside, nitric oxide, arachidonate, linoleate, oleate, and superoxide dismutase. However, the degree of activation was dependent upon the concentration of enzyme protein assayed.**

Guanylate cyclase [GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2] catalyzes the formation of cyclic GMP and exists in both soluble and particulate fractions of homogenates (1–4). The soluble and particulate forms are different in several respects (5), including their antigenic properties (6).

A number of agents increase guanylate cyclase activity in broken cell preparations and cyclic GMP levels in tissues. Azide, nitroprusside, nitroglycerin, and nitrosamines activate both particulate and soluble forms of the enzyme (5, 7–9). The common pathway for activation by these agents may be the formation of nitric oxide (10). Activation by hydroxyl radical (11) and unsaturated fatty acids (12, 13) has also been described. The precise mechanism(s) by which guanylate cyclase is activated by these different agents is unknown.

The properties of guanylate cyclase activated by many of these agents are different from those of the native enzyme. For example, activated enzyme may use Mg^{2+} as cofactor as effectively as Mn^{2+} (14) and can catalyze the formation of cyclic AMP (15).

Identification of the precise mechanism(s) by which agents activate guanylate cyclase as well as a study of the molecular changes occurring during activation will require highly purified enzyme. Purification of particulate guanylate cyclase from sea urchin sperm has been reported (16), and partial purifications of soluble guanylate cyclase from rat liver (15, 17), bovine lung (18), and human platelets (19) have been described. Bacterial guanylate cyclase has been partially purified from *Caulobacter crescentus* (20) and *Escherichia coli* (21).

In this communication we report the purification of soluble guanylate cyclase from rat liver and describe some of the properties of the purified enzyme. Some of these observations have recently been presented in abstract form (22).

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MATERIALS AND METHODS

Livers were removed from male Sprague–Dawley rats (150–200 g) and placed in cold 20 mM Tris·HCl buffer (pH 8.0) containing 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol (buffer I). All subsequent procedures were at 4°C. Livers were homogenized in 4 vol of buffer I with a Waring blender for 30 sec followed by two strokes with a glass–Teflon homogenizer. Homogenates were centrifuged at 105,000 × *g* for 60 min. Supernatant fractions were adjusted to pH 5.0 with 1 M HCl and centrifuged at 12,000 × *g* for 20 min. The pH 5.0 precipitate was dissolved in 20 mM Tris·HCl buffer (pH 7.6)/1 mM EDTA/1 mM dithiothreitol (buffer II) and recentrifuged at 12,000 × *g* for 20 min. The supernatant fraction was brought to 20% saturation with ammonium sulfate and centrifuged at 12,000 × *g* for 20 min. The resulting supernatant fraction was brought to 50% saturation with ammonium sulfate and recentrifuged. The 20–50% ammonium sulfate precipitate was suspended in buffer II and dialyzed against 200 vol of the same buffer. The dialyzed preparation was applied to a DEAE-Sephacel column (5 × 40 cm) equilibrated with buffer II. Enzyme was eluted with a linear NaCl gradient (0–0.5 M). Guanylate cyclase eluted between 100 mM and 200 mM NaCl. Fractions containing activity were pooled, concentrated by ammonium sulfate precipitation (0–60%), and applied to a Sepharose 6B column (5 × 100 cm) equilibrated and eluted with buffer II. Guanylate cyclase eluted with a relative elution volume V_e/V_o of 1.85. Fractions with activity were pooled and applied to a column (2.5 × 5.5 cm) of agarose-hexane (5–10 μmol of hexane per ml of resin) equilibrated with 20 mM Tris·HCl buffer (pH 7.6)/10 mM dithiothreitol/1 mM EDTA. The column was washed with equilibration buffer until the absorbance at 280 nm was zero. The column was eluted with 200 mM NaCl in the same buffer. Fractions with activity were pooled, then desalted and concentrated to 15 ml in an Amicon ultrafiltration apparatus fitted with an XM50 (43 mm) filter.

Guanylate cyclase was further purified by preparative polyacrylamide gel electrophoresis at 4°C in a Buchler Polyprep 200. A 5% polyacrylamide gel (65 ml) was used with a 2.5% stacking gel (volume equal to volume of sample) layered above. The upper and lower buffers were 50 mM and 150 mM Tris·glycine (pH 8.2), respectively, each containing 1 mM dithiothreitol. The elution buffer was 20 mM Tris·HCl (pH 7.6)/25% sucrose/1 mM EDTA/10 mM dithiothreitol. The preparation after chromatography on hexane-agarose was adjusted to contain 20 mM Tris·HCl (pH 7.6), 1 mM EDTA, 10 mM dithiothreitol, 10% sucrose, and 0.1 mM bromphenol blue. Electrophoresis was at 30 mA for 15 hr. At this stage of purification many, but not all, preparations yielded a single protein band on analytical polyacrylamide gel electrophoresis that was coincident with activity. However, preparative electrophoresis fractions with activity were routinely pooled and subjected to a second preparative electrophoresis at 40 mA for 15 hr in a 7.5% polyacrylamide gel. All other conditions were identical

to the first electrophoresis. Fractions with activity were pooled and stored at −70°C.

Analytical polyacrylamide gel electrophoresis was carried out at 4°C with 8–18 μg of sample protein by using a 5% polyacrylamide separating gel (6 × 75 mm) and a 2.5% polyacrylamide stacking gel (6 × 4 mm). Electrophoresis was for 150 min at 3 mA per gel tube in a 50 mM Tris·glycine buffer (pH 8.2). Gels were stained for protein with Coomassie blue or cut into 3-mm sections and assayed for guanylate cyclase activity.

Guanylate cyclase activity was determined as described previously (14, 15). Incubations (100 μl for 10 min at 37°C) contained 50 mM Tris·HCl buffer (pH 7.6), 4 mM $MnCl_2$ or $MgCl_2$, and 1 mM GTP. A nucleoside triphosphate-regenerating system was not utilized in the experiments reported here. In some incubations with crude or partially purified enzyme 10 mM theophylline was included. Theophylline was omitted in incubations of purified enzyme and 0.02% bovine serum albumin was included. Nitric oxide gas was introduced into some reaction mixtures as described (10). Cyclic GMP formed was determined by radioimmunoassay (23) as described (3). All values represent the means of duplicate incubations from representative experiments.

Protein was measured by the method of Lowry *et al.* (24). Unsaturated fatty acids were from Sigma, the prostaglandin endoperoxide analogues U-44069 and U-44619 were from Upjohn, and agarose-hexane resin was from P-L Biochemicals. All other materials were obtained as reported previously (3, 4, 7, 8, 10, 11, 14, 15).

RESULTS

Purification of Guanylate Cyclase. The purification of soluble guanylate cyclase from rat liver is summarized in Table 1. Purified guanylate cyclase had an apparent specific activity with Mn^{2+} as the cation cofactor of 276 nmol of cyclic GMP/mg per min. This represents a 9200-fold purification with a 5.4% recovery. The apparent specific activity with Mg^{2+} as the cation cofactor was 23.8 nmol of cyclic GMP/mg per min and represents a 7400-fold purification with a 4.3% recovery. These data must, however, be interpreted with caution because, as discussed below, guanylate cyclase specific activity is altered with enzyme concentration.

After the pH 5.0 precipitation step, there was an increase in total guanylate cyclase activity that was most striking when Mg^{2+} was the cofactor (Table 1). The cation requirements for guanylate cyclase change when the enzyme is activated by some agents, such that Mg^{2+} may become as effective as Mn^{2+} (5, 13).

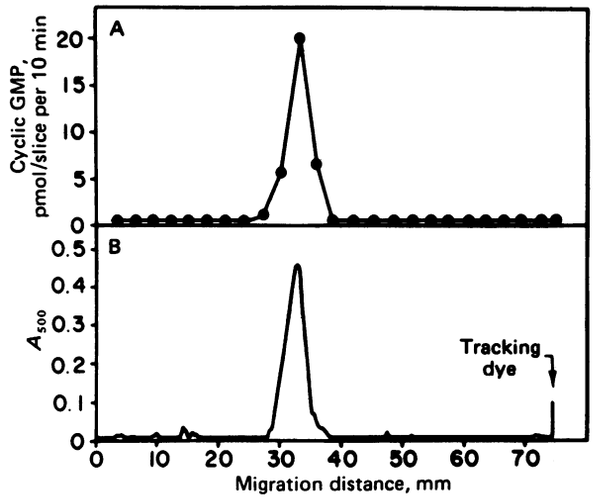

FIG. 1. Polyacrylamide gel electrophoresis of purified guanylate cyclase. (*A*) Activity profile of cyclic GMP formation by 3-mm slices of gels incubated for 10 min at 37°C in 200 μl of 50 mM Tris·HCl (pH 7.6)/1 mM GTP/4 mM $MnCl_2$/0.02% bovine serum albumin. (*B*) Absorbance at 500 nm of a stained gel run with 18 μg of protein.

Activation of the enzyme during purification may be reflected by a decrease in the Mn^{2+}/Mg^{2+} activity ratio (Table 1). The source and mechanism of activation during purification are not apparent at this time.

Concentrations of creatine phosphate (7.5 mM) and theophylline (10 mM) usually employed in the guanylate cyclase assay (3, 15) inhibited the purified enzyme activity 100% and 40%, respectively (data not shown). All enzyme activities after preparative electrophoresis were determined in the absence of theophylline and regenerating system because they were not required. Due to the low protein concentration in the assay of purified enzyme (2–200 ng per 100 μl), 0.02% bovine serum albumin was included in the reaction mixture. Under these conditions activity was linear with time for at least 20 min.

In the presence of 10 mM dithiothreitol and 25% sucrose the purified enzyme was stable for months when stored at −70°C. However, activity was rapidly lost when the enzyme was stored without thiols.

Electrophoresis of purified guanylate cyclase on 5% polyacrylamide gels revealed one major protein band coincident with guanylate cyclase activity (Fig. 1). Similar results were obtained with 5% polyacrylamide gels run at pH 6 or 10.5 as well as polyacrylamide gels of 7.5%, 10%, or 12% run at pH 8.2

Table 1. Purification of rat liver guanylate cyclase

		Cyclic GMP formed*				
		Mn^{2+}		Mg^{2+}		
Fraction	Total protein, mg	Total activity, nmol/min	Specific activity, nmol/mg per min	Total activity, nmol/min	Specific activity, nmol/mg per min	Activity ratio, Mn^{2+}/Mg^{2+}
1. 105,000 × *g* supernate	37,440	1123	0.03	119.8	0.0032	9.4
2. pH 5.0 precipitate	9,607	1345	0.14	230.6	0.024	5.8
3. Ammonium sulfate precipitate	5,024	1256	0.25	266.3	0.053	4.7
4. DEAE-Sephacel	474	436	0.92	71.1	0.15	6.1
5. Sepharose 6B	147	269	1.83	42.6	0.29	6.3
6. Hexane-agarose	36.3	153.9	4.24	21.8	0.6	7.1
7. Preparative electrophoresis, 5% gel	6.3	95.2	15.1	10.7	1.7	8.9
8. Preparative electrophoresis, 7.5% gel	0.22	60.7	276.0	5.2	23.8	11.6

Guanylate cyclase was purified from 600 g of rat liver.

* Samples obtained in steps 1 through 8 of the purification procedure were assayed with 43.4, 13.3, 23.2, 4.4, 4.0, 7.5, 0.5, and 0.21 μg of protein per 100 μl of reaction mixture, respectively.

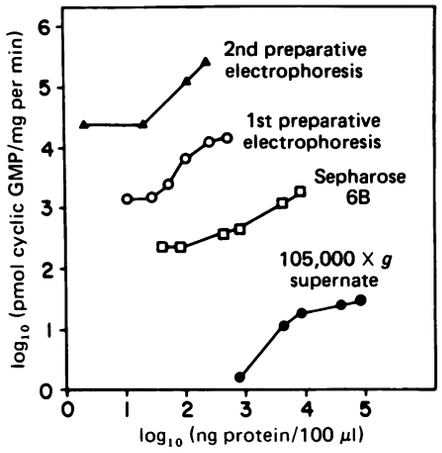

FIG. 2. Specific activity of guanylate cyclase at different stages of purification. Guanylate cyclase activity was assayed with Mn^{2+} as cofactor at the protein concentrations indicated. Enzyme preparations were: 105,000 × *g* supernatant fraction (●); Sepharose 6B enzyme (□); 5% gel preparative electrophoresis enzyme (○); purified 7.5% gel preparative electrophoresis enzyme (△). All reaction mixtures contained 0.02% bovine serum albumin. Theophylline at 10 mM was included in the assays of the 105,000 × *g* supernatant and Sepharose 6B enzymes.

(data not shown). Thus, under a variety of electrophoretic conditions purified guanylate cyclase exhibited a single protein band.

With Sepharose 6B chromatography, soluble liver guanylate cyclase had a molecular weight of about 200,000 and a Stokes radius of 46 Å. The molecular weight and radius obtained with electrophoresis on different percent polyacrylamide gels (25) were 150,000 and 35 Å, respectively.

Guanylate Cyclase Specific Activity with Enzyme Concentration. Guanylate cyclase activity was not linear with enzyme concentration. Shown in Fig. 2 are the log values for specific activities of guanylate cyclase at various stages of purification determined with various amounts of enzyme. In general, a 10- to 20-fold change in enzyme concentration results in approximately a 10-fold change in the specific activity. This phenomenon occurs with Mn^{2+} or Mg^{2+} as cofactor. The time course of the guanylate cyclase reaction is linear for at least 20

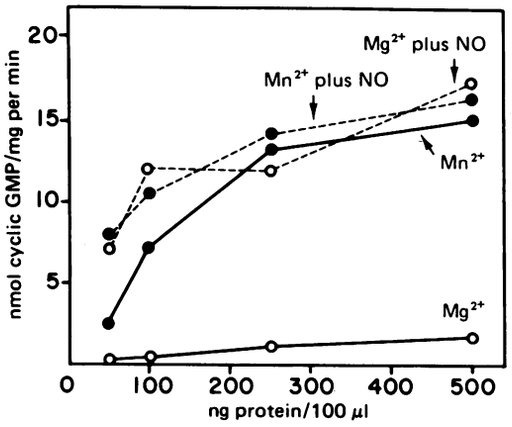

FIG. 3. Effect of nitric oxide on guanylate cyclase activity at various protein concentrations. Guanylate cyclase purified through the 5% gel preparation electrophoresis step was assayed at the protein concentrations indicated with either Mn^{2+} (●) or Mg^{2+} (○) as cofactor. In some incubations 167 μl of nitric oxide gas was vented over incubation mixtures as described previously (10) (broken lines). All reaction mixtures contained 0.02% bovine serum albumin.

Table 2. Effects of fatty acids on purified guanylate cyclase

Addition	% of basal activity			
	10 μM	25 μM	50 μM	100 μM
Na arachidonate	158	205	184	130
Na linoleate	163	231	163	37
Na oleate	137	242	321	ND
U-44069	126	126	158	121
U-44619	137	116	110	131

Cyclic GMP formation by purified guanylate cyclase was determined with 30 ng of protein/100 μl with the agents listed at the concentrations indicated, using Mg^{2+} as cofactor. Basal activity was 4.1 nmol of cyclic GMP/mg per min. ND, not detectable.

min with both high and low enzyme concentrations (data not shown). Concentrations of 105,000 × *g* supernatant protein below 800 ng/100 μl could not be assayed due to the sensitivity limits of the radioimmunoassay for cyclic GMP. However, for other enzyme fractions there is a tendency for specific activity to level off at low enzyme concentrations (Fig. 2). Thus far, our most highly purified preparations have had specific activities in the low protein range (2–30 ng/100 μl) of 24–34 nmol of cyclic GMP/mg per min with Mn^{2+} as cofactor and 3–4 nmol of cyclic GMP/mg per min with Mg^{2+}. The highest specific activities obtained to date are as reported in Table 1. Purified guanylate cyclase activity decreases when stored at 4°C. For this reason specific activities with concentrations of purified guanylate cyclase higher than 200 ng/100 μl have been difficult to interpret due to instability of the enzyme preparation during procedures used to concentrate enzyme at 4°C.

Activation of Purified Guanylate Cyclase. Various concentrations of a guanylate cyclase preparation were assayed with and without exposure to nitric oxide gas (Fig. 3). At 50 ng of protein/100 μl, nitric oxide increased Mn^{2+} and Mg^{2+} activities 2-fold and 36-fold, respectively. After activation with nitric oxide, activities with Mn^{2+} and Mg^{2+} were similar. At 500 ng of protein/100 μl, little or no effect of nitric oxide was seen with Mn^{2+} as cofactor and only a 10-fold increase in activity occurred with Mg^{2+} as cofactor.

Nitric oxide and sodium nitroprusside also increased cyclic GMP formation by purified enzyme with Mg^{2+} as cofactor but had little or no effect with Mn^{2+} as cofactor (data not shown). Purified guanylate cyclase with Mg^{2+} as cofactor could be activated by the unsaturated fatty acids arachidonate, linoleate, and oleate and to a lesser extent by the stable prostaglandin endoperoxide analogues U-44069 and U-44619, which possess a methylene substitution for oxygen in the endoperoxy ring (Table 2). The activity with Mn^{2+} as the cofactor was either slightly inhibited or unaffected by fatty acids. Superoxide dismutase produced a small and reproducible activation of guanylate cyclase with Mg^{2+} as cation, and this effect was enhanced by the addition of nitrate reductase (data not shown). These experiments have been complicated by the presence of sucrose and dithiothreitol in the enzyme preparation, because sugars are capable of scavenging hydroxyl radicals (26) and thiols are known inhibitors of superoxide dismutase (27).

DISCUSSION

Soluble guanylate cyclase purified from rat liver exhibited a single band on polyacrylamide gels. Interpretation of the purification is complicated by nonproportionality of activity with enzyme concentration. Depending upon the enzyme concentration in the guanylate cyclase assay, a purification of 1000- to 100,000-fold could be reported (Fig. 2). The total activity recovered in the final preparation could likewise range from 0.5% to 96%. We have chosen to report our results on the pu-

rification shown in Table 1 on the basis of maximal activities we have observed for the 105,000 × *g* supernatant and purified fractions. Specific activities for 105,000 × *g* supernatant preparations plateau at about 30–40 pmol of cyclic GMP/mg per min (Fig. 2). It is not known what the maximal specific activity for the purified preparation may be because procedures used to concentrate the preparation at 4°C resulted in a loss of activity.

The maximal specific activity reported here exceeds that of other partially purified preparations of guanylate cyclase from rat liver (15, 17), bovine lung (18), human platelets (19), and *C. crescentus* (20). It is also greater than that observed with an apparently highly purified soluble enzyme from *E. coli* (21). Only the specific activity reported for purified particulate guanylate cyclase from sea urchin sperm (16) is greater. It is not known whether guanylate cyclase from other sources exhibits the same behavior with enzyme concentration as does the liver enzyme. However, we have observed similar results with highly purified soluble guanylate cyclase from rat lung (unpublished observations).

Our experiments have indicated that the increase in specific activity seen with increased enzyme concentration may represent activation of guanylate cyclase. This activation apparently differs from the spontaneous activation of guanylate cyclase described by Böhme *et al.* (28) because the latter was prevented by dithiothreitol. The phenomenon described here occurs in the presence of 1 mM dithiothreitol, and thiol concentrations up to 10 mM have had no effect in preventing the process.

Purified guanylate cyclase could be activated by agents known to alter guanylate cyclase activity in cruder systems. Thus, activation of guanylate cyclase with nitroprusside, nitric oxide, unsaturated fatty acids, stable prostaglandin endoperoxide analogues, and hydroxyl radical has no apparent requirements for other proteins or factors. The reason why these agents increase cyclic GMP formation to a greater extent with Mg^{2+} as cofactor is unclear. The degree of activation of guanylate cyclase with Mn^{2+} as cofactor by agents such as nitric oxide decreases more rapidly throughout the purification than with Mg^{2+} as cofactor. The activation seen with nitric oxide also decreases with increasing enzyme concentration (Fig. 3). Whether these observations are in part responsible for the decreased activation of Mn^{2+} guanylate cyclase activity by nitric oxide and other compounds is unclear. To complicate matters further, cyclic AMP formation by purified guanylate cyclase with Mg^{2+} or Mn^{2+} as cofactor can be increased 3-fold and 5-fold, respectively, by nitric oxide. In addition, arachidonate inhibits cyclic AMP formation with Mg^{2+} as cation but causes a 2-fold increase with Mn^{2+} (data not shown).

Guanylate cyclase is apparently a unique enzyme that can be altered by oxidative and reductive processes. Purification of the soluble enzyme from a mammalian source has proven to be a complex problem due to enzyme instability and activation during purification. The availability of a highly purified mammalian guanylate cyclase should permit additional physicochemical and kinetic studies of the inactive and active enzyme. Pure enzyme can also be used to elucidate the mechanism of redox and free radical regulation of guanylate cyclase activity.

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