PURIFICATION AND CHARACTERIZATION OF GUANYLATE CYCLASE FROM *CAULOBACTER CRESCENTUS**

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SUMMARY: Guanylate cyclase has been purified 60-fold from cell extracts of the bacterium *Caulobacter crescentus*. It has a molecular weight of approximately 140,000 and is dependent upon Mn^{2+} for activity. Enzymic activity is unaffected by cyclic AMP, cyclic GMP or $N^6,O^{2'}$-dibutyryl cyclic AMP but is stimulated by $N^2,O^{2'}$-dibutyryl cyclic GMP. The partially purified preparation of guanylate cyclase does not contain detectable adenylate cyclase activity.

The cell cycle of the bacterium *Caulobacter crescentus* is associated with a series of morphogenetic events occurring in a fixed spatial and temporal sequence (1,2). Studies of the mechanisms which regulate the timing of gene expression and the localization and assembly of gene products have implicated the cyclic 3',5'-adenosine and guanosine monophosphates (cyclic AMP and cyclic GMP) in the control of the cell cycle (3,4).

We have shown that the cell cycle of *C. crescentus* can be arrested by transferring cultures grown in glucose to medium containing lactose as the sole carbon source (3). This block in growth and development, just prior to the formation of flagella, pili, and cell division, can be overcome by the addition of $N^6,O^{2'}$-dibutyryl cyclic AMP but not cyclic GMP or the dibutyryl derivative of cyclic GMP. The addition of dibutyryl cyclic GMP, however, prevented the formation of polar flagella during spontaneous rescue of growth in the blocked cultures. Furthermore, dibutyryl cyclic GMP blocked surface structure formation without affecting the rate of growth of mid-log phase

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cultures using mannose as carbon source (Kurn and Shapiro, unpublished). Schmidt and Samuelson (4) observed that cyclic GMP stimulated stalk elongation in mutants of C. crescentus that form abnormally long stalks, and that both cyclic AMP and dibutyryl cyclic AMP inhibited this effect of cyclic GMP. In view of the cited studies which suggest that cyclic AMP and cyclic GMP are involved in regulating the growth and development of C. crescentus, and the presence of both cyclic AMP (3) and cyclic GMP (Kurn and Shapiro, unpublished) in this bacterium, we have purified and characterized the guanylate cyclase from C. crescentus.

MATERIALS AND METHODS

Materials [α-^{32}P] guanosine 5'-triphosphate (GTP), [γ-^{32}P] GTP and [α-^{32}P] adenosine 5'-triphosphate (ATP) were obtained from the International Chemical and Nuclear Corp., [^{3}H] cyclic GMP and [^{3}H] cyclic AMP were purchased from Schwarz BioResearch. $Na_4{}^{32}P_2O_7$ was a product of New England Nuclear. Creatine phosphate, creatine phosphokinase, $N^2,O^{2'}$-dibutyryl cyclic GMP, $N^6,O^{2'}$-dibutyryl cyclic AMP, nucleoside triphosphates, transferrin (human), beef heart cyclic nucleotide phosphodiesterase and chromatographic Alumina (neutral) were products of Sigma Chemical Co. Fructose 1,6-diphosphatase and alcohol dehydrogenase (yeast) were from Worthington Biochemical Corp. and bovine serum albumin was from Miles Laboratories, Inc.

The cyclic AMP-specific phosphodiesterase purified from Blastocladiella emersonii was a generous gift from Dr. Philip M. Silverman.

Assay of Guanylate Cyclase Enzyme activity was determined by a modification of the method described by White et al (5). The incubation mixture contained 30mM Tris-HCl buffer, pH 7.6, 15mM $MnCl_2$, 3.6mM [α-^{32}P] GTP (20-40 cpm/pmole), 8mM cyclic GMP, 10mM mercaptoethanol, 3.7mM creatine phosphate, 15μg creatine phosphokinase and enzyme in a final volume of 100μl. 2-mercaptoethanol and a GTP regenerating system were required for optimal activity (see Table 4). The reaction was carried out for 15 min at 37° and then stopped by freezing in a dry ice-acetone bath, followed by boiling for 3 min. After cooling on

ice, 0.8 ml of 2mM sodium pyrophosphate, pH 7.6, containing 80,000 cpm of cyclic [^{3}H] GMP was added. The tubes were mixed vigorously, centrifuged and each supernatant was decanted onto a column (8 x 110mm) containing approximately 3 gm of neutral alumina oxide which had been equilibrated with 0.05M Tris-HCL buffer, pH 7.6. One ml of each 3 ml fraction was added to 9 ml of Triton-toluene scintillation fluid (3 parts Triton, 7 parts toluene and 4 gm/liter Omnifluor). The amount of ^{3}H and ^{32}P in the aliquot was measured and the amount of cyclic [^{32}P] GMP formed in each assay was corrected for recovery of cyclic [^{3}H] GMP (about 60%). Purified cyclic GMP was generally eluted between 15 and 18 ml.

Assay of Adenylate Cyclase Activity was measured (a) under the conditions described above for guanylate cyclase, except that 3.6mM [α-^{32}P] ATP (50 cpm/pmole) and 8mM cyclic AMP were used instead of GTP and cyclic GMP, or (b) in a reaction mixture containing 2mM dithiothreitol, 3.6mM [α-^{32}P] ATP (50 cpm/pmole), 8mM cyclic AMP, 3.7mM creatine phosphate, 15ug creatine phosphokinase and enzyme in a total volume of 50ul. In the second assay the reaction was terminated as described for the guanylate cyclase assay except that after boiling and cooling, 50ul each of $ZnSO_4$ (0.25mM) and $Ba(OH)_2$ (0.25mM) were added and the resultant precipitate was removed by centrifugation. Cyclic [^{32}P] AMP was isolated from the supernatant fluid by paper chromatography as described for the cyclic nucleotide phosphodiesterase assay in the legend to Table 2.

RESULTS

Purification of Guanylate Cyclase C. crescentus CB13 was grown to mid-log phase in nutrient broth (1) and harvested by centrifugation. Cells, 60 gm wet weight, were ground with alumina (1:2, w/w) and resuspended in a solution containing 20mM Tris-HCl, pH 7.6, 7mM mercaptoethanol, 3mM $MnCl_2$, 5mM $MgSO_4$, 0.05mM EDTA and 20% glycerol (buffer G). After centrifugation for 15 min at 10,000 rpm in a Sorval GSA rotor, the supernatant (step 1, Table 1) was

Table 1. Purification of Guanylate Cyclase from C. crescentus

Step	Fraction	Volume (ml)	Protein (mg)	Units (pmoles/min)	Specific activity (units/mg)	Recovery (%)
I	Extract	120	6,240	118,560	19	100
II	100,000 x g supernatant fluid	100	3,000	96,000	32	81
III	Ammonium sulfate fraction	35	980	64,680	66	55
IV	DEAE-cellulose eluate	82	87	17,574	202	15
V	G-200 gel filtration eluate	11	27	27,720	1,026	23

One unit of guanylate cyclase activity is defined as that amount of enzyme required to catalyze the formation of 1 pmole of cyclic GMP in 1 min at 37°. The specific activity is the number of units per milligram of protein; protein was measured by the method of Lowry et al (13).

treated with DNase (10µg/ml) for 20 min at 4°, followed by centrifugation for 90 min at 30,000 rpm (step II). The supernatant fluid was slowly brought to 25% saturation with solid ammonium sulfate. The precipitate was discarded and sufficient ammonium sulfate was added to the supernatant to bring the solution to 50% saturation. The resulting precipitate was then collected by centrifugation, dissolved in 15 ml of buffer G, dialyzed overnight against two changes of buffer G (step III) and applied to a DEAE-cellulose column (1.7 x 30 cm) pre-equilibrated with the same buffer. After the column was washed with 150 ml of buffer, a 400 ml linear gradient of NaCl (0.02-0.25M) in buffer G was applied. The flow rate was 10 ml/hr and fractions of 4 ml were collected. The eluate (~0.08M) containing enzymic activity was concentrated by precipitation with ammonium sulfate (60% saturation) and then applied, in 3 ml of buffer G (step IV), to a Sephadex G-200 column (Fig. 1).

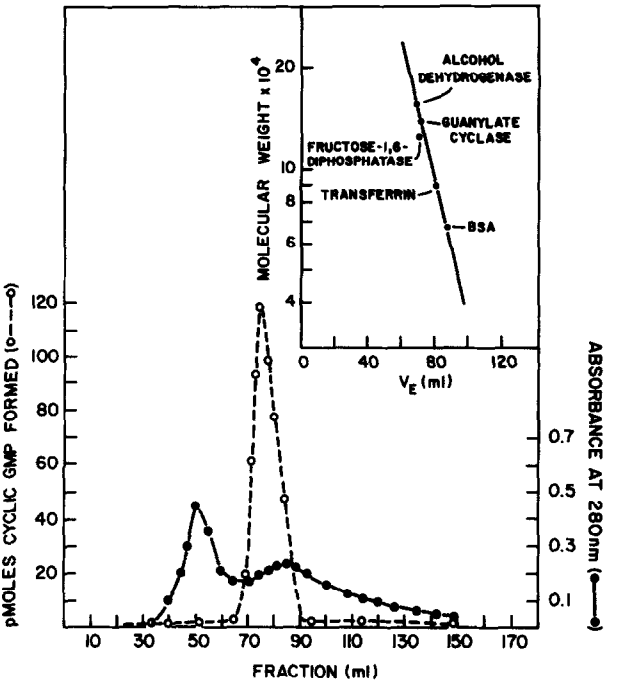

Fig. 1. Elution of guanylate cyclase from a calibrated column of Sephadex G-200 (1.7 x 46 cm). The Sephadex G-200 column was calibrated with proteins of known molecular weight (see Methods). Alcohol dehydrogenase and fructose 1,6-diphosphatase activities were assayed according to Vallee and Hoch (16) and Pontremoli (17), respectively. V_E, elution volume.

Fractions containing guanylate cyclase activity were pooled and dialyzed against buffer G containing 60% glycerol (step V). The concentrated eluate (2 mg/ml) could be stored at -70° for a month without loss of activity. A summary of the enzyme purification appears in Table 1. The final purification was about 60-fold with a recovery of 23%. All of the studies to be reported in this communication were performed with the most purified fraction (step V, Table 1).

Gel filtration (step V, Table 1) yielded an enzyme preparation low in cyclic nucleotide phosphodiesterase but containing GTPase and pyrophosphatase activities (Table 2). We could not detect any adenylate cyclase activity in the purified preparation of guanylate cyclase using either of the assays outlined in Methods. The guanylate cyclase eluted from the Sephadex column in

Table 2. Other Enzyme Activities in Guanylate Cyclase Preparations

Fraction	GTPase	Inorganic pyrophosphatase	Cyclic GMP phosphodiesterase	Cyclic AMP phosphodiesterase
	(nmoles/mg/min)		(pmoles/mg/min)	
I Extract	0.06	--	0.01	0.46
II 100,000 x g supernatant fluid	1.0	--	0.15	0.56
III Ammonium sulfate fraction	0.84	--	0.03	0.09
IV DEAE-cellulose eluate	1.5	120.8	0.09	0.09
V G-200 gel filtration fraction	0.06	6.4	0.09	0.09

Activities of GTPase and pyrophophatase were determined by measuring the release of inorganic phosphate from GTP and PP_i, respectively. The reaction mixtures contained either 0.1mM [γ-^{32}P]GTP (48 cpm/pmole), or 0.5mM $Na_4{}^{32}P_2O_7$ (660 cpm/nmole) and 0.01M $MgSO_4$, 2.5mM DTT, 0.015M Tris-HCl pH 7.4, in a total volume of 200ul. The GTPase assays were incubated for 5 min at 37° and the pyrophosphatase assays were incubated for 10 min at 37°. P_i (0.5mM) was added as carrier and the released inorganic phosphate was measured using the solvent extraction method of Martin and Doty (14). Phosphodiesterase activity was measured as described previously (15). The reaction mixture contained 1.5uM cyclic GMP (1,436 cpm/pmole) or 1.5uM cyclic AMP (3,458 cpm/pmole), 10mM Tris-HCl pH 8.0, 5mM $MgSO_4$, 7.5mM mercaptoethanol in a total volume of 100ul. The incubations were carried out at 30° for 40 min and were stopped by boiling in a water bath for 2 min. An aliquot was applied to Whatman No. 1 paper. Nucleotides were separated by descending chromatography for 16 hr in ammonium acetate/ethanol (30:70). The area containing cyclic GMP or cyclic AMP, 5'-GMP or 5'-AMP, guanosine or adenosine were detected by UV adsorption, cut out and counted in the Triton-toluene scintillation fluid.

a volume consistent with a molecular weight of approximately 140,000 (Fig. 1).

Identification of Cyclic GMP The radioactive product of the guanylate cyclase reaction was eluted from alumina and further identified by co-chromatography with marker cyclic GMP in two solvent systems (ammonium acetate/ethanol 30:70 and isopropanol/concentrated NH_4OH/0.1M H_3BO_3 60:10:30). Treatment of the product with beef heart cyclic nucleotide phosphodiesterase resulted in the quantitative appearance of 5'-[^{32}P] GMP, whereas treatment with a cyclic AMP-specific

Table 3. Identification of the Product of the Guanylate Cyclase Reaction

Treatment	Cyclic [^{32}P] GMP (cpm)	5'-[^{32}P] GMP (cpm)
None	177	44
Phosphodiesterase (bovine heart)	29	203
Phosphodiesterase *Blastocladiella emersonii*	204	48

Standard guanylate cyclase reactions (see Methods) were carried out with 100µg of enzyme and then applied to alumina oxide columns as described in Methods. The eluate (500µl) containing the ^{32}P-labeled product was incubated with 7mM $MgSO_4$ and 20µg of beef heart cyclic nucleotide phosphodiesterase (0.2-0.5 units/mg) for 30 min at 30°. One unit of enzyme converted 1µmole of the 3',5'-cyclic nucleotide to the 5'-mononucleotide per min at 30°. The reaction was terminated by boiling, the precipitate removed by centrifugation, and 250µl of the supernatant fluid applied to paper and chromatographed as described in the legend to Table 2. Areas corresponding to carrier cyclic GMP and 5'-GMP were identified by their adsorption of UV light, cut out and their radioactivity determined. One ml of the same eluate from alumina oxide was concentrated by evaporation to 0.5 ml and incubated with 100µg of *B. emersonii* phosphodiesterase (15-20 units/mg) for 30 min at 37°. One unit is defined as that amount of enzyme required to convert 1 nmole of cyclic AMP to 5'-AMP per min at 37°. The reaction was terminated and and processed as described above. The recovery of ^{32}P after chromatography was 95% of the radioactivity applied. Under the conditions described, 80% of added standard cyclic [^{3}H] GMP (2µM) was hydrolyzed by the bovine heart diesterase and <0.1% by the diesterase from *B. emersonii*. The enzyme from *B. emersonii* completely hydrolyzed cyclic [^{3}H] AMP (2µM) under identical conditions.

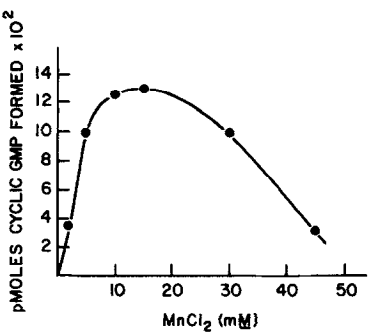

Fig. 2. Effect of Mn^{2+} on the synthesis of cyclic GMP. Assays were performed as described in Methods using 100µg of protein in each reaction.

Table 4. Requirements for Cyclic GMP Formation

Reaction mixture	Cyclic GMP formation (pmoles/15 min)
Complete	963
Minus $MnCl_2$	< 9
Minus $MnCl_2$, plus $MgCl_2$ (15mM)	< 9
Plus $MgCl_2$ (15mM)	578
Plus $CaCl_2$ (15mM)	323
Plus $ZnCl_2$ (15mM)	< 9
Plus NaF (2mM)	575
Plus NaF (10mM)	< 9
Plus sodium pyrophosphate (2mM)	338
Plus sodium pyruvate (5mM)	205
Minus GTP regenerating system	462
Minus 2-mercaptoethanol	255

The complete reaction mixture was as described in Methods and contained 100μg of enzyme.

phosphodiesterase isolated from Blastocladiella emersonii (6) did not alter the chromatographic behavior of the reaction product (Table 3).

Characterization of the Reaction Catalyzed by Guanylate Cyclase The rate of the reaction was constant with time for 25 min at 3.6mM GTP and was dependent upon enzyme concentration between 10 and 50ug of protein. Activity was optimal between pH 7.6 and pH 8.3. The enzyme activity was dependent on Mn^{2+} with optimal rates obtained between 10 and 15mM (Fig. 2). Other divalent metal ions, Mg^{2+}, Ca^{2+}, and Zn^{2+} inhibited the reaction when present in concentra-

tions equal to Mn^{2+}(Table 4). The addition of carrier cyclic GMP had no effect upon enzymic activity during any phase of the purification. Fluoride, an activator of the adenylate cyclase of eukaryotic cells (7,8), inhibited the formation of cyclic GMP, as did pyrophosphate and pyruvate, an activator of the adenylate cyclase from Brevibacterium liquifaciens (9). Caffeine (4mM) inhibited activity 40% (data not shown). The nucleoside triphosphates at 3.6mM and $N^6,O^{2'}$-dibutyryl cyclic AMP had little effect on cyclic GMP formation whereas $N^2,O^{2'}$-dibutyryl cyclic GMP stimulated the cyclic GMP formation (Table 5). Maximal stimulation (2-3 fold) occurred when $N^2,O^{2'}$-dibutyryl cyclic GMP was added at concentrations equal to GTP. The stimulatory effect of

Table 5. Effect of Nucleotides on Cyclic GMP Formation

	Addition	Concentration (mM)	Cyclic GMP formation (pmoles/15 min)
A)	None		986
	$N^6,O^{2'}$-dibutyryl cyclic AMP	4.0	1,083
	Cyclic AMP	2.0	1,080
	$N^2,O^{2'}$-dibutyryl cyclic GMP	1.8	868
		2.7	1,300
		3.6	1,786
	Sodium butyrate	2.0	1,115
		4.0	1,153
B)	None		306
	ATP	1.8	224
	$N^2,O^{2'}$-dibutyryl cyclic GMP	1.8	1,074
		3.6	1,070

A) Enzyme assays were performed as described in Methods, using 100µg of enzyme.

B) The amount of [α-^{32}P] GTP in the reaction mixture was decreased from 3.6mM to 1.8mM. The concentration of the other components were as described in Methods.

$N^2,O^{2'}$-dibutyryl cyclic GMP was apparent whether or not carrier cyclic GMP was present during the assay. $N^2,O^{2'}$-dibutyryl cyclic GMP had no stimulatory effect on the crude guanylate cyclase activity (step I and II, Table 1).

DISCUSSION

Cyclic GMP is found in a wide variety of eukaryotic and prokaryotic cells (10). It has been suggested that at least some of the effects of cyclic GMP in eukaryotic cells are antagonistic to those promoted by cyclic AMP (10). Little is known about the biological role of cyclic GMP in bacteria. In Escherichia coli, cyclic GMP inhibits the binding of cyclic AMP to the cyclic AMP-receptor protein thereby antagonizing the cyclic AMP-induced synthesis of β-galactosidase in vitro (11).Even less is known about bacterial guanylate cyclases. Clark et al were able to identify activity in a particulate fraction from Bacillus lichenformis (12), but did not purify it. The guanylate cyclase from Caulobacter crescentus has many properties in common with the enzyme studied in eukaryotes; under our conditions of cell breakage it is soluble (or solubilized), it is not stimulated by F^-, an agent known to activate the adenylate cyclases of eukaryotes, it requires Mn^{2+} for activity, and it does not use ATP as substrate for the cyclization reaction. It is interesting that the dibutyryl derivative of cyclic GMP activates the purified guanylate cyclase. This raises the possibility that the physiological effects of exogenous dibutyryl cyclic GMP on assembly of polar structures may be mediated by the endogenous synthesis of cyclic GMP.

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