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Adenosine 3′:5′-Cyclic Monophosphate Control of the Enzymes of Glutamine Metabolism in *Escherichia coli*

(glutaminases/glutamate synthase/glutamine synthetase/glutamate dehydrogenase/cyclic AMP)

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ABSTRACT **The effect of cAMP on the intracellular levels of five enzymes concerned with the interconversion of glutamate and glutamine in *E. coli* has been examined. Cyclic AMP added to the culture medium increases the levels of glutamate dehydrogenase (EC 1.4.1.4) and glutamine synthetase (EC 6.3.1.2); it decreases the levels of glutamate synthase (EC 1.4.1.X), and glutaminase A (EC 3.5.1.2). Cyclic AMP did not affect the level of glutaminase B (EC 3.5.1.2). These alterations in enzyme levels by cAMP require cyclic AMP receptor protein, since the levels of these enzymes were unchanged by cAMP in a mutant lacking this receptor. Chloramphenicol also abolished the effects of cAMP, a result that implies protein synthesis is necessary for these changes in enzyme levels to occur. The reciprocal effects of cAMP on the levels of these enzymes may play an important role in the cellular regulation of nitrogen metabolism.**

The interconversion of glutamate and glutamine lies at a crossroads where nitrogen and carbohydrate metabolic pathways intersect. Glutamine may provide its amide nitrogen for the biosynthesis of amino sugars, amino acids, nucleotides, or cofactors. Glutamate may donate its carbon skeleton for energy generation in the tricarboxylic acid cycle or its amino nitrogen for the biosynthesis of amino acids (1, 2).

Since cAMP is known to enhance the synthesis of many enzymes in bacteria (3, 4), we studied its effect on five of the enzymatic activities that are concerned with the cellular regulation of glutamate and glutamine pools in *Escherichia coli*. Results of this study disclose the existence of a system of reciprocal control by cAMP in which both positive and negative regulation of enzyme levels are observed. Cyclic AMP increases the levels of glutamate dehydrogenase and glutamine synthetase, which catalyze reactions 1 and 2, respectively:

$$\text{2-oxoglutarate} + NH_3 + \text{NADPH} \rightleftharpoons \text{glutamate} + \text{NADP} \quad 1$$

$$\text{glutamate} + NH_3 + \text{ATP} \rightleftharpoons \text{glutamine} + \text{APP} + P_i \quad 2$$

In contrast, cAMP diminishes the levels of glutamate synthase (5) and glutaminase A, which participate in reactions 3 and 4, respectively:

$$\text{2-oxoglutarate} + \text{glutamine} + \text{NADPH} \rightleftharpoons 2\ \text{glutamate} + \text{NADP} \quad 3$$

$$\text{glutamine} + H_2O \rightleftharpoons \text{glutamate} + NH_3 \quad 4$$

Cyclic AMP has no effect on the level of glutaminase B, a constitutive enzyme that also catalyzes reaction 4.

Abbreviations: CRP, cAMP receptor (catabolite-gene activator protein).

MATERIALS AND METHODS

Materials. Cyclic AMP was obtained from Sigma, St. Louis, Mo. and P-L Biochemicals, Milwaukee, Wis. The L-[*U*-^{14}C]glutamine was obtained from New England Nuclear and purified over Dowex 1-Cl. The remaining chemicals were of the purest grades commercially available.

Bacterial Growth. E. coli K12—1100 and two mutants K12-5333 (*crp*) and K12-5336 (*cya*), all requiring thiamin, were the generous gift of Dr. Ira Pastan (6, 7). All cultures were started from single colonies isolated on MacConkey's agar. Both the *crp* and *cya* mutants were easily identified, since they could not ferment lactose. Bacteria were grown in liquid medium containing 1.7 mM $MgSO_4$, 43 mM NaCl, 14 mM K_2SO_4, 150 mM potassium phosphate (pH 7.4), 30 mM glucose, and 40 mM NH_4Cl, unless otherwise noted. The pH of the medium remained above 6.7 during the growth. The culture medium was not supplemented with glutamate and/or histidine, since these amino acids did not affect growth of the *cya* mutant under our experimental conditions. The doubling time of the *cya* mutant was about 1.5 times longer than that reported for growth on glutamate- and histidine-supplemented medium (7).

The cells were grown for four generations on minimal medium; a 1:10 innoculum was used for each 40-ml culture, which was grown at 37° in a 300-ml triple-baffle (Bellco) flask equipped with a side arm on a New Brunswick gyrotory shaker oscillating 100 times per min. Growth was followed with a Klett Summerson colorimeter equipped with a no. 66 filter. A galvanometric reading of 100 Klett units corresponded to an absorbancy of 0.200 as measured on a Gilford 240 spectrophotometer at 660 nm. The cells were harvested after 1.5–3 hr in stationary phase, unless otherwise noted, by cooling the culture with ice and sedimenting the cells at 10,000 × *g* for 5 min at 4°. The cells were washed with distilled water, recentrifuged, and frozen in liquid nitrogen.

Preparation of Extracts. The frozen cells were thawed in 1 ml of 10 mM imidazole·Cl (pH 7.1) at 37° (water bath), and were sonicated in an ice bath with a Branson sonifier equipped with a microtip. The cells were given three 15-sec intervals of sonication. During sonication the temperature was maintained at less than 17°. The extracts were centrifuged at 45,000 × *g* for 30 min at 4°, and the supernatants (5–20 mg of protein per ml) were assayed for enzymatic activities.

Assays. Glutaminases A (pH 5) and B (pH 7) (EC 3.5.1.2) were assayed by measurement of the conversion of [^{14}C]-glutamine to [^{14}C]glutamate (2,8). [^{14}C]Glutamate was isolated chromatographically on Dowex-1-Cl and measured

in Bray's solution (9) with a Beckman LS 250 liquid scintillation counter. Glutaminase A assay solution (0.1 ml) contained 30 mM L-glutamine and 100 mM sodium acetate (pH 5); glutaminase B assay solution (0.1 ml) contained 30 mM L-glutamine and 100 mM potassium phosphate (pH 7.1). Blanks contained no enzyme. Identical results were obtained when glutaminases A and B were assayed by measurement of the NH_4^+ produced under the above assay conditions. In this case, the reaction was stopped with trichloroacetic acid (final concentration 5%). Samples were centrifuged at 300 × *g* for 10 min, and an aliquot of the supernatant was assayed for NH_4^+ content. Ammonia was determined from the initial rate of NADH oxidation (37°) by bovine glutamate dehydrogenase (10). All spectrophotometric assays were performed with a Gilford 300 N spectrophotometer equipped with a thermostated cuvette (1-cm light path).

Glutamate synthase (EC 1.4.1.X) activity was determined by following the rate of NADPH oxidation at 30° (5)*. The assay mixture (1.0 ml) contained 0.16 mM NADPH, 5.0 mM 2-oxoglutarate, 5.0 mM L-glutamine, and 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.5). The blanks contained no L-glutamine. *E. coli* glutamate dehydrogenase (EC 1.4.1.4) activity was measured with an assay mixture (1.0 ml) containing 0.16 mM NADPH, 5.0 mM 2-oxoglutarate, 100 mM NH_4Cl, and 50 mM potassium HEPES buffer (pH 7.5). The blanks contained no NH_4^+.

Transferase activity of glutamine synthetase (EC 6.3.1.2) was assayed by measurement of the γ-glutamylhydroxamate formed at 37° in 15 min. The assay mixture (0.5 ml) contained 150 mM L-glutamine, 40 mM NH_4OH, 20 mM arsenic acid, 0.4 mM ADP, 50 mM 2,4-dimethylglutaric acid, 300 mM KCl, and 0.4 mM $MnCl_2$. The pH was adjusted to 7.57 with triethanolamine. Under these assay conditions, the enzyme activity is not altered by the state of adenylylation (11). The reaction was stopped and the color was developed by the addition of 1.5 ml of 3.5% $FeCl_3$, 2% Cl_3CCOOH, and 0.25 N HCl. The absorbancy was measured at 540 nm in a Gilford 300N spectrophotometer.

Protein was determined by the biuret method, with crystalline bovine serum albumin as standard (12).

RESULTS

Table 1 shows the effects of cAMP on the levels of glutaminase A, glutamate synthase, glutamate dehydrogenase, glutamine synthetase, and glutaminase B in wild-type *E. coli* K12-1100 grown in culture medium containing either glycerol or glucose, and either high (40 mM) or low (6 mM) concentrations of NH_4Cl. In cells grown on glucose and high NH_4Cl, the levels of glutaminase A and of glutamate synthase were 5 and 1.5 times greater, respectively, than in cells grown on glycerol and high NH_4Cl. Addition of cAMP to the culture medium had no effect on the levels of either enzyme in cells grown on glycerol, but the levels of these two enzymes in cells grown on glucose were reduced to the same values observed in cells grown with glycerol. When growth on glucose is limited by low concentrations of NH_4Cl, the level of glutaminase A is reduced, but the level of glutamate synthase is increased. Addition of cAMP to the low NH_4Cl medium leads to reduction of both enzyme levels. In contrast to the effects of cAMP noted above, the levels of glutamate dehydrogenase and glutamine synthetase were not significantly altered by inclusion of cAMP in the culture medium. Cells grown on glycerol contained a higher level of glutamate dehydrogenase than cells grown on glucose. As noted (1), the level of glutamine synthetase is greatest when growth on glycerol is limited by availability of NH_4^+. Glutaminase B level did not change under any of the conditions examined.

TABLE 1. *Enzyme activities in wild-type E. coli K12-1100: The effect of cAMP*

Medium	cAMP added (mM)	Units/mg of: Glutaminase A	Glutamate synthase	Glutamate dehydrogenase	Glutamine synthetase	Glutaminase B
1*	0	0.091	0.070	0.877	0.193	0.063
1	5	0.100	0.065	0.963	0.210	0.075
2†	0	0.466	0.110	0.561	0.198	0.060
2	5	0.103	0.072	0.529	0.212	0.056
3‡	0	0.075	0.160	0.471	0.603	0.073
3	5	0.028	0.107	0.491	0.669	0.068

One unit of enzyme is the amount that catalyzes conversion of 1.0 μmol of substrate to product per min under the standard assay conditions.
* 60 mM glycerol–40 mM NH_4Cl.
† 30 mM glucose–40 mM NH_4Cl.
‡ 30 mM glucose–6 mM NH_4Cl.

A more exaggerated effect of cAMP could be demonstrated in studies with the adenylate cyclase-deficient mutant K12-5336 (7). When the mutant was grown on minimal medium containing 30 mM glucose and either high or low concentrations of ammonia, the addition of 5 mM cAMP decreased the levels of glutaminase A and glutamate synthase more than 60% and almost doubled the levels of glutamate dehydrogenase and glutamine synthetase (Table 2). Glutaminase B level was unaltered by cAMP.

Curves relating growth rate and enzyme levels of the adenylate cyclase mutant to the concentration of cAMP in the culture medium are shown in Fig. 1. The growth rate was doubled by 1 mM cAMP. The levels of glutaminase A and glutamate synthase were decreased by cAMP over a narrow concentration range (1–5 mM); half-maximal decrease in the level of these two enzymes occurred at 3.6 mM cAMP. This concentration of cAMP is similar to that required to overcome catabolite repression of β-galactosidase *in vivo* (13, 14). As depicted in Fig. 1*B*, the increase in glutamate dehydrogenase and glutamine synthetase levels required about 10% of the cAMP concentration needed to decrease glutaminase A and glutamate synthase. The half-maximal increase of glutamate dehydrogenase occurred at 0.16 mM cAMP and of glutamine synthetase at 0.4 mM. Presumably, the level of cAMP in wild-type *E. coli* is much higher than that in the adenylate cyclase mutant; thus, exogenous cAMP may have little effect on the levels of glutamate dehydrogenase and glutamine synthetase in wild-type *E. coli*.

The temporal effect of cyclic AMP on cellular growth and enzyme levels is shown in Fig. 2. *E. coli* K12-5336 was grown for four generations on a glucose–NH_4^+ minimal medium, and subsequently diluted 1:10 into paired flasks at time 0. After 5 hr of logarithmic growth, 5 mM cyclic AMP was added to one of the paired flasks (Fig. 2*A*). Cells were harvested at various times after the addition of cAMP, and enzyme activities were determined. In all cases, more than 60% of the observed decrease or increase in enzyme levels occurred within 2.5 hr (about one generation time) after addition of cyclic AMP to cultures (Fig. 2*B*). 5 mM cAMP depressed both the levels of glutaminase A and glutamate synthase, while it

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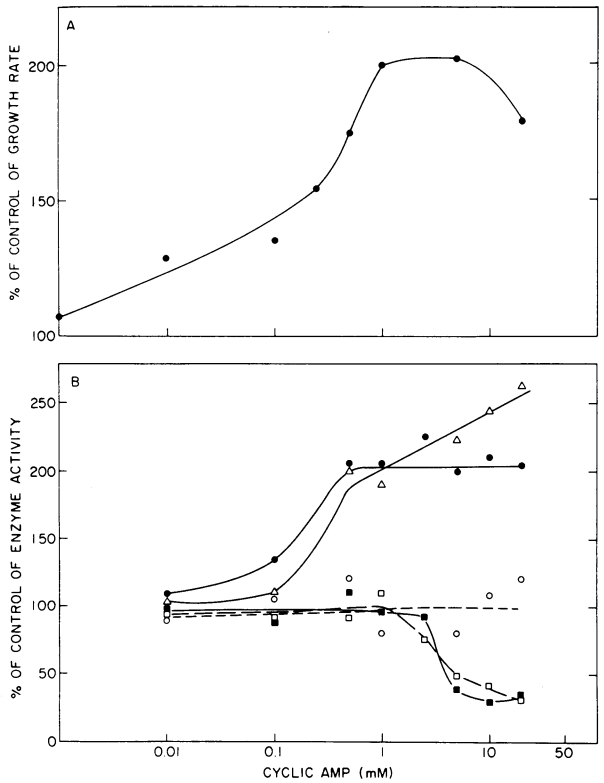

FIG. 1. Curves relating growth rate and enzyme levels to the concentration of cAMP in the growth medium. *E. coli* K12-5336 were grown on minimal medium containing 30 mM glucose and 40 mM NH_4Cl. (*A*) Increase in growth rate due to cAMP. % of control = 100 × [(generations per hr + cAMP)/(generations per hr − cAMP)]. (*B*) Alterations in the specific activities (units/mg) of glutaminase A (■——■), glutamate synthase (□——□), glutamate dehydrogenase (●——●), glutamine synthetase (△——△), and glutaminase B (○——○) in response to various concentrations of cAMP in the growth medium. % of control = 100 × [(specific activity + cAMP)/(specific activity − cAMP)].

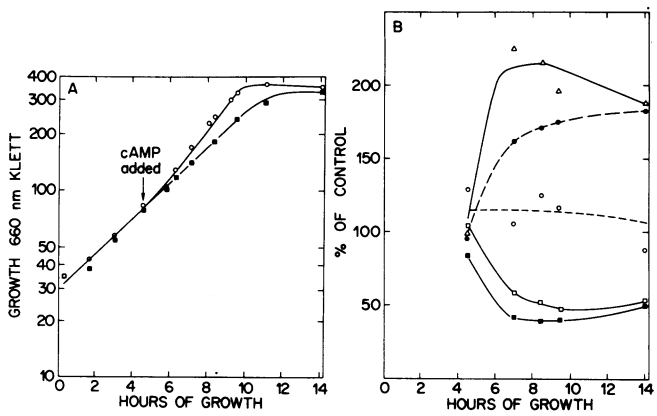

FIG. 2. Effect of cAMP on *E. coli* K12-5336. (*A*) Growth of *E. coli* deficient in adenylate cyclase (■——■). 5 mM Cyclic AMP added at the *arrow* (○——○). (*B*) Changes in specific activities (units/mg) of glutaminase A (■——■), glutamate synthase (□——□), glutamate dehydrogenase (●——●), glutamine synthetase (△——△), and glutaminase B (○——○) at various times after addition of cAMP.

increased the levels of glutamate dehydrogenase and glutamine synthetase; glutaminase B level was not altered.

In cultures harvested immediately after the addition of cAMP, enzyme levels were not significantly affected (denoted as 0 hr in Table 3). After 5 hr in the presence of cAMP, glutaminase A and glutamate synthase were depressed to 39 and 46% of their original levels, while glutamine synthetase and glutamate dehydrogenase levels were almost doubled. The glutaminase B level was not significantly altered. The effects of cAMP on enzyme levels were completely abolished by chloramphenicol, an inhibitor of protein synthesis. In addition, mixture of extracts from cultures grown in the presence and absence of cAMP gave expected average values, indicating that a specific metabolite activator or inhibitor was not responsible for the observed enzyme activities. cAMP does not alter the catalytic activities of purified glutamate synthase. 5 mM cAMP does activate glutaminase B, but cAMP in the growth medium does not alter the level of glutaminase B (as shown). Indeed, the time course, the action of chloramphenicol, and the mixing experiments all indicate that protein synthesis is necessary for cAMP to alter the levels of glutaminase A, glutamate synthase, glutamate dehydrogenase, and glutamine synthetase.

The mode of action of cAMP in the control of bacterial enzyme synthesis has been extensively examined by use of lactose and galactose operons (15–17). These studies have shown that cAMP binds to cyclic AMP receptor protein, which regulates the initiation of mRNA transcription by RNA polymerase. Cyclic AMP did not alter the levels of the five enzymes examined in mutants lacking receptor protein (Table 4). This observation strongly suggests that the presence of the receptor protein is necessary for cAMP to alter the level of glutaminase A, glutamate synthase, glutamate dehydrogenase, and glutamine synthetase. Mixing of extracts from mutants lacking receptor protein and wild-type *E. coli* gave expected average values, indicating that a specific activator or inhibitor was not responsible for differences observed between the two types.

TABLE 2. *Enzyme activities in E. coli K12-5336: The effect of cAMP on a mutant deficient in adenylate cyclase*

	Components added to growth medium (mM)							
Units/mg of:	cAMP (0)	NH_4Cl (40)	cAMP (5)	NH_4Cl (40)	cAMP (0)	NH_4Cl (6)	cAMP (5)	NH_4Cl (6)
Glutaminase A	0.482		0.090		0.160		0.044	
Glutamate synthase	0.075		0.075		0.377		0.148	
Glutamate dehydrogenase	0.486		0.808		0.497		0.750	
Glutamine synthetase	0.129		0.279		0.579		1.090	
Glutaminase B	0.064		0.066		0.078		0.066	

TABLE 3. *Chloramphenicol prevention of cAMP alteration of enzyme synthesis in E. coli K12-5336*

Enzyme	Time of harvest (hr) 0	5	5 (plus chloramphenicol)*
Glutaminase A	84†	39	88
Glutamate synthase	104	46	107
Glutamate dehydrogenase	96	175	108
Glutamine synthetase	99	196	110
Glutaminase B	128	125	83

* 100 μg/ml of chloramphenicol added.
† Enzyme levels given as % of control value.

TABLE 4. *Enzyme activities in E. coli K12-5333: The effect of cAMP on a mutant deficient in cAMP receptor protein*

Enzyme	Enzyme activity from cells grown with: Control	+5 mM cAMP
Glutaminase A	0.661*	0.696
Glutamate synthase	0.187	0.185
Glutamate dehydrogenase	0.564	0.569
Glutamine synthetase	0.190	0.183
Glutaminase B	0.091	0.079

* Data given as units/mg of enzyme.

A comparison of the specific activities of the five enzymes examined in the wild-type *E. coli* and two mutants (Tables 1, 2, and 4) shows that the differences in levels are smaller in several cases than might be expected. This deviation from expected values may in part be due to the alterations in cellular metabolites that undoubtedly occur in bacteria deficient in adenylate cyclase or cAMP receptor. As illustrated in Tables 1 and 2, the concentration of NH_3 in the medium can cause 4-fold changes in the levels of some of these enzymes, while other studies have shown even larger changes that are dependent upon the glutamate concentration in the medium (1, 5, 19, 20).

Table 5 shows the specificity of the adenylate nucleotide response in *E. coli* K12-5536. Only cAMP reduced glutaminase A and glutamate synthase levels to less than 50% of their levels in its absence, and only cAMP stimulated the synthesis of glutamate dehydrogenase and glutamine synthetase. ATP doubled the glutamate synthase level. This effect was not seen in the mutant lacking receptor protein. The explanation for this increase is not apparent, since the addition of 5 mM ATP to the growth medium would not be expected to greatly alter intracellular ATP levels, and 5 mM ATP does not significantly alter the activity of purified glutamate synthase*.

Variations in certain culture medium components did not alter the observed responses to cAMP. The potassium phosphate concentration was varied from 50 to 150 mM. The addition of a mixture containing trace elements (18 μM $CaCl_2$, 248 μM $FeCl_2$, 252 μM $ZnSO_4$, 256 μM $CuSO_4$, 304 μM $CoCl_2$, and 216 μM Na EDTA) also had no effect.

DISCUSSION

We have demonstrated that cAMP exerts both positive and negative control on the levels of enzymes in *E. coli*. The increase in glutamate dehydrogenase and glutamine synthetase brought about by cAMP may be added to a list of 14 proteins known to be under positive control by cAMP (3, 4). The decrease of glutamate synthase and glutaminase A levels caused by cAMP may represent a new type of negative control by cAMP in bacteria.

Other enzymes in bacteria known to be under positive control by cAMP are subject to "catabolite repression" by glucose (4, 13, 14). This "catabolite repression" is attributed to a lowering of intracellular cAMP levels by glucose (4, 21, 22). From studies on the lactose, galactose, and arabinose operons, it appears that cAMP exerts its effect on enzyme synthesis mainly at the level of mRNA transcription (15–17, 23, 24). After cAMP complexes with a specific binding protein, the cAMP–binding protein complex acts at a promoter region of DNA to facilitate the initiation of mRNA transcription by RNA polymerase (4, 15–17, 25).

The requirement of cAMP for the synthesis of enzymes that are catabolite repressible is variable. For example, β-galactosidase is very low in *E. coli* mutants deficient in adenylate cyclase, whereas the level of galactokinase is only moderately lowered in these mutants (7, 18). As shown here, the synthesis of glutamine synthetase and glutamate dehydrogenase is not tightly coupled to cAMP, since both enzymes are present in the mutants deficient in adenylate cyclase or receptor protein. It is noteworthy that addition of cAMP increased the levels of glutamate dehydrogenase and glutamine synthetase in the mutant deficient in adenylate cyclase, but had no demonstrable effect on their levels in the wild type (Tables 1 and 2). This finding may be explained by the fact that a much lower concentration of cAMP is needed to affect the levels of these two enzymes than is needed to diminish the levels of glutaminase A and glutamate synthase (Fig. 1*B*), or is needed to abolish catabolite repression of β-galactosidase *in vivo* (14, 15). Perhaps the endogeneous level of cAMP in the wild type is sufficiently high to maxi-

TABLE 5. *Specificity of the alterations by cAMP of enzyme activities*

Nucleotide added (5 mM)	Enzyme levels (% of control): Glutaminase A	Glutamate synthase	Glutamate dehydrogenase	Glutamine synthetase	Glutaminase B
cAMP	50	47	173	221	87
ATP	94	182	81	135	122
ADP	112	144	83	106	99
5′-AMP	95	112	121	112	117
3′-AMP	102	115	92	105	85
3,′5′-GMP	99	111	101	138	95

mally affect the levels of glutamine synthetase and glutamate dehydrogenase, but is insufficient to affect the levels of the other enzymes. It remains to be established whether or not the difference in sensitivity of these enzymes to cAMP involves different molecular mechanisms. Cyclic AMP elevates the level of glutamine synthetase in neural retina of chick embryos (26).

In contrast to the many enzymes under positive control by cAMP, the levels of glutaminase A and glutamate synthase are diminished, rather than increased, by the addition of cAMP to the culture medium of cells growing on glucose. It is significant that in the absence of exogenous cAMP the level of glutaminase A and glutamate synthase are much lower in cells grown on glycerol than in cells grown on glucose. The addition of cAMP to the glycerol medium had no effect on the levels of either enzymes, whereas addition of cAMP to glucose medium decreased the levels of both enzymes to those found in cells grown with glycerol. These results could be explained if the endogeneous level of cAMP in cells grown with glycerol is sufficiently high to produce maximal depression of the two enzyme levels. This interpretation is consistent with the fact that the intracellular concentration of cAMP is much higher in cells grown on glycerol than those grown on glucose (21, 22).

Although the molecular basis of negative control of glutaminase A and glutamate synthase by cAMP remains to be established, it is clear that cAMP receptor protein is involved since the levels of these enzymes were not altered by exogenous cAMP in a mutant deficient in this protein. Examples of negative control in mammals by cAMP have been observed in the regulation of glucokinase (27) and glucose-6-phosphate dehydrogenase (28).

Both positive and negative effects of cAMP have been found in the regulation of mammalian glycogen metabolism (29). Cyclic AMP stimulates the activity of a protein kinase that phosphorylates glycogen synthase, specifically inactivating the synthase. The same protein kinase phosphorylates phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase to activate the phosphorylase. These covalent modifications proceed in the absence of protein synthesis. It is unlikely that the reciprocal effects of cAMP reported in this communication could be explained by a similar mechanism, since these changes in four enzymes concerned with glutamine metabolism require cAMP receptor protein and are abolished by chloramphenicol, an inhibitor of protein synthesis.

Thus, three classes of control mechanisms regulate the synthesis of the five enzymes examined that are concerned with the metabolism of glutamate and glutamine in *E. coli:* (*i*) generalized control by cAMP (3, 4), (*ii*) specific control by repression and induction (30), and (*iii*) constitutive control, where enzyme synthesis is independent of nutritional conditions (31). The generalized regulation by cAMP causes only a 2- to 3-fold change in the enzyme levels reported above, in contrast to specific derepression, which may alter the level of enzyme synthesis up to 20-fold and obscure the generalized regulation by cAMP.

In addition to the generalized, specific, and constitutive controls of enzyme synthesis noted above, at least three additional modes of regulation are also operative in enzymatic formation of glutamate and glutamine in *E. coli:* (*i*) allosteric feedback inhibition by specific end products or energy metabolites (1, 2)*, (*ii*) covalent modification of glutamine synthetase by adenylylation (1), and (*iii*) divalent cation modulation of enzyme activity directly and through complex formation with ATP (1, 2).

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