The Escherichia coli adenylyl cyclase complex: Stimulation by GTP and other nucleotides

ARTICLE *in* PROTEIN SCIENCE · APRIL 2008

Impact Factor: 2.85 · DOI: 10.1002/pro.5560020402 · Source: PubMed

2 AUTHORS, INCLUDING:

Alan Peterkofsky
National Institutes of Health
168 PUBLICATIONS **4,865** CITATIONS

SEE PROFILE

Protein Science (1993), *2*, 498-505. Cambridge University Press. Printed in the USA.

The *Escherichia coli* adenylyl cyclase complex: Stimulation by GTP and other nucleotides

ALAN PETERKOFSKY AND NATAN GOLLOP

Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, Bethesda, Maryland 20892

(RECEIVED October 13, 1992; REVISED MANUSCRIPT RECEIVED December 17, 1992)

Abstract

Escherichia coli cells permeabilized by treatment with low concentrations of toluene contain an adenylyl cyclase activity that can be stimulated 3.6–7.6-fold by GTP. The stimulatory effect of GTP is maximal at concentrations of the nucleotide in the physiological range (above 0.7 mM). Studies of the dependence of velocity on substrate (ATP) concentration indicate that the velocity vs. substrate plots are sigmoid in the absence of GTP but hyperbolic in the presence of GTP, suggesting an allosteric regulatory site that can be occupied by either ATP or GTP. Replacement of ATP by AMPPNP as substrate results in velocity vs. substrate plots that are hyperbolic in the absence or presence of GTP, although GTP increases the V_{max} by a factor of 2.2; these findings indicate that AMPPNP specifically occupies the substrate site and GTP exclusively occupies the regulatory site. A test of the capacity of other guanine nucleotides to stimulate adenylyl cyclase activity showed that 2′-deoxy-GTP was almost as effective as GTP, but that GDP, GMP, ppGpp, and 3′,5′-cGMP were not stimulatory effectors; GTP-γ-S and GMPPNP stimulated adenylyl cyclase activity but to a lesser degree than did GTP. In addition to the previous indication that ATP can occupy the regulatory site on adenylyl cyclase, it was found that CTP and UTP were potent stimulators. Thus, all the naturally occurring RNA precursor nucleoside triphosphates are capable of stimulating adenylyl cyclase activity. In contrast, PPPi inhibits adenylyl cyclase activity. Additional studies showed that there is only one regulatory site for all the nucleotide stimulators and that the nucleotide occupying the substrate site can influence the properties of the regulatory site. These observations suggest a mechanism by which adenylyl cyclase activity can be regulated by the availability of nucleic acid precursors.

Keywords: allosteric regulation; AMPPNP; GTP; nucleotides; signal transduction

GTP activation is the signature of all eukaryotic adenylyl cyclases. Hormone-dependent activation of adenylyl cyclase is mediated by the binding of GTP to a heterotrimeric regulatory protein (G protein) (Gilman, 1987, 1989). In general, the binding of GTP to the protein promotes a response (stimulatory via G_s and inhibitory via G_i), and the subsequent hydrolysis of GTP terminates the response. In the case of *Escherichia coli*, an analogy has been drawn for the regulation of adenylyl cyclase activity to that of a hormone-dependent system (Harwood & Peterkofsky, 1976). Extracellular glucose or other sugar substrates of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) provoke an inhibitory response on the enzyme activity (Harwood & Peterkofsky, 1975). A major low molecular weight effector of this response is inorganic orthophosphate (Liberman et al., 1985). The state of phosphorylation of PTS proteins is also involved in the activity regulation (Peterkofsky, 1977; Peterkofsky et al., 1989).

With a view to evaluating the possibility that the adenylyl cyclase of *E. coli* might be similar to the enzyme in eukaryotic cells in eliciting a response to GTP, Stein et al. (1985) tested the effect of some guanine nucleotides on the activity of the enzyme. They reported a stimulation by GTP that averaged about 37%. In the present study, these observations have been substantially extended and characterized. Under the appropriate incubation conditions, activity stimulations by GTP have been in the range of 3.6–7.6-fold. It is established here that there is a unique binding site on the adenylyl cyclase complex that can respond to all four normally occurring ribonucleoside triphosphates and that the interaction of the complex with a nucleotide modulator explains the allosteric kinetics characteristic of the physiologic complex of *E. coli* adenylyl cyclase with the variety of factors that regulate its activity (Liberman et al., 1985; Peterkofsky et al., 1989).

Reprint requests to: Alan Peterkofsky, National Institutes of Health, Building 36, Room 4C-11, Bethesda, Maryland 20892.

Results

Stimulation of adenylyl cyclase activity by GTP

It was previously demonstrated (Stein et al., 1985) that GTP stimulated adenylyl cyclase activity in permeabilized cells of *E. coli*; the degree of stimulation was cited to be an average of 37%. As a result of decreasing the substrate concentration in the adenylyl cyclase assay, we have been able to substantially increase the stimulatory effect of GTP (see Fig. 1). When the substrate (ATP) concentration in the assay was 0.1 mM, the addition of 1 mM GTP resulted in a stimulation of the activity by 7.6-fold. In other experiments, the stimulation of activity has ranged from 3.6- to 7.6-fold.

The concentration dependence for stimulation of adenylyl cyclase activity by GTP was studied (Fig. 2). The curve shows that concentrations of GTP greater than 0.7 mM did not further increase the stimulation. The concentration of GTP that gives half-maximal stimulation of adenylyl cyclase activity is 0.06 mM. No inhibition of the adenylyl cyclase activity could be detected at 2 mM GTP, a concentration that is 20 times greater than that of the substrate, indicating that GTP does not compete with ATP at the active site of the enzyme in these permeabilized cell preparations. As pointed out in the Discussion, this behavior is quite different from that observed with cell extracts or purified preparations of the enzyme.

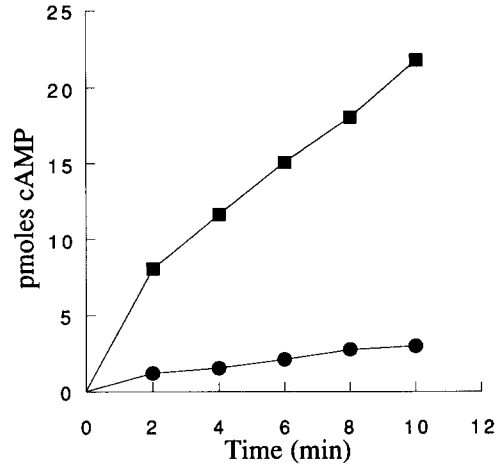

Fig. 1. Effect of GTP on adenylyl cyclase activity in toluene-treated cells of *E. coli*. A culture of strain 647, grown under standard conditions, was washed and resuspended in 100 mM potassium phosphate buffer, pH 8.5, and then treated with toluene (see Materials and methods). Reaction mixtures (1.5 mL) for measurement of adenylyl cyclase activity (ATP concentration was 0.1 mM) were set up and were either deficient in (●) or supplemented with (■) 1 mM GTP. At the indicated times, aliquots (0.1 mL) were removed for the determination of the amount of [^{32}P]cAMP formed. Each 0.1-mL aliquot of reaction mixture contained 0.044 mg of protein.

GTP alters the kinetic properties of adenylyl cyclase

In agreement with previous results (Liberman et al., 1985), the velocity vs. substrate concentration curve for adenylyl cyclase activity measured in permeabilized cells is sigmoid (Fig. 3, filled circles). This kinetic behavior may indicate an allosteric site on the enzyme complex. It is noteworthy that, when the reaction mixtures are supplemented with 1 mM GTP, the velocity vs. substrate concentration curve is hyperbolic (Fig. 3, filled squares), suggesting that GTP can occupy a regulatory site on the enzyme complex to desensitize the enzyme.

When AMPPNP was substituted for ATP as the adenylyl cyclase substrate, the maximum basal activity was approximately 25 times lower. Most importantly, the kinetic plot shown in Figure 4 (filled circles) indicates that the velocity vs. substrate concentration curve is hyperbolic. The interpretation of the observation that the velocity vs. substrate concentration curve is sigmoid with ATP (Fig. 3) but hyperbolic with AMPPNP (Fig. 4) is described in the Discussion in the framework of the argument that ATP can serve as both a substrate and an activator, while AMPPNP can serve only as a substrate. Consistent with the expectation that GTP exclusively occupies the regulatory site (see previous discussion relating to Fig. 2), the velocity vs. substrate concentration experiment carried out in the presence of 1 mM GTP also produces a hyperbolic plot (Fig. 4, filled squares). In this case, the addition of GTP decreases the K_m for AMPPNP (from 0.58 to 0.31 mM) but increases the V_{max} from 1.42 to 3.12 nmol/mg/h.

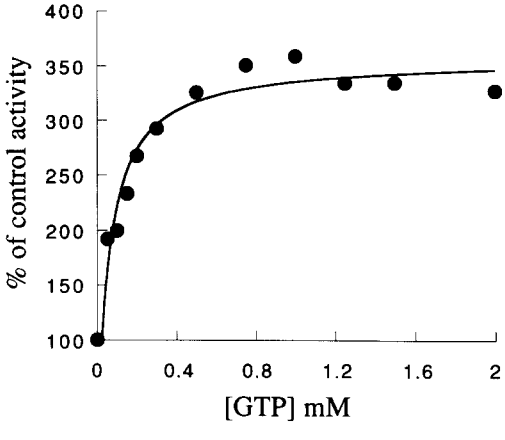

Fig. 2. Effect of GTP concentration on adenylyl cyclase activity in toluene-treated cells of *E. coli*. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity in the presence of the indicated concentrations of GTP. The ATP concentration was 0.1 mM. The specific activity in the absence of added GTP was 2,142 pmol cAMP formed/mg protein/h. In the presence of 1 mM GTP, the specific activity was increased to 7,678 pmol cAMP formed/mg protein/h. The Enzfitter program was used to calculate V_{max} (354% of control activity) and K_m (0.06 mM). Using these values, the curve was fit to the Michaelis-Menten equation and plotted with the Kaleidagraph program.

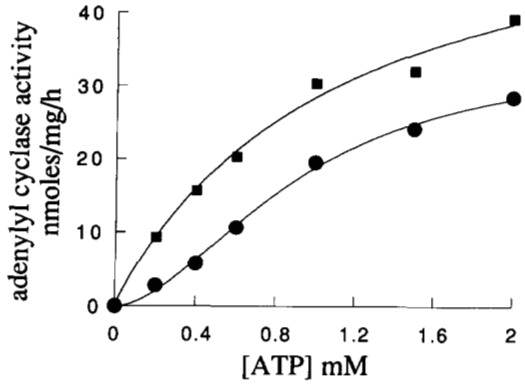

Fig. 3. Effect of GTP on the kinetics of adenylyl cyclase activity using ATP as substrate. Toluene-treated cells of strain 647 were tested for adenylyl cyclase activity at the indicated concentrations of ATP. ●, GTP absent; ■, GTP (1 mM) present. The values of V, K, and n were calculated using the Enzfitter program. Using these values (absence of GTP: V_{max} = 35.1, K_m = 0.86, Hill coefficient = 1.84; presence of GTP: V_{max} = 59.5, K_m = 1.10), the curves were fit to the Michaelis-Menten equation for the data generated in the presence of GTP and to the Hill equation for the data generated in the absence of GTP using the Kaleidagraph program.

Nucleotide specificity for stimulation of adenylyl cyclase activity

A variety of nucleotides (at a concentration of 1 mM) were tested for their capacity to effect a stimulation of adenylyl cyclase activity (Fig. 5). Whereas GTP promoted a fivefold stimulation of the activity, GDP and GMP did not stimulate. In contrast, 2′-deoxy-GTP (383% of control activity) was almost as effective as GTP (489% of control activity). GTP-γ-S and GMPPNP are nonhydrolyzable analogs of GTP. The activity in the presence of 1 mM GTP-γ-S was double that of the control and that in the presence of 1 mM GMPPNP was 126% that of the control. These data suggest that stimulation of adenylyl cyclase activity does not require a transfer of the γ-phosphate of the nucleoside triphosphate, but that the nonhydrolyzable analogs are not as effective as is GTP. The nucleoside tetraphosphate, ppGpp, and the cyclic nucleotide, 3′,5′-cGMP, were both incapable of substituting for GTP in the stimulatory activity. Surprisingly, CTP (394% of control activity) and UTP (338% of control activity) were also very effective in promoting the stimulation of adenylyl cyclase activity. These data indicate that the requirement for stimulation of adenylyl cyclase activity is the addition of any ribo- or deoxyribonucleoside triphosphate.

The data of Figure 5 indicated that a variety of nucleoside triphosphates stimulated the enzyme activity but that nucleoside diphosphates were inactive. The question therefore arose whether the triphosphate portion of the nucleotide was sufficient by itself to promote the stimulatory effect. Inorganic tripolyphosphate (PPPi), as well as inorganic pyrophosphate (PPi) as a control, was tested as a possible stimulator of adenylyl cyclase activity. The data shown in Figure 6A indicate that PPPi completely inhibits adenylyl cyclase activity at a 1 mM concentration, a concentration at which GTP shows significant stimulation of activity (see Fig. 1). Since PPi shows only a low level of inhibition under these conditions, it appears that the effect of PPPi is quite specific. The experiment shown in Figure 6B indicates that the inhibition profile produced

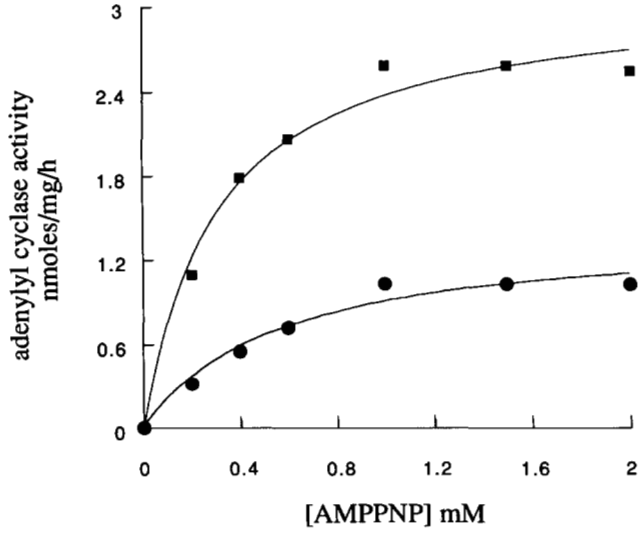

Fig. 4. Effect of GTP on the kinetics of adenylyl cyclase activity using AMPPNP as substrate. Toluene-treated cells of strain 647 were tested for adenylyl cyclase activity at the indicated concentrations of AMPPNP. ●, GTP absent; ■, GTP present. The curves were fit to the Michaelis-Menten equation as described in the legend to Figure 3.

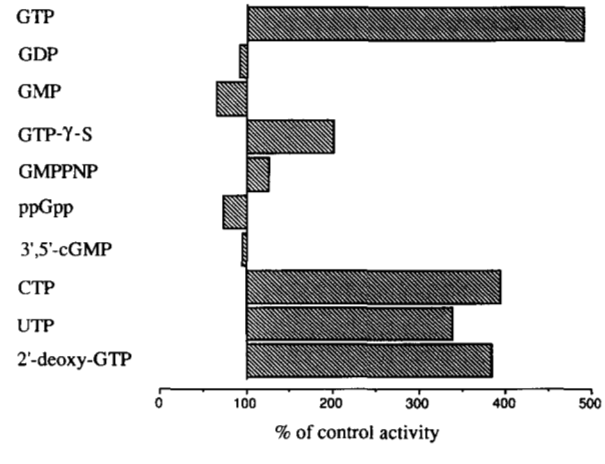

Fig. 5. Nucleotide specificity for stimulation of adenylyl cyclase activity. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity using 0.1 mM ATP as substrate. The indicated effectors were included in reaction mixtures at a final concentration of 1 mM. The specific activity in the absence of added effectors was 710 pmol cAMP formed/mg protein/h.

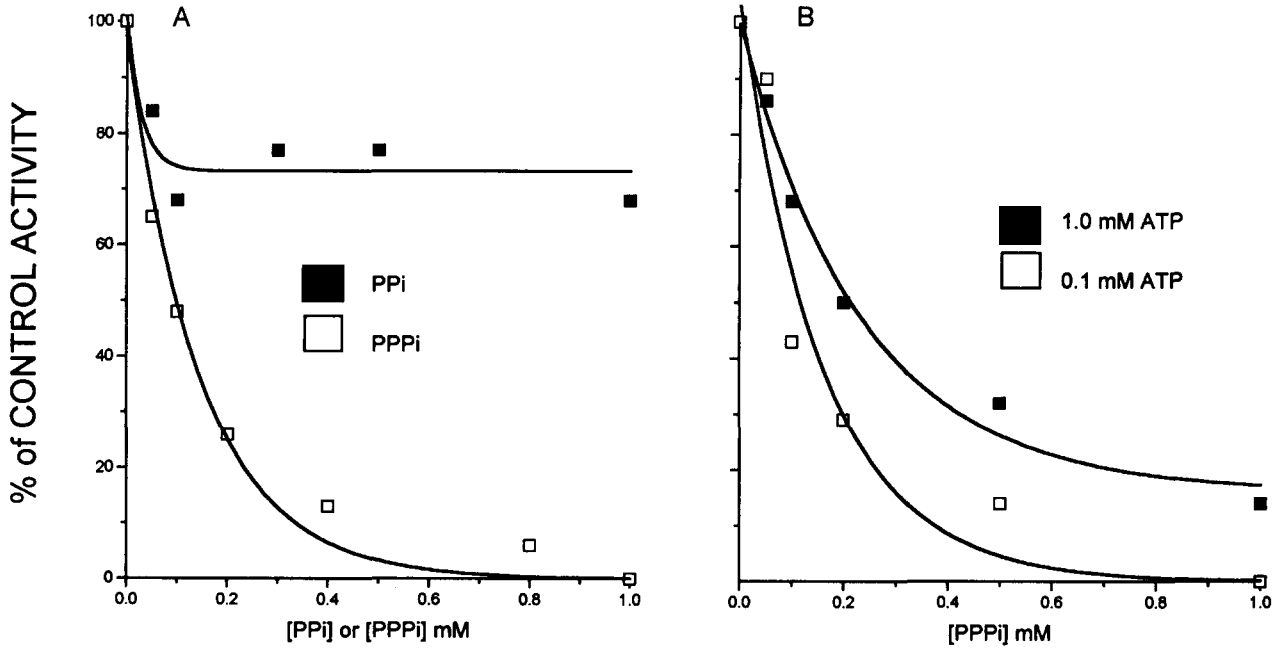

Fig. 6. Inhibition of adenylyl cyclase activity by tripolyphosphate. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity using 0.1 mM ATP as substrate in panel A and either 0.1 mM or 1 mM ATP as substrate, as indicated, in panel B. Where indicated, reaction mixtures were supplemented with the specified concentrations of either inorganic tripolyphosphate or inorganic pyrophosphate. The data were fit to an exponential decay curve using Origin software (MicroCal, Inc., Northampton, Massachusetts). **A:** Specific activity in the absence of added effectors was 861 pmol cAMP formed/mg protein/h. **B:** Specific activities in the absence of added inorganic tripolyphosphate were 913 pmol cAMP formed/mg protein/h (0.1 mM ATP) and 9,565 pmol cAMP formed/mg protein/h (1 mM ATP).

by PPPi is inversely proportional to the concentration of ATP in incubation mixtures. Because ATP may occupy both the catalytic and regulatory site of the adenylyl cyclase complex, it is impossible to conclude from these data whether the site of action of PPPi is at one or both of these sites. In any event, because PPPi does not promote activity stimulation, that due to nucleotides such as GTP must involve the base and/or sugar moieties in addition to the tripolyphosphate portion of the molecules.

To determine whether the stimulation of adenylyl cyclase activity by nucleotides was additive (Fig. 7), the effect of nucleotide combinations was evaluated. The activity in the presence of 2 mM GTP was 567% of the control and that in the presence of 2 mM CTP was 300% of the control. When both GTP and CTP were included in the reaction mixtures, the activity was intermediate (467% of the control) between the individual stimulations rather than showing an additive stimulation. These data are consistent with the idea that there is only one site for stimulation of activity by nucleotides. A similar result was obtained with UTP, which gave 283% of the control activity by itself and 350% of the control activity in the presence of GTP. The conclusion from these experiments was that the stimulation of adenylyl cyclase activity by nucleotides is not cumulative.

The data of Figure 5 suggested that GMPPNP and GTP-γ-S supported a low level of stimulation of adenylyl cyclase activity relative to GTP. The effect of these two nucleotides on the stimulation of adenylyl cyclase activity by GTP was further explored (Fig. 8). GMPPNP at a concentration of 0.1 mM resulted in a stimulation of activity of 57%; increasing the concentration to 1 mM led to a smaller stimulation (14% in this experiment and 26% in the experiment of Fig. 5), suggesting that GMPPNP saturated the binding site at a concentration of 0.1 mM, resulting in a modest stimulation of activity. The concentration dependence for stimulation by GTP was different

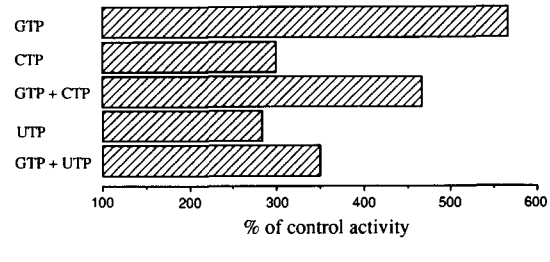

Fig. 7. The stimulation of adenylyl cyclase activity by nucleoside triphosphates is not cumulative. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity with 0.1 mM ATP as substrate. Where indicated, the specified nucleoside triphosphates were added to final concentrations of 2 mM. The specific activity in the absence of added nucleoside triphosphates was 508 pmol cAMP formed/mg protein/h.

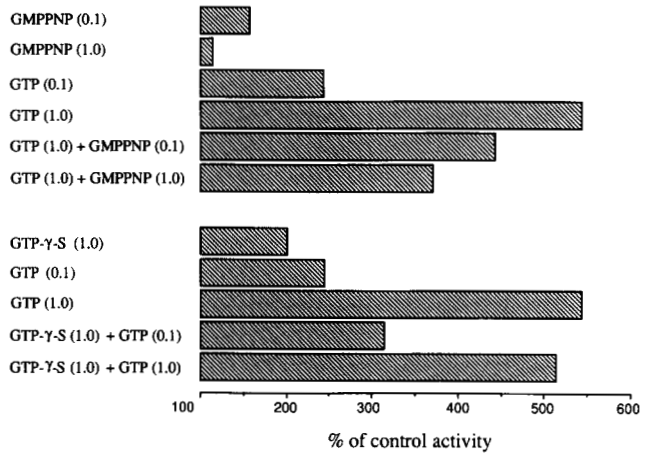

Fig. 8. Effect of GMPPNP and GTP-γ-S on the GTP stimulation of adenylyl cyclase activity. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity using 0.1 mM ATP as substrate. Where indicated, the effectors GTP, GMPPNP, or GTP-γ-S were added alone or in combination at the concentrations designated in parentheses. The control specific activity was 493 pmol cAMP formed/mg protein/h.

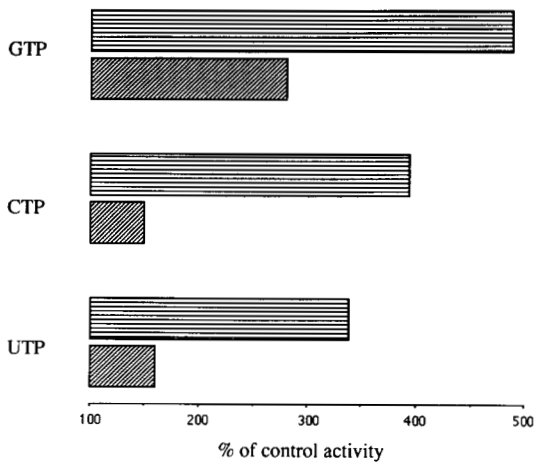

Fig. 9. Specificity for nucleotides for stimulation of adenylyl cyclase activity using ATP or AMPPNP as substrates. Toluene-treated cells of strain 647 were assayed for adenylyl cyclase activity using either 0.1 mM ATP (bars shaded with horizontal stripes) or 0.1 mM AMPPNP (bars shaded with cross-hatching) as substrates. Where indicated, the effectors GTP, CTP, or UTP were included in assays at a final concentration of 1 mM. The specific activities in the absence of added effectors were (pmol cAMP formed/mg protein/h): 710, ATP as substrate; 154, AMPPNP as substrate.

from that of GMPPNP in two ways: 0.1 mM GTP resulted in a 143% stimulation of activity, and 1 mM GTP gave a 443% stimulation. Therefore, the affinity of GTP for the binding site appears to be lower than that of GMPPNP, but the degree of activation is higher. When assays were carried out with a mixture of GTP (at 1 mM) and GMPPNP (at either 0.1 or 1 mM), the degree of stimulation was less than that observed with GTP alone (443% of the control activity in the presence of GTP and 0.1 mM GMPPNP; 371% of the control activity in the presence of GTP and 1 mM GMPPNP).

GTP-γ-S (1 mM) addition resulted in a 100% stimulation of the activity compared to a 443% stimulation by a comparable concentration of GTP. A mixture of 1 mM GTP-γ-S and 0.1 mM GTP resulted in an almost additive stimulation (214% observed, while complete additivity should give 243% stimulation). A mixture of 1 mM of both nucleotides resulted in a 414% stimulation compared to a stimulation of 443% by GTP alone and 100% by GTP-γ-S alone. A reasonable interpretation of these results is that GTP-γ-S binds to the same site as GTP but that it stimulates adenylyl cyclase activity not as well as does GTP and does not compete effectively with GTP for binding to the allosteric site.

The specificity for nucleotide stimulation documented in Figure 5 used ATP as the adenylyl cyclase substrate. Because the data of Figure 4 suggested that AMPPNP was an alternate substrate that produced kinetic curves different from ATP, a comparison of the nucleotide specificity using ATP and AMPPNP was made (Fig. 9). When ATP was the substrate, the stimulation by CTP was 76% as great as with GTP; in contrast, when AMPPNP was the substrate, the stimulation by CTP was only 28% as great as with GTP. A similar difference was observed with UTP. When ATP was the substrate, the stimulation by UTP was 61% as great as with GTP; when AMPPNP was the substrate, the stimulation with UTP was only 33% as great as with GTP.

Potentiation by inorganic orthophosphate of the stimulation of adenylyl cyclase activity by GTP

It has previously been demonstrated (Harwood & Peterkofsky, 1975) that the activity of adenylyl cyclase in permeable cell preparations is substantially stimulated by the addition of concentrations of Pi in the range of 15–30 mM; these are typical cellular concentrations of this ion. An examination was carried out of a possible interrelationship between the effect of Pi and the newly observed effect of GTP on adenylyl cyclase activity. The results of this study, documented in Figure 10, indicate a physiological connection between these two factors. It can be seen that, at low ATP concentrations (0.1 mM), the stimulation of adenylyl cyclase activity by Pi requires the presence of GTP. When the concentration of ATP is higher (1 mM), the stimulation of adenylyl cyclase activity by Pi is significantly enhanced by the presence of GTP (Pi supplemented incubations result in 184% of control activity in the absence of GTP and 500% of control activity in the presence of GTP). An additional aspect to the data is that, in the presence of Pi, stimulation of adenylyl cyclase activity by GTP is relatively greater at the lower than at the higher ATP concentrations.

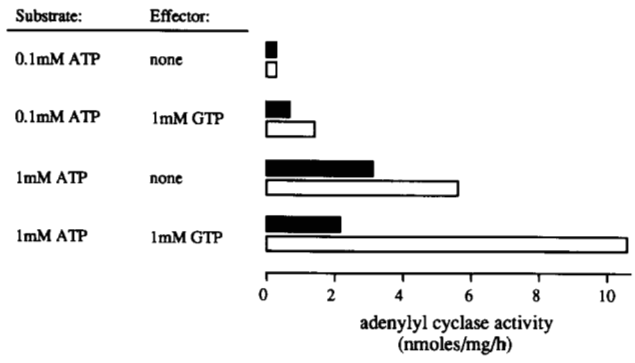

Fig. 10. Potentiation by inorganic orthophosphate of the stimulation of adenylyl cyclase activity by GTP. Toluene-treated cells of strain 647 were tested for adenylyl cyclase activity at substrate concentrations of either 0.1 mM or 1 mM ATP with or without 1 mM GTP, as indicated. Shaded bars correspond to incubations without added Pi and open bars to incubations supplemented with Pi (20 mM; added as potassium phosphate buffer, pH 8.5).

Discussion

In typical eukaryotic adenylyl cyclases, hormonal activation signals are mediated by GTP (Gilman, 1987). It is noteworthy that the concentration range in which GTP is effective is micromolar. For example, thyroid membranes (Wolff & Cook, 1973) contain an adenylyl cyclase in which the basal and thyrotropin-stimulated activity is stimulated two- to fourfold at concentrations of GTP ranging from 1 to 30 μM. Because cellular concentrations of GTP are significantly higher than the micromolar range, it is unlikely that a variation in GTP concentrations in eukaryotic cells plays a role in the regulation of adenylyl cyclase activity.

In the case of *E. coli* adenylyl cyclase, the stimulatory effects of GTP or other nucleotides require concentrations that are in the millimolar range. The data presented here show that the GTP stimulatory effect saturates at approximately 0.7 mM. It is therefore useful to consider the possibility that adjustments in the cellular pools of nucleoside triphosphates are important in regulating adenylyl cyclase activity. It has been reported that the intracellular concentration of ATP is 3.3×10^{-3} M and that of GTP is 1.3×10^{-3} M (Gallant & Harada, 1969). Amino acid starvation of *E. coli* is accompanied by cessation of both protein and RNA synthesis; under these circumstances, the GTP pool size decreases about 40% and that of ATP about 30%. The relevant ramification of these pool size fluctuations is that it would be expected that intracellular levels of cAMP might decrease as a result of the desaturation of both the catalytic and regulatory sites of the adenylyl cyclase enzyme complex. This response should be an important device by which the adenylyl cyclase enzyme complex can communicate with a metabolic condition in cells.

In the experimental system used in these studies (permeabilized cells), GTP behaves as an allosteric effector of adenylyl cyclase activity. This is in marked contrast to the previously reported action of GTP on adenylyl cyclase activity when the enzyme is separated from other cellular components. The studies of Yang and Epstein (1983) with highly purified enzyme showed that ATP inhibits at high concentrations; they suggested the presence of an allosteric site on the enzyme in which ATP inhibits. Further, because GTP was shown to inhibit noncompetitively with ATP, they proposed an interaction of this nucleotide at the putative allosteric site. Different properties of adenylyl cyclase in crude cell-free extracts were reported by Tao and Huberman (1970). They showed that GTP was about 10% as good a substrate as ATP as well as a competitive inhibitor of ATP. It is most likely that the properties of adenylyl cyclase described here for permeable cells represent the closest approximation to the normal physiological behavior of the enzyme.

The studies presented here show that when ATP is used as a substrate, kinetic plots typical of an allosteric enzyme (sigmoid velocity vs. substrate plots) are obtained. In the presence of added GTP (1 mM), the apparent kinetics become typically Michaelis–Menten. Therefore, under conditions of rapid growth where nucleotide levels are high, the enzyme should behave noncooperatively as a desensitized enzyme complex. According to this analysis, the allosteric properties of adenylyl cyclase may only be taken advantage of when the cells are placed under a stress condition, such as carbon source starvation. The significance of the response of the allosteric regulatory site to all the naturally occurring ribonucleoside triphosphates that serve as precursors of RNA may relate to a possible coupling of protein, RNA, and cAMP synthesis alluded to above.

Cyclic GMP and ppGpp are guanine nucleotides that might be expected to be good candidates for regulatory signalling molecules. When these compounds were tested as possible stimulators of adenylyl cyclase activity, they were found to be inactive.

Inorganic orthophosphate has been acknowledged as an important regulator of adenylyl cyclase activity (Harwood & Peterkofsky, 1975). The data of Figure 10 indicate that effective stimulation by Pi requires that the allosteric regulatory site of the adenylyl cyclase complex be loaded with a nucleotide. The net result is that there is a double layer of cooperativity in the capability of the enzyme complex to react to these low molecular weight ligands. This aspect of the regulation of the enzyme complex should substantially widen the window of activity exhibited by the adenylyl cyclase complex in the absence or presence of these regulators.

The data in this paper argue that the catalytic site of the adenylyl cyclase complex binds ATP but not other nucleotides. The experiment shown in Figure 2 indicates that the stimulatory effect of GTP plateaus at GTP concen-

trations in excess of 0.5 mM with no indication of an inhibition, consistent with the interpretation that GTP does not compete with ATP for binding to the substrate site. The data of Figure 5 show that CTP and UTP also stimulate adenylyl cyclase activity. The concentration dependence for stimulation by these nucleotides (data not shown) also shows no indication for competition. Thus, the major specificity determinant for the catalytic site appears to be for the adenine ring. There is also some selectivity for the triphosphate moiety of the substrate since AMPPNP is only 1/25 as good a substrate as is ATP (Fig. 4).

The activating effects by the variety of nucleotides shown here allow for some distinctions between the specificity for binding to the catalytic site compared to the regulatory site of the adenylyl cyclase complex. Nucleoside di- or monophosphates fail to activate, whereas triphosphates do (Fig. 5). Since GMPPNP activates approximately 20-fold less than does GTP, and GTP-γ-S is about one-fourth as effective an activator as is GTP, there is a relatively high specificity for activation associated with the triphosphate moiety (Fig. 5).

It is clear from the data of Figure 5 that activation of adenylyl cyclase is not specific for the base moiety of the nucleoside triphosphate activator, since CTP and UTP are also effective activators. It is also likely that ATP can serve as both a substrate and an activator. The data of Figure 4 show that, when AMPPNP is used as a substrate, the stimulation by GTP does not change dramatically as the concentration of AMPPNP is increased (the stimulation by GTP at the lowest concentration studied [0.2 mM] is 1.4 times as great as at the highest concentration used [2.0 mM]). We propose an interpretation of these data that AMPPNP competes very little with GTP at the activator site, so that the degree of stimulation by GTP is relatively unaffected by AMPPNP. This is consistent with the data, mentioned above, that the activator site interacts poorly with a nucleotide containing a -PPNP moiety. In contrast, the data of Figure 3 show that, when ATP is used as a substrate, the stimulation by GTP changes significantly as the concentration of ATP is increased (the stimulation by GTP at the lowest concentration studied [0.2 mM] is 2.5 times as great as at the highest concentration used [2.0 mM]). A possible interpretation of these data is that ATP can serve as both a substrate and an effective activator, although GTP is a somewhat better activator than ATP. This is consistent with the data, mentioned above, that all nucleoside triphosphates appear to serve as activators. This interpretation provides a basis for understanding the sigmoid kinetics observed with ATP (Fig. 3) but hyperbolic kinetics observed with AMPPNP. It should be emphasized that these data do not distinguish between two alternative models: (1) the model presented here in which ATP stimulates adenylyl cyclase activity by virtue of its association with a unique allosteric activator site (that also binds other nucleotides) either on the adenylyl cyclase protein or on some associated protein or (2) the model involving a classical positive cooperativity associated with the binding of ATP to the catalytic site of a multisubunit enzyme.

The stimulatory effects of GTP described here are demonstrable in permeable but not broken cells. Previous studies have implicated a variety of factors (PTS proteins, EF-Tu, CRP, Pi, and sugars) in the in vivo regulation of adenylyl cyclase activity. The system is therefore quite complex. The experiments in this study do not characterize the localization of the allosteric regulatory site on the adenylyl cyclase complex. Studies to be reported elsewhere (in prep.) suggest that nucleotide regulation of the adenylyl cyclase complex involves an interaction of the activating nucleotide with one of the PTS proteins.

Materials and methods

Materials

All of the nucleotides used in this study were obtained from Boehringer-Mannheim (Indianapolis, Indiana). The ppGpp was a gift from Michael Cashel. [α^{32}P]ATP (~30 Ci/mmol) was from New England Nuclear. [α^{32}P]AMPPNP (~25 Ci/mmol) was from ICN. All other chemicals were of analytical grade.

Escherichia coli

Strain 647 is a recA derivative of strain LBE2041 (Van de Klundert et al., 1978) (obtained from L. Bosch). The strain has been transformed with a plasmid (pDIA100) (Roy et al., 1983) that expresses the gene for adenylyl cyclase. As a result, the level of adenylyl cyclase is approximately 10-fold higher in this strain than in the parent strain. The higher level of adenylyl cyclase activity permits more accurate determination of the enzyme activity.

Methods

Growth conditions

Bacteria were grown in salts medium (Vogel & Bonner, 1956) supplemented with 0.8% Difco nutrient broth and ampicillin (30 μg/mL). Cultures were harvested in midlogarithmic phase (A_{650} approximately 0.4), washed, and resuspended in 100 mM potassium phosphate, pH 8.5.

Adenylyl cyclase assay

The washed cells were treated with 1% toluene for 10 min to permeabilize the cells. Assays for adenylyl cyclase were performed immediately after permeabilization as described previously (Harwood & Peterkofsky, 1975). The assay mixture contained bicine buffer, pH 8.5 (20 mM), $MgCl_2$ (10 mM), dithiothreitol (1 mM), and radioactive substrate, either ATP or AMPPNP, at a concentration of 0.1 mM or as indicated in the figure legends.

Protein was estimated by the method of Lowry et al. (1951). The ranges of activities (expressed as pmol/mg/h) reported in the various experiments are from 330 to 2,142 (absence of GTP) and 1,480 to 7,678 (presence of GTP). This variability, alluded to previously (Harwood & Peterkofsky, 1975), reflects the different degrees of exposure of the enzyme by toluene treatment from experiment to experiment. It is important to note, however, that although there is a variation in enzyme-specific activity of 5.2–6.5-fold, the variation in the degree of GTP stimulation of adenylyl cyclase activity is only 2-fold.

Computer-assisted calculation of kinetic constants for adenylyl cyclase

The kinetic constants V_{max}, K_m, and the Hill coefficient were calculated from the data generated by adenylyl cyclase assays using the Enzfitter program (version 1.03). Using these constants, enzyme activity vs. substrate concentration plots were generated using the Kaleidagraph program (version 2.1; Synergy Software, Reading, Pennsylvania).

References

Gallant, J. & Harada, B. (1969). The control of ribonucleic acid synthesis in *Escherichia coli*. III. The functional relationship between purine ribonucleoside triphosphate pool sizes and the rate of ribonucleic acid accumulation. *J. Biol. Chem. 244*, 3125–3132.

Gilman, A.G. (1987). G proteins: Transducers of receptor generated signals. *Annu. Rev. Biochem. 56*, 615–649.

Gilman, A.G. (1989). Transmembrane signaling, G proteins and adenylyl cyclase. *Harvey Lect. 85*, 153–172.

Harwood, J.P. & Peterkofsky, A. (1975). Glucose-sensitive adenylate cyclase in toluene-treated cells of *Escherichia coli*. *J. Biol. Chem. 250*, 4656–4662.

Harwood, J.P. & Peterkofsky, A. (1976). Regulation of *Escherichia coli* adenylate cyclase by glucose: A model for the study of receptor-coupled adenylate cyclase systems. In *Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules* (Beers, R.F., Jr. & Bassett, E.G., Eds.), pp. 393–410. Raven Press, New York.

Liberman, E., Reddy, P., Gazdar, C., & Peterkofsky, A. (1985). The *Escherichia coli* adenylate cyclase complex: Stimulation by potassium and phosphate. *J. Biol. Chem. 260*, 4075–4081.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193*, 265–275.

Peterkofsky, A. (1977). Regulation of *Escherichia coli* adenylate cyclase by phosphorylation-dephosphorylation. *Trends Biochem. Sci. 2*, 12–14.

Peterkofsky, A., Svenson, I., & Amin, N. (1989). Regulation of *Escherichia coli* adenylate cyclase activity by the phosphoenolpyruvate: sugar phosphotransferase system. *FEMS Microbiol. Rev. 63*, 103–108.

Roy, A., Haziza, C., & Danchin, A. (1983). Regulation of adenylate cyclase synthesis in *Escherichia coli*: Nucleotide sequence of the control region. *EMBO J. 2*, 791–797.

Stein, J.M., Kornberg, H.L., & Martin, B.R. (1985). Effects of GTP, GDP[bS] and glucose on adenylate cyclase activity of *E. coli* B. *FEBS Lett. 182*, 429–434.

Tao, M. & Huberman, A. (1970). Some properties of *Escherichia coli* adenyl cyclase. *Arch. Biochem. Biophys. 141*, 236–240.

Van de Klundert, J.A.M., Van der Meide, P.H., Van de Putte, P., & Bosch, L. (1978). Mutants of *Escherichia coli* altered in both genes coding for the elongation factor Tu. *Proc. Natl. Acad. Sci. USA 75*, 4470–4473.

Vogel, H.J. & Bonner, D.M. (1956). Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *J. Biol. Chem. 218*, 97–106.

Wolff, J. & Cook, G.H. (1973). Activation of thyroid membrane adenylate cyclase by purine nucleotides. *J. Biol. Chem. 248*, 350–355.

Yang, J.K. & Epstein, W. (1983). Purification and characterization of adenylate cyclase from *Escherichia coli* K12. *J. Biol. Chem. 258*, 3750–3758.