JOURNAL OF BACTERIOLOGY, Sept. 1981, p. 931-934
0021-9193/81/090931-04$02.00/0

Vol. 147, No. 3

Guanylate Cyclase Activity in *Escherichia coli* Mutants Defective in Adenylate Cyclase

V. MACCHIA,[1] G. CAPUTO,[1] E. MANDATO,[1] A. ROCINO,[1] SANKAR ADHYA,[2] AND IRA PASTAN[2]*

Centro di Endocrinologia ed Oncologia Sperimentale del CNR, % Istituto di Patologia Generale, II Facoltà di Medicina e Chirurgia, Università degli Studi, Naples, Italy,[1] and Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205[2]

Received 3 March 1981/Accepted 8 June 1981

Guanylate cyclase, which catalyzes the synthesis of guanosine 3',5'-monophosphate, has been assayed in several strains of *Escherichia coli*. They include wild-type cells and mutants defective in adenylate cyclase, which is responsible for the synthesis of adenosine 3',5'-phosphate. Our results demonstrate that adenylate cyclase and guanylate cyclase are two different enzymes in *E. coli* and suggest that the gene that encodes adenylate cyclase also plays a regulatory role in the synthesis of guanylate cyclase.

Cyclic GMP has been found in a wide variety of organisms (7). The nucleotide has been implicated in the intracellular modulation of several biological processes in both eucaryotic and procaryotic cells (3, 5, 6, 10, 11, 17, 18). Cyclic GMP was first detected in *Escherichia coli* cells by Bernlohr et al. (2), who showed that the levels of cyclic GMP tend to change in the opposite directions from cyclic AMP. They suggested that the two nucleotides might function antagonistically. Later, Gonzales and Peterkofsky (8) measured the changes in cyclic AMP and cyclic GMP concentrations in *E. coli* under a wide variety of conditions of growth and observed no consistent pattern in the way the levels of the two nucleotides changed. More recently, Shibuya et al. (19) showed that five independent adenylate cyclase-deficient mutants (*cya*) of *E. coli* accumulated exceedingly low levels of cyclic GMP and that the ability to form both cyclic AMP and cyclic GMP was simultaneously restored by transduction of an intact *cya*$^+$ locus to one of the above *cya* mutants. On the basis of these data, they suggested that the synthesis of both cyclic GMP and cyclic AMP is mediated by the same enzyme, adenylate cyclase.

To gain further information on the role of cyclic GMP and to study whether cyclic GMP and cyclic AMP provide opposite regulatory influences or independently regulate separate sets of cellular functions in *E. coli*, we have been studying the enzymes of cyclic GMP synthesis and degradation. We previously reported (14) the occurrence in *E. coli* of a guanylate cyclase distinct from adenylate cyclase. The guanylate cyclase which was purified about 1,000-fold was specific for GTP and did not catalyze the formation of cyclic AMP from ATP. In this paper, the regulation of cyclic GMP synthesis has been examined by measuring the levels of guanylate cyclase activity in mutants of *E. coli* defective in adenylate cyclase (*cya*).

MATERIALS AND METHODS

Cyclic GMP, GTP, and GDP were purchased from Boehringer Mannheim Corp.; thin-layer plates (Silica Gel F_{254}) and polyethyleneimine cellulose and aluminum oxide for column chromatography were purchased from Merck & Co.; and cyclic [^{3}H]GMP (2.4 Ci/mmol) and [α-^{32}P]GTP (1.3 Ci/mmol) were purchased from Amersham Corp.

The following strains of *E. coli* were used: B, K-12, 1100, and 500. The latter two are derivatives of K-12. 1100 is HfrH *thi* and 500 is F$^-$ *str his relA*. 5336 (*cya-1*) and 1039 (*cya-39*) are two independent adenylate cyclase-deficient mutants of strains 1100 and 500, respectively. 5336R and 2059 are their corresponding *cya*$^+$ revertants.

Preparation of *E. coli* extracts. The *E. coli* cells were grown in Luria broth containing 1% glucose and 0.01 M $MgSO_4$ at 32°C to early stationary phase, collected by centrifugation, washed once with chilled 0.01 M $MgSO_4$, and then frozen. The frozen *E. coli* cells were resuspended in a buffer solution containing 0.05 M Tris-hydrochloride (pH 7.8), 0.01 M $MgCl_2$, 0.01 M dithiothreitol, and 5 μg of DNase per ml and disrupted by passage through a French press at 5,000 lb/in^2. Extracts were centrifuged at 20,000 × *g* for 20 min to remove cell debris, and the supernatant liquid was centrifuged for 1 h at 105,000 × *g*.

Guanylate cyclase activity was measured on the high-speed supernatant fraction and on the pooled sediments from the 20,000 × *g* and 105,000 × *g* centrifugations.

Guanylate cyclase assay. Guanylate cyclase activity was assayed by measuring the conversion of [α-^{32}P]GTP to cyclic [^{32}P]GMP (13). Each reaction tube

(final volume, 0.07 ml) contained 0.1 mM GTP, 0.1 mM $MnCl_2$, 50 mM Tris-hydrochloride (pH 7.6), 3.3 mM creatine phosphate, 12 μg of creatine kinase and [α-^{32}P]GTP (0.5×10^6 cpm).

At the end of the incubation at 37°C, 0.1 ml of a solution containing 3 mM GTP, 1.5 mM cyclic GMP, 0.4 mg of sodium pyrophosphate per ml, 50 mM Tris-hydrochloride (pH 7.6), and 0.01 μCi of cyclic [^{3}H]-GMP was added to stop the reaction. To each tube was added 1 ml of 50 mM Tris-hydrochloride (pH 7.6); the contents were blended in a Vortex mixer, and the tubes were centrifuged for 10 min at $2,500 \times g$. The supernatants were applied to columns of aluminum oxide prepared as previously described (14). The first 3-ml eluate from the column containing the cyclic GMP was collected, mixed with 6 ml of Instagel (Packard Instruments), and counted in a liquid scintillation counter. The cyclic [^{3}H]GMP serves to determine the recovery of cyclic GMP during the procedure; recovery ranged from 80 to 90%. Guanylate cyclase was also measured by a chromatographic method. Aliquots of the reaction mixture were chromatographed on a silica gel plate using isopropyl alcohol-water-ammonium hydroxide (7:2:1) as a solvent. Unlabeled cyclic GMP, GTP, and GDP were used as standards. The spots were located under UV light, scraped from the plates, and counted in a naphthalene dioxane fluor (4).

Protein was determined by the method of Lowry and co-workers (12). The data reported in this paper are based upon assays in which the enzymatic activities were linear with respect to the incubation time and the amount of enzyme present in the reaction mixture.

RESULTS

The total guanylate cyclase activity of extracts from four different strains of *E. coli* was measured and ranged in a typical experiment from 4 to 18 pmol of cGMP synthesized per mg of protein per 10 min. The origin of this variation among strains is unknown. Approximately 90 to 95% of the total guanylate cyclase activity in each of the extracts could be recovered in the soluble fraction (Table 1). In crude extracts of all strains tested, the guanylate cyclase activity was very low. This was due to the significant destruction of GTP by GTP-degrading enzymes. The presence of creatine phosphate and creatine kinase, added to regenerate GTP, improved the guanylate cyclase activity (Table 2). The presence of Mn^{2+} was required to detect guanylate cyclase activity. Cyclic GMP and caffeine were not required in the assay because hydrolysis of cyclic GMP by phosphodiesterase was undetectable in strains 1100 and 500 and very low in strains B and K-12 (9). Fluoride slightly inhibited guanylate cyclase activity.

To examine the relationship between cyclic AMP and cyclic GMP synthesis, the guanylate cyclase activity was measured in two independent adenylate cyclase (*cya*)-deficient mutants, 5336 and 1039. Both soluble and particulate guanylate cyclase activities were fivefold higher in strain 5336 than in the parental wild-type 1100 (Table 3). In the second *cya* mutant, 1039, guanylate cyclase activity was threefold higher than that of the parental wild-type 500. We have isolated *cya*$^+$ revertants of 5336 and 1039 by selecting for Lac$^+$ revertants on MacConkey lactose indicator plates and then screening for a Sor$^+$ Ara$^+$ Mal$^+$ phenotype. The adenylate cyclase-positive characters of some of these revertants were transducible with the *cya*$^+$ gene by bacteriophage P1 at greater than 90% efficiency. One such revertant from each *cya* strain, 5336R from 5336 and 2059 from 1039, was further characterized. The guanylate cyclase activity in 5336R and 2059 was similar to that in the parental wild-type strains 1100 and 500, respectively (Table 3). These data indicate that the *cya*$^+$ gene does not encode guanylate cyclase and that cyclic AMP is not required for the synthesis of guanylate cyclase. These conclusions agree with our previous observation (14) that guanylate cyclase and adenylate cyclase are different enzymes.

Although the assay method employing aluminum oxide has been reported (14) to be specific for cyclic GMP, we also identified the products of the reaction by a chromatographic method. After a 10-min incubation, the reactants

TABLE 1. *Subcellular distribution of guanylate cyclase activity in various strains of E. coli*

Strain	Guanylate cyclase activity (pmol of cyclic GMP accumulated per mg of protein per 10 min)		
	Total extract	Sediment	Supernatant
B	18.0 ± 2.0[a]	1.3 ± 0.1	17.3 ± 2.1
K-12	12.5 ± 1.7	0.9 ± 0.06	11.8 ± 1.8
1100	8.0 ± 1.8	0.6 ± 0.05	8.0 ± 1.7
500	4.0 ± 1.4	0.3 ± 0.04	3.8 ± 1.3

[a] Mean ± standard deviation from six experiments in which each sample was assayed in triplicate.

TABLE 2. *Requirements for cyclic GMP formation*

Reaction mixture	Cyclic GMP formation (pmol/mg of protein per 10 min)			
	B	K-12	1100	500
Tris-hydrochloride + GTP + $MnCl_2$	17	12	8	4
Minus $MnCl_2$	2	1	1	1
Plus GTP regenerating system	26	18	12	9
Plus cGMP and caffeine	26	18	11	9
Plus NaF	21	14	10	8

TABLE 3. *Guanylate cyclase activity in mutants of E. coli*

Strain	Genotype	Guanylate cyclase activity (pmol/mg of protein per 10 min)	
		Supernatant	Sediment
1100	Wild type	11.8 ± 1.8[a]	0.7 ± 0.06
5336	*cya*	58.9 ± 5.1	3.8 ± 0.2
5336R	*cya*$^+$	9.5 ± 1.4	0.6 ± 0.04
500	Wild type	9.1 ± 1.3	0.5 ± 0.03
1039	*cya*	30.6 ± 2.7	1.9 ± 0.13
2059	*cya*$^+$	9.0 ± 1.2	0.6 ± 0.03

[a] Average of six assays ± standard deviation.

and the products were directly separated by thin-layer chromatography. As mentioned before, the amount of guanylate cyclase present in the *cya* mutants was much higher than that formed in the *cya*$^+$ parents and revertants.

DISCUSSION

This paper presents evidence that in *E. coli*, as in mammalian cells, the synthesis of cyclic AMP and cyclic GMP is controlled by two different enzymes. The evidence is based on the observations that in two different adenylate cyclase-deficient mutants, the guanylate cyclase is higher than that in the parental wild-type strains as well as in their *cya*$^+$ revertants. In the latter revertants, the ability to synthesize cyclic AMP was restored and the guanylate cyclase was lowered to normal levels.

Tao and Huberman (21) found that *E. coli* adenylate cyclase could form cyclic GMP from GTP. However, the preparation which they used was only partially purified and could easily have been contaminated with guanylate cyclase. We found that guanylate cyclase that was 1,000-fold purified only formed cyclic GMP from GTP and did not form cyclic AMP from ATP (14). Adenylate cyclase has been purified to homogeneity from *Brevibacterium liquefaciens* and shown to be specific for ATP (20).

Our data also suggest that the guanylate cyclase is subjected to some type of cellular regulation either by adenylate cyclase or by cyclic AMP. The change in the activity of guanylate cyclase in *cya* strains appears to be a change in enzyme levels. The level of several proteins has been found to be elevated in adenylate cyclase or cyclic AMP receptor protein-defective mutants of *E. coli* (1, 15, 16, 22). Included in this group is adenylate cyclase itself, which is elevated in *crp* mutants (15, 22). It is believed that cyclic AMP and cyclic AMP receptor protein play a repressive role in the expression of the corresponding genes. The increased level of guanylate cyclase in *cya* mutants suggests that cyclic AMP may play a similar regulatory role in its synthesis.

Very recently, two important physiological roles have been ascribed to cyclic GMP in *E. coli*. Cook et al. (5) have suggested that cyclic GMP regulates the bacterial cell cycle; Black et al. (3) showed the involvement of cyclic GMP in intracellular signaling in the chemotactic response of *E. coli*. Shibuya et al. (19) reported that *cya* mutants have lower levels of both cyclic AMP and cyclic GMP than do wild-type cells. Inconsistent with this finding is the report of Black et al. (3) that both the intracellular level of cyclic GMP and its fluctuation in response to external stimuli are normal in a *cya* deletion strain. The intracellular levels of cyclic GMP may be modulated at the level of its synthesis or its excretion. Shibuya et al. (19) have shown that cyclic GMP production does not occur continuously but at a definite phase of cell cycles and is rapidly excreted in the medium. Therefore, measurements of cyclic GMP levels at any one time may not represent the level of activity of guanylate cyclase.

LITERATURE CITED

1. **Adhya, S., and W. Miller.** 1979. Modulation of the two promoters of the galactose operon of *Escherichia coli*. Nature (London) **279**:492–494.
2. **Bernlohr, R. W., M. K. Haddox, and N. D. Goldberg.** 1974. Cyclic guanosine 3′:5′-monophosphate in *Escherichia coli* and *Bacillus lichenformis*. J. Biol. Chem. **249**:4329–4331.
3. **Black, R. A., A. C. Hobson, and J. Adler.** 1980. Involvement of cyclic GMP in intracellular signalling in the chemotactic response of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **77**:3879–3883.
4. **Bray, G. A.** 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–281.
5. **Cook, W. R., V. F. Kalb, Jr., A. A. Peace, and R. W. Bernlohr.** 1980. Is cyclic guanosine 3′,5′-monophosphate a cell cycle regulator? J. Bacteriol. **141**:1450–1453.
6. **George, W. J., J. B. Polson, A. G. O'Toole, and N. D. Goldberg.** 1970. Elevation of guanosine 3′,5′-cyclic phosphate in rat heart after perfusion with acetylcholine. Proc. Nat. Acad. Sci. U.S.A. **66**:398–403.
7. **Goldberg, N. D., R. F. O'Dea, and M. K. Haddox.** 1973. Cyclic GMP. Adv. Cyclic Nucleotide Res. **3**:155–223.
8. **Gonzales, J. E., and A. Peterkofsky.** 1975. Diverse directional changes of cGMP relative to cAMP in *E. coli*. Biochem. Biophys. Res. Comm. **67**:190–197.
9. **Gulletta, E., G. Caputo, and V. Macchia.** 1979. Cyclic 3′,5′ adenosine monophosphate phosphodiesterase in mutants of *Escherichia coli* altered in cAMP metabolism. Bull. Mol. Biol. Med. **4**:80–89.
10. **Hadden, J. W., E. M. Hadden, M. K. Haddox, and N. D. Goldberg.** 1972. Guanosine 3′:5′-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. Proc. Nat. Acad. Sci. U.S.A. **69**:3024–3027.
11. **Jimenez de Asua, L., D. Clingan, and P. S. Rudland.** 1975. Initiation of cell proliferation in cultured mouse fibroblasts by prostaglandin $F_{2\alpha}$. Proc. Nat. Acad. Sci.

U.S.A. **72**:2724–2728.

12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
13. **Macchia, V., A. Rocino, E. Gulletta, E. Consiglio, and S. Varrone.** 1978. Guanylate cyclase chez *Escherichia coli*. II. Identification et caractéristiques d'un inhibiteur de l'enzyme. C.R. Soc. Biol. Paris **172**:868–873.
14. **Macchia, V., S. Varrone, M. Weissbach, D. C. Miller, and I. Pastan.** 1975. Guanylate cyclase in *Escherichia coli*. J. Biol. Chem. **250**:6214–6217.
15. **Potter, K., G. Chaloner-Larsson, and H. Yamazaki.** 1974. Abnormally high rate of cyclic AMP excretion from an *Escherichia coli* mutant deficient in cyclic AMP receptor protein. Biochem. Biophys. Res. Comm. **57**:379–385.
16. **Prusiner, S., R. E. Miller, and R. C. Valentine.** 1972. Adenosine 3′:5′-cyclic monophosphate control of the enzymes of glutamine metabolism in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **69**:2922–2926.
17. **Rudland, P. S., D. Gospodarowicz, and W. E. Seifert.** 1974. Activation of guanyl cyclase and intracellular cyclic GMP by fibroblast growth factor. Nature (London) **250**:741–773.
18. **Seifert, W. E., and P. S. Rudland.** 1974. Possible involvement of cyclic GMP in growth control of cultured mouse cells. Nature (London) **248**:138–140.
19. **Shibuya, M., Y. Takebe, and Y. Kaziro.** 1977. A possible involvement of cya gene in the synthesis of cyclic guanosine 3′:5′-monophosphate in E. coli. Cell **12**:521–528.
20. **Takai, K., Y. Kurashina, C. Suzuki-Hori, H. Okamoto, and O. Hayaishi.** 1974. Adenylate cyclase from *Brevibacterium liquefaciens*. J. Biol. Chem. **249**:1965–1972.
21. **Tao, M., and A. Huberman.** 1970. Some properties of *Escherichia coli* adenylate cyclase. Arch. Biochem. Biophys. **141**:236–240.
22. **Wayne, P. K., and O. M. Rosen.** 1974. Cyclic 3′:5′-adenosine monophosphate in *Escherichia coli* during transient and catabolite repression. Proc. Nat. Acad. Sci. U.S.A. **71**:1436–1440.