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Binding of Adenosine 3′:5′-Cyclic Phosphate to G Factor of *Escherichia coli*, and Its Effects on GTPase, RNase V, and Protein Synthesis

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Abstract. Unique among adenine nucleotides tested by filter binding assays, 3′:5′-cyclic AMP binds to the G translocation factor. Binding is dependent on the presence of GTP, and is inhibited by GDP, by the analog 5′-β,γ-methylene GTP, and by the antibiotic fusidic acid. The cAMP seems to be released during the ribosome-dependent translocation of charged tRNA catalyzed by G factor. Bound cAMP inhibits GTPase and ribosome-associated degradation of messenger RNA, but does not inhibit protein synthesis. cAMP might thereby regulate the ratio of productive to degradative transits of ribosomes on messenger RNA, and this may account for some part of its profound effect on levels of specific bacterial messenger RNA species.

The experiments of Perlman and Pastan[1–2] have extended the sphere of influence of 3′:5′-cyclic AMP (cAMP) from higher cells (in which it is believed to function as a "secondary messenger" in the response to diverse hormones)[3] to bacterial cells, in which it is normally present.[4, 5] Somehow cAMP can overcome catabolite repression of degradative enzymes[6] by an interaction at or near the promoter locus, the site at which RNA polymerase is thought to begin transcription.[7–8]

In the case of β-galactosidase, cAMP seems to act at the level of transcription;[9] but it also has an apparent effect on translation in the synthesis of tryptophanase, increasing the yield of enzyme from a given amount of mRNA.[10] While the effect on transcription suggests an interaction with RNA polymerase, the results with tryptophanase hint at a possible further reaction at the ribosome level. Here we report an interaction of cAMP with a component of the translation apparatus, factor G.

G factor catalyzes ribosome-dependent GTPase, an enzyme with an extraordinary reaction in which the ribosome serves as a cofactor. This reaction is thought to provide the motive force for translocation,[11–12] the process by which successive transfer RNA molecules are shifted on the ribosome as it moves along messenger RNA. Since G factor GTPase is essential for ribosome movement, it is required both for protein synthesis[11–12] and for the ribosome-associated activity, RNase V, that degrades mRNA in cell extracts.[13–15]

We have found that 3′:5′-cAMP binds to G factor, and appears to be released again during the ribosome-dependent function of G in translocation. A variety

of other nucleotides and nucleosides, including 3′:5′-cyclic GMP, failed to react in this manner.

Results. Binding of cAMP dependent on GTP and G factor: Binding was studied by the membrane-filter binding technique[16–17] to detect complex formation and dissociation in a variety of conditions. High concentrations of GTP were required to detect cAMP binding (Fig. 1*a*, *b*). Probably a ternary complex of GTP and cAMP with G is formed (see below), and high concentrations of GTP are required because of its low affinity for G factor.[18] That the binding of cAMP is a function of GTP concentration was further confirmed by measuring the ratio of bound to free ligand at increasing concentrations of cAMP or GTP. At 1 mM GTP, the apparent association constant of cAMP for G factor is of the order of $1 \times 10^5\ M^{-1}$; binding decreases progressively at lower levels of GTP.

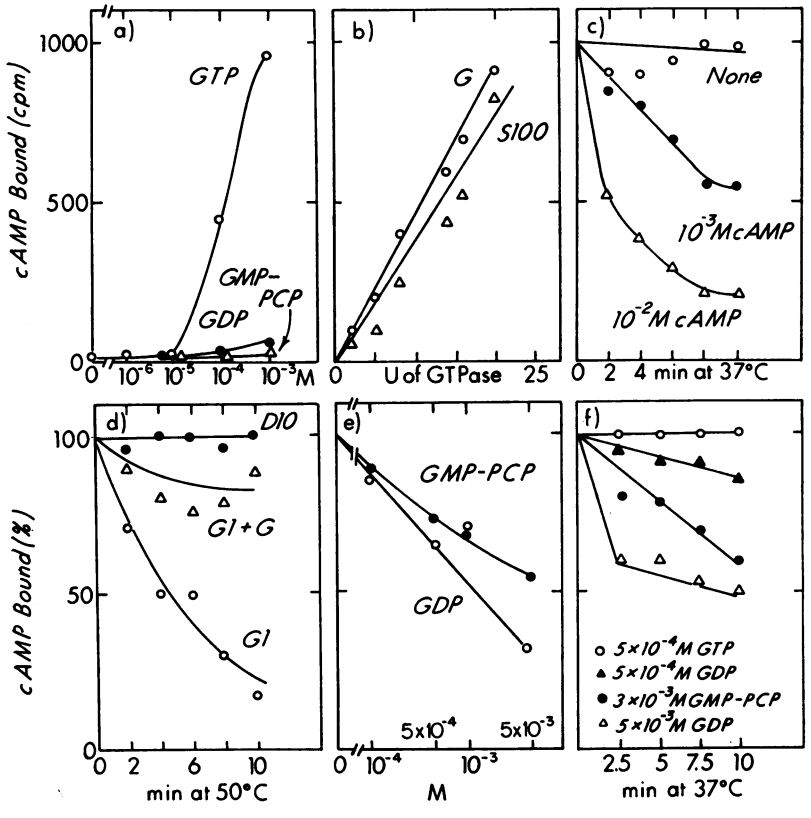
The binding to G factor appears to account for much of the binding of cAMP in cell extracts tested with the filter assay. If units of G factor are defined by ribosome-dependent GTPase activity, then per unit of GTPase, the cAMP binding capacity of the crude cell extract S-100 (ref. 19) or purified G factor is very nearly the same (Fig. 1*b*). Strong support for this view is also provided by experiments with an *E. coli* mutant having a temperature-sensitive G factor, strain G1.[21] In S-100 from the mutant (Fig. 1*d*), the inactivation of G factor at high temperature is accompanied by a parallel loss of cAMP binding capacity. Extracts or S-100 of a control strain, D10[22] (Fig. 1*d*) show no such loss of activity. Moreover, addition of purified G factor can maintain the binding capacity of the temperature-inactivated mutant strain (Fig. 1*d*). Even after the complex of G, GTP, and cAMP has been formed, it can in great part be disrupted by heating when prepared with extracts of the mutant but not of the wild-type strain.

A further indication that G factor is responsible for cAMP binding came from the observation that binding was inhibited by fusidic acid (data not shown). This antibiotic is known to inhibit the GTPase activity of G factor.[23] Thus, fusidic acid could block the proposed binding cycle of Figure 4 between steps 1 and 2 (see *Discussion*). As expected, however, in extracts of mutants resistant to fusidic acid, this agent did not inhibit binding of cAMP.

cAMP binding also shows an absolute requirement for Mg^{2+} ions, with an optimum at about 0.01 *M*; but no requirement for added monovalent cations was observed. (The Mg^{2+} may be necessary for the filter binding assay, rather than an intrinsic requirement of the binding reaction.[17] Binding in presence of the required factors is reversible, since cAMP can be displaced from the complex by added unlabeled cAMP (Fig. 1*c*). We have eluted bound ^{3}H-cAMP from filters and shown that it moves with carrier cAMP in two-dimensional paper chromatography (results to be published).

Binding is specific for both cAMP and GTP. None of the other adenine derivatives tested were detectably bound, including 5′-AMP, ADP, ATP; 3′-AMP; and 2′:3′-cyclic AMP. Nor would GDP or the analog 5′-β,γ-methylene GTP (GMP-PCP)[24] replace GTP in the binding reaction. Even more striking, GDP and GMP-PCP strongly inhibit binding and can cause dissociation of

FIG. 1.—cAMP binding to G factor. Binding was measured by trapping the complex on a Millipore filter. The standard binding asaay mixture contained, in a volume of 50 μl, 0.1 *M* Tris-HCl, pH 7.5; 0.01 *M* magnesium acetate; 0.05 *M* KCl; 0.25 μCi ^{3}H-cAMP (12.7 Ci/mmole, from Schwarz Bioresearch; under the conditions used, this corresponds to 70,000 cpm and 0.4 μM); 1 mM GTP, except where indicated; 5 μl of S-100 (a crude cell extract[19]) or an equivalent number of ribosome-dependent GTPase units of purified G; and other nucleotides or additives as mentioned. After 10 min at 37°C, the sample was diluted to 3 ml and passed through a Millipore filter, which was washed and counted by the procedures of ref. 12. Counts per minute bound to the filter are reported in panels *a* to *c;* in *d* to *f*. 100% binding [i.e., the binding in a control, without heating (*d*) or inhibitors (*e* and *f*)] corresponds to about 1000 cpm.



(*a*) Binding as a function of added GTP, GDP, or 5′-β, γ-methylene GTP (GMP-PCP), with 5 μl of S-100 per assay.

(*b*) Binding to increasing amounts of G factor or amounts of S-100 containing equivalent amounts of G. G was measured as relative ribosome-dependent GTPase in arbitrary units;[12] 20 units correspond to about 50 μg of S-100 protein, or 0.3 μg of purified G. G was purified on DEAE-Sephadex[12] and G-150 Sephadex to a specific activity corresponding to 60% pure enzyme.[20]

(*c*) Exchangeability of unlabeled for labeled bound cAMP. Complex was formed under the standard conditions for 10 min; then 5 μl was added, containing unlabeled cAMP, to give the indicated final concentration, and incubation at 37°C was continued for the times shown before dilution and filtration.

(*d*) Binding after heating at 50°C. S-100 of strain D10; of strain G1; or of strain G1 supplemented with about an equivalent of G factor purified from strain D10 was heated at 50°C for the times indicated, and then added to the other components for the standard binding assay.

(*e*) Inhibition of cAMP binding by GDP or GMP-PCP. The complex was formed under the standard conditions, with 1 mM GTP always present, but with the added concentrations of GDP or GMP-PCP indicated.

(*f*) Reversibility of cAMP binding by GDP or GMP-PCP. After complex formation for 10 min at 37°C, 5 μl of GDP or GMP-PCP was added to the final indicated concentration. Incubation was then continued at 37°C for the indicated times.

bound cAMP from the complex (Figs. 1*e*, *f*). Very probably, G factor is one of those enzymes in which binding at one site is influenced by binding of an effector at another site.[25] Replacement of bound GTP by GDP also provoked the dissociation of bound cAMP.

Release of bound cAMP: The inhibition and disruption of cAMP binding by GDP suggested that cAMP binding would be reversed when the G factor catalyzes conversion of GTP to GDP. This supposition (the closing of the cycle at steps III–V in Fig. 4; see *Discussion*) was supported by the type of experiment summarized in Figure 2. First a complex of cAMP and G was formed in S-100, and various additional requirements for translocation were then added. cAMP was released at a maximal rate when all of them were present, including K^+ and Mg^{2+} ions, ribosomes, mRNA, and charged tRNA. It thus appears that when GTP is hydrolyzed during translocation, any cAMP bound to G factor will be released. Particularly noteworthy is the requirement for charged tRNA for cAMP release (Fig. 2); uncharged tRNA showed much less capacity to release cAMP from its complex under the same conditions (Fig. 2). This implies that the release of bound cAMP has a special relationship to the productive translocation that is linked to protein synthesis (see *Discussion*).

Effects of bound cAMP: A connection between cAMP and protein synthesis is again implied in direct measurements of the effects of cAMP on ribosome function (Fig. 3): bound cAMP inhibits ribosome-dependent GTPase (Fig. 3*a*) and ribosome-dependent RNase (RNase V; Fig. 3*c*); but it has, if anything, a

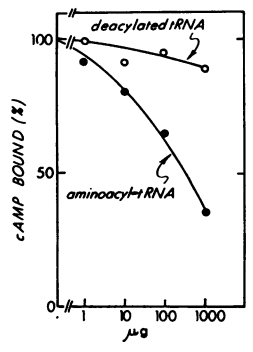


FIG. 2.

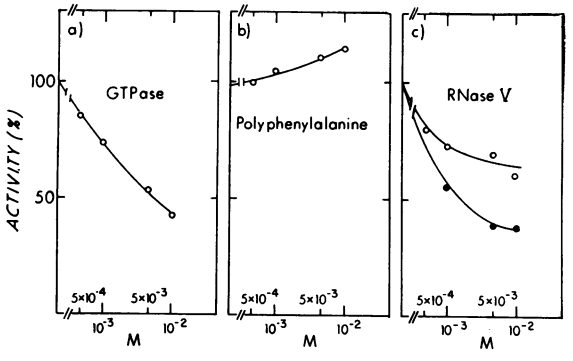


FIG. 3.

FIG. 2.—Dissociation of bound cyclic AMP by components of the translocation cycle. An example is shown in which cAMP was bound in 50 μl of a standard assay mixture (Fig. 1), with or without various additional components: 50 μg of washed ribosomes[8] of strain D10, 5 μg of poly U, or charged or uncharged tRNA. The total mixture was then incubated with 5 μl of buffer, or with 5 μl containing other additives, for another 10 min at 37°C before filtration. In the trial shown, the initial mixture contained S-100, poly U, and ribosomes; charged or uncharged tRNA was then added at the final indicated levels per milliliter for the additional 10 min. tRNAwas stripped according to ref. 30 and some of it was then recharged according to ref. 31.

FIG. 3.—Effect of various levels of cAMP on (*a*) ribosome-dependent GTPase;[12] (*b*) polyphenylalanine synthesis;[19] or (*c*) RNase V attack on poly U (—○—) or purified T4-specific mRNA (—●—); the gift of Dr. D. E. Kennell.[13] Under the standard assay conditions used, 100% would correspond to 5000 cpm P_i in panel (*a*); 6000-cpm of ^{14}C-polyphenylalanine in panel (*b*); and 300-cpm of ^{3}H-nucleotides in panel (*c*).

slight stimulatory effect on protein synthesis (Fig. 3*b*). It should be noted, however, that these effects required relatively high concentrations of cAMP, much higher than those required to saturate G (Fig. 1), either because cAMP has an effect on some component other than G factor—which we think unlikely—or because the relevant affinity constant is much reduced in these conditions. These levels of cAMP are similar to those that stimulate polyphenylalanine formation on reticulocyte ribosomes.[26]

Discussion. Based on the results reported here, Figure 4 suggests a tentative

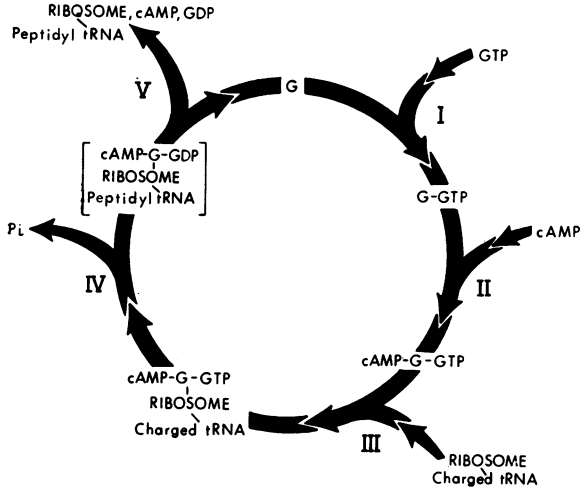


FIG. 4.—Suggestion for a 3,: 5,-cyclic AMP-G factor binding cycle.

Step I. GTP binds to the G factor inducing a conformational change that permits *Step II.* cAMP binds to the G factor, and the ternary complex arrives at *Step III.* Here G factor, still bearing GTP and cAMP, joins to the ribosome, which will already be bound to mRNA, peptidyl-tRNA, and charged tRNA, freshly bound from a complex with GTP and T factor.[32] *Step IV* is translocation. Peptide bond formation takes place at the a site, and the peptidyl-tRNA is shifted to the p site. At the same time, GTP is hydrolyzed to GDP and Pi. *Step V.* With the formation of GDP, bound cAMP is released from the ribosome (along with GDP and G factor[33]).

In the generally accepted "translocation cycle" during protein synthesis, the ribosome that comes off at step V interacts with a complex of the next tRNA, T factor, and GTP, and reenters the cycle shown at *Step III.* To this customary cycle, the reactions shown add *Step II* and the release of cAMP at *Step V.*

cAMP-G factor binding cycle. cAMP binds reversibly to G factor in presence of GTP (steps I and II), and is released during translocation of aminoacyl-tRNA on ribosomes with the attendant cleavage of GTP (steps III to V).

Much of Figure 4 is speculative. For example, we have not yet shown whether cAMP is chemically modified when it is released after GTPase action; and while binding is certainly mediated by G factor, we have not proven that no other protein is involved. However, the conditions for maximal release (Figs. 1 and 2) are certainly very similar to those for the hydrolysis of GTP during translocation.

Recent studies with ribosome-associated RNase V to detect translocation by the breakdown of the substrate mRNA suggest that a part of GTPase activity (perhaps "uncoupled" GTPase[12, 27]) is actually associated with a form of translocation that does not require peptide bond formation.[15] There seem to be at least two modes of translocation: one, a "productive" movement involved in protein synthesis; the other, an "abortive" movement that produces no protein but can support RNase V action. Unlike productive translocation, which proceeds only with charged tRNA, abortive translocation can perform steps IV and V with uncharged or even with periodate-oxidized tRNA.[15] Thus, G factor GTPase can be associated with a productive movement of ribosomes that is dependent on charged tRNA, or with an abortive movement more characteristic of (and possibly required for) RNase V action.

Since release of bound cAMP is far greater with charged than with uncharged tRNA (Fig. 2), it seems possible that cAMP does not affect productive translocation (Fig. 3*c*), but acts by specifically inhibiting abortive translocation leading to a reduction of RNase V activity (Fig. 3).

Any connection between this notion and the effects of cAMP *in vivo* is very tenuous, since our results have been obtained with poly U and T4 phage mRNA, while the regulatory action of cAMP *in vivo* seems to be specific for catabolic enzymes. Binding of cAMP to G factor probably occurs in growing cells, since the intracellular concentration of GTP and cAMP are about 1.3×10^{-3} M[28] and 10^{-5} M,[4] sufficient to support the binding reported here. But the significance is unclear. Mutants that lack adenyl cyclase[1]—and therefore contain little cAMP—can grow; thus, cAMP binding to G factor is probably not an indispensable requirement for cell growth. On the other hand, mutants lacking cyclase do grow more slowly than the parental strain, and cAMP has a growth-enhancing effect that might in part be exerted through binding to G factor.

It seems very unlikely that the effects of cAMP on β-galactosidase synthesis are mediated through G factor. A cAMP-binding protein distinct from G has been isolated by M. Emmer, R. L. Perlman, B. deCrombrugghe, and I. Pastan (personal communication). They have shown that in mutants with low levels of this protein, cAMP has no effect on β-galactosidase synthesis. In extracts of the mutant, β-galactosidase synthesis is dependent on the addition of the specific binding protein. Furthermore, the stimulation of synthesis by cAMP and its binding to the specific protein are both inhibited by 3′:5′-cyclic GMP, whereas cyclic GMP does not inhibit cAMP binding to G factor (results to be published).

One can nevertheless imagine possible connections between cAMP binding to G factor and its effects on translation yields of mRNA.[10] For example, events at the 5′-start of an mRNA[13–15, 29] may determine its survival and translation yield through a ribosome-dependent reaction.[13–15] As ribosomes join to appropriate molecules of mRNA, cAMP could regulate the number of productive starts: the more cyclic AMP bound to G factor on the ribosomes, the less abortive transits of mRNA would begin, the less breakdown of mRNA would be initiated, and the greater the net amount of mRNA or protein for the corresponding operons.

Note added in proof: In extracts of some *E. coli* K12 strains, cAMP binding is relatively resistant to fusidic acid; however, from these strains mutants have been derived that show greatly increased sensitivity. The results with fusidic acid will be reported separately.

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