Journal of Supramolecular Structure 9:219–230 (1978)
Transmembrane Signaling 233–244

The Escherichia coli Adenylate Cyclase Complex: Activation by Phosphoenolpyruvate

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A model for the regulation of the activity of Escherichia coli adenylate cyclase is presented. It is proposed that Enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) interacts in a regulatory sense with the catalytic unit of adenylate cyclase. The phosphoenolpyruvate (PEP)-dependent phosphorylation of Enzyme I is assumed to be associated with a high activity state of adenylate cyclase. The pyruvate or sugar-dependent dephosphorylation of Enzyme I is correlated with a low activity state of adenylate cyclase. Evidence in support of the proposed model involves the observation that Enzyme I mutants have low cAMP levels and that PEP increases cellular cAMP levels and, under certain conditions, activates adenylate cyclase, Kinetic studies indicate that various ligands have opposing effects on adenylate cyclase. While PEP activates the enzyme, either glucose or pyruvate inhibit it. The unique relationships of PEP and Enzyme I to adenylate cyclase activity are discussed.

Key words: adenylate cyclase, Escherichia coli; adenylate cyclase, interaction with transport proteins; adenylate cyclase, phosphoenolpyruvate activation, sugar transport system, regulatory complex with E. coli; sugar transport system

Our previous studies concerned with the mechanism by which the activity of E. coli adenylate cyclase is inhibited by sugars have indicated that, in toluene-treated cells, adenylate cyclase is inhibited by glucose but not by glucose 6-phosphate [1]. Since the pathway for glucose utilization in E. coli involves the coupled transport and phosphorylation of glucose, it is clear that the inhibition of adenylate cyclase by glucose is dependent on some aspect of the transport mechanism rather than on subsequent metabolism of glucose 6-phosphate. Figure 1 outlines the mechanism by which certain sugars such as glucose are transported into E. coli. This system, which is referred to as the phosphoenolpyruvate:sugar phosphotransferase system (PTS), is energized by phosphoenolpyruvate

Received Feburary 26, 1978; accepted July 26, 1978.

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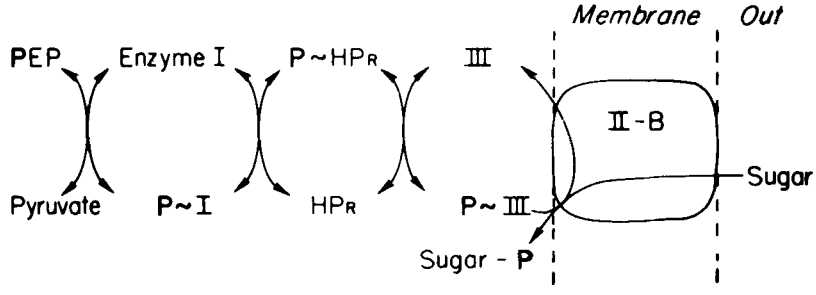

Fig 1. The phosphoenolpyruvate:sugar phosphotransferase system (PTS). The phosphate group derived from phosphoenolpyruvate (PEP) is transferred sequentially to the cytoplasmic proteins Enzyme I (EI), HPr, then to III. Finally, there is a transfer of phosphate to a specific sugar coupled to the transport of the sugar through the membrane. This final step of the PTS is catalyzed by one of a family of membrane-bound, sugar-specific Enzymes II (EII's).

(PEP). The phosphate group of PEP is passed through a chain of soluble proteins (Enzyme I, HPr, and III) which are not sugar-specific. Then there is a coupled phosphorylation and transport of sugar catalyzed by a membrane-bound sugar-specific Enzyme II [2].

We have pursued the hypothesis that some PTS protein serves as a regulator of adenylate cyclase activity [3]. In a preliminary study [4] we noted that a leaky Enzyme I mutant had an aberrant cAMP metabolism. It had low intracellular cAMP levels. Toluene-treated cells of this strain had low adenylate cyclase activity which could be substantially stimulated by assay in the presence of PEP. We hypothesized that these observations are consistent with a model for regulation of adenylate cyclase activity involving the PTS protein Enzyme I. It was suggested that when Enzyme I is phosphorylated, adenylate cyclase is active and when adenylate cyclase is dephosphorylated adenylate cyclase is inhibited. The present studies were designed to characterize further the effect of PEP on adenylate cyclase and the connection of the PTS to the mechanism for regulation of the enzyme. The data further provide support for the originally proposed model and explain the unique dependence of adenylate cyclase in the leaky Enzyme I mutant on PEP.

METHODS

Bacterial strains used in this study have been described previously [4, 5]. A wild-type E. coli, strain 1100, is isogenic with a leaky HPr mutant, strain 1101, and a leaky Enzyme I mutant, strain 1103. Strain 95, also known as N2679 [6], carries a deletion of the gene for Enzyme I. All strains were grown in a New Brunswick gyrotory shaker at 37° in salts medium [7] supplemented either with 1% nutrient broth or with 0.1% glucose, as indicated. Adenylate cyclase in toluene-treated cells was determined as previously described [1]. Assays for intracellular cAMP levels have also been described [8]. Measurements of PTS-dependent phosphorylation of α-methylglucoside were carried out by a previously described method [12].

TABLE I. Intracellular cAMP Levels in E. coli

Strain	Genotype	Intracellular cAMP (μM)
1100	Wild-type	4.81
1101	HPr$^-$ (leaky)	4.04
1103	EI$^-$ (leaky)	0.71
95	EI$^-$ (deletion)	0.64

The indicated strains were grown in salts medium [7] supplemented with 1% nutrient broth. At logarithmic phase, aliquots of the cultures were processed for determinatin of intracellular cAMP as described [8]. Data adapted from [4] and [10].

RESULTS

Intracellular cAMP levels in E. coli are in part a reflection of the activity of adenylate cyclase [8]. Table I shows the levels of this nucleotide measured in strains carrying mutations in the PTS. It can be seen that a leaky mutant in the HPr protein has a normal cAMP concentration. However, mutations in Enzyme I, whether leaky or deletion, have low cAMP levels. As previously suggested [4], the correlation of low cAMP levels with defective Enzyme I led to the idea that adenylate cyclase activity is regulated by the PTS.

Another indication that PTS activity influences adenylate cyclase comes from the study shown in Figure 2. The addition of PEP, the energy source for the PTS, to growing cells of either a wild-type or a leaky Enzyme I mutant produces a transient increase in cellular cAMP. Our interpretation of this finding is that an increase in the concentration of some phosphorylated intermediate of the PTS results in a stimulation of adenylate cyclase activity.

We previously showed that the adenylate cyclase activity in toluene-treated cells of a leaky Enzyme I mutant was substantially stimulated by assay in the presence of PEP [4]. The data in Figure 3 further characterize this phenomenon. Cultures of wild-type E. coli (strain 1100) or strains carrying leaky mutations in HPr (strain 1101) or Enzyme I (strain 1103) were harvested at various culture densities. The cells were then tested for adenylate cyclase activity in toluene-treated cells in the absence or presence of PEP (1 mM). In the wild-type strain adenylate cyclase activity decreases with increasing cell density (Fig 3, top panel). Only a small part of the decrease in adenylate cyclase activity can be explained by a deficit in the phosphorylation state of the enzyme, since assay in the presence of PEP results in only a small stimulation of activity at cell densities above 0.7 A_{650}. In the leaky HPr mutant, strain 1101 (Fig 3, center panel), the relationship of adenylate cyclase activity to culture density is opposite that seen with the wild-type and there is no effect of added PEP at any stage of growth. The most unique behavior is associated with the leaky Enzyme I mutant, strain 1103 (Fig 3, bottom panel). The basal activity of the enzyme is generally lower than wild-type with a peak of activity at approximately $A_{650} = 0.3$. In contrast to the other two strains, at all stages of growth the adenylate cyclase activity in strain 1103 is markedly stimulated by assay in the presence of PEP. It is possible that the leaky Enzyme I mutant is characterized by having a high dose of enzyme that is in a relatively inactive form; assay in the presence of PEP may allow full expression of this activity.

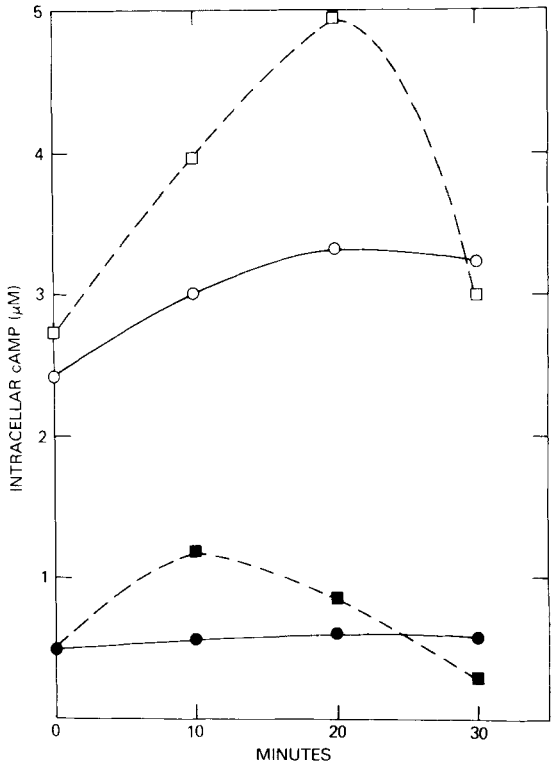

Fig 2. Phosphoenolpyruvate-dependent increase in cellular cAMP. E. coli strains 1100 (wild-type) or 1103 (leaky Enzyme I mutant) were grown on salts medium [7] supplemented with 1% nutrient broth. At A_{650} = 0.5, the cultures were divided and PEP (final concentration 10 mM) was added to one portion. Shaking at 37° was continued for the next 30 min. At the indicated times, aliquots (30 ml) were removed for the estimation of intracellular cAMP, as described elsewhere [8]. □- - -□, strain 1100 plus PEP; ○—○, strain 1100 minus PEP; ■ - - - ■, strain 1103 plus PEP; ●—●, strain 1103 minus PEP.

The nature of the growth-condition-dependent expression of adenylate cyclase activity in the leaky Enzyme I mutant was further explored by the experiments outlined in Table II. Experiment I shows that the lower adenylate cyclase activity characteristic of high culture densities is not due to the accumulation of an inhibitor in the medium. If medium recovered from previous growth of cells (used medium) was resupplemented with 1% nutrient broth, strain 1103 grew at the same rate as in fresh medium. When grown in either fresh or used medium to a density of 0.3 A_{650}, adenylate cyclase activity was present at comparable basal levels and was substantially stimulated by assay in the presence of PEP. Experiment II shows that old cells are devoid of adenylate cyclase activity, but that a short period of growth is sufficient to regenerate the normal level of activity. Therefore, some component of the adenylate cyclase complex may be unstable in stationary phase cells. It cannot be ascertained from these experiments whether that component is the adenylate cyclase catalytic unit or some PTS protein.

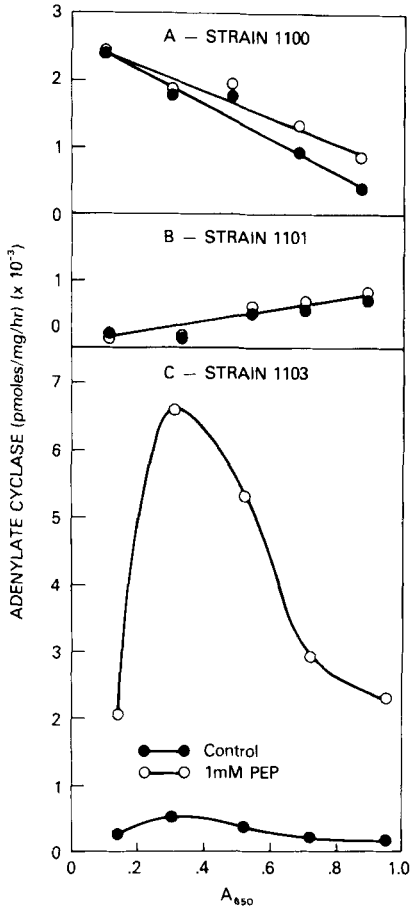

Fig 3. Effect of growth stage on adenylate cyclase activity. Strains 1100 (wild-type), 1101 (leaky HPr mutant), and 1103 (leaky Enzyme I mutant) were grown in salts medium [7] supplemented with 1% nutrient broth. At the culture densities indicated, aliquots were removed, centrifuged, and washed. The cell suspensions were then permeabilized with toluene and assayed for adenylate cyclase activity in the absence or presence of 1 mM PEP, as described elsewhere [1].

The data of Figure 4 suggest that the growth-phase-dependent differences in adenylate cyclase activity in strain 1103 may be due to changes in the state of the Enzyme I protein. A profile of the effect of PEP concentration on adenylate cyclase activity in early compared to late logarithmic phase cells was established. It is apparent that not only is the activity of adenylate cyclase lower at late logarithmic phase but also the pattern of activation by PEP is different. Since it appears that PEP activation of adenylate cyclase is mediated by Enzyme I, these data suggest that the capability of Enzyme I to interact with PEP varies with the stage of growth.

TABLE II. Effect of Growth Condition on Adenylate Cyclase Activity in a Leaky Enzyme I Mutant

	Adenylate cyclase activity (pmoles/mg hr)	
	– PEP	+ PEP
Experiment I		
a) Growth in fresh medium	230	2000
b) Growth in used medium	276	3538
Experiment II		
a) Overnight culture	0	0
b) Overnight culture diluted to A_{650} = 0.3 with fresh medium	0	0
c) Overnight culture diluted to A_{650} = 0.15 with fresh medium and grown to A_{650} = 0.3	240	4920
d) Overnight culture diluted to A_{650} = 0.03 with fresh medium and grown to A_{650} = 0.3	360	6120

E. coli Strain 1103 (leaky Enzyme I mutant) was grown in salts medium [7] supplemented with nutrient broth. In all cases, except for Experiment IIa, cells were harvested at A_{650} = 0.3 and processed for assay of adenylate cyclase in toluene-treated cells as described [1]. Where indicated, assays were carried out in the presence of PEP (final concentration = 1 mM).

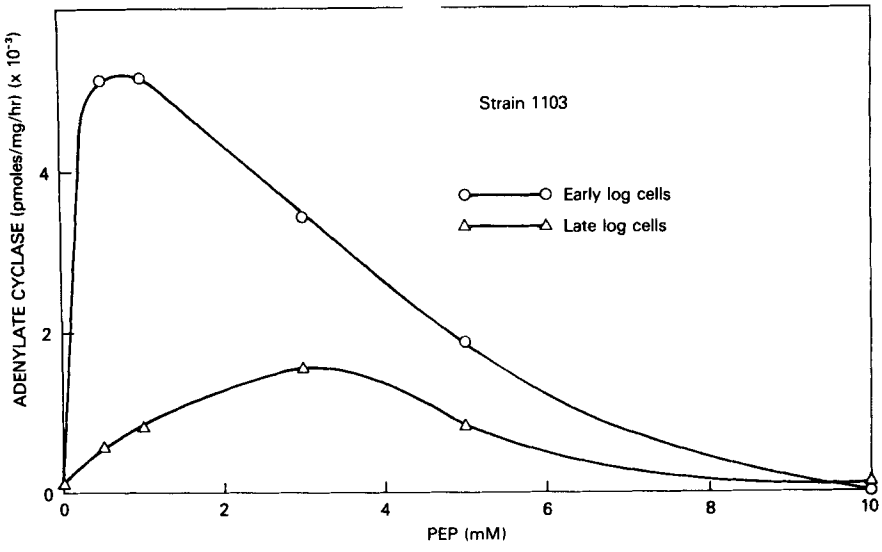

Fig 4. Effect of phosphoenolpyruvate concentration on adenylate cyclase activity. Strain 1103 (leaky Enzyme I mutant) was grown in salts medium [7] supplemented with nutrient broth. Aliquots of the culture were processed at early logarithmic phase (A_{650} = 0.29) and at late logarithmic phase (A_{650} = 0.68). Washed cells were permeabilized with toluene and tested for adenylate cyclase activity in the presence of the indicated concentrations of PEP.

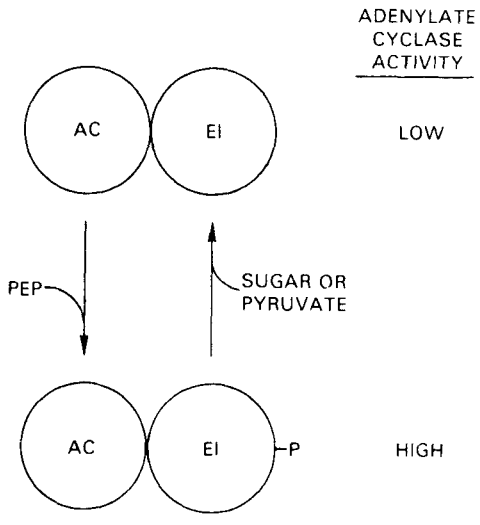

Fig 5. A phosphorylation–dephosphorylation model for the regulation of adenylate cyclase activity. Adenylate cyclase (AC) is represented to be functionally complexed with Enzyme I (EI) of the PTS. The complex in which EI is dephosphorylated has low AC activity, whereas the complex in which EI is phosphorylated is characterized by high AC activity. EI phosphorylation is stimulated by PEP, whereas EI dephosphorylation is stimulated either by pyruvate or sugar substrates of the PTS.

On the basis of the data presented here and elsewhere [4], a model for the regulation of adenylate cyclase activity by the state of phosphorylation of the PTS Enzyme I is proposed (see Figure 5). It is suggested that Enzyme I forms a functional complex with adenylate cyclase. The PEP-dependent phosphorylation of Enzyme I converts the complex to an activated state. The PEP-dependent reaction can be directly reversed by pyruvate, leading to a low-activity state of the enzyme. In the presence of other components of the PTS, a sugar substrate of the PTS can also act as phosphate acceptor and convert the complex to a low activity state. The following experiments were designed to test this model further.

The possibility was considered that the PEP-dependence for high adenylate cyclase activity in the leaky Enzyme I mutant might be due to a requirement for PEP to maintain the level of ATP, the substrate for the adenylate cyclase reaction. This might be accomplished by a pyruvate kinase ATP regeneration reaction or by some unspecified protection of ATP against degradation. The data of Figure 6 indicate that this possibility can be eliminated. Toluene-treated cells of the wild-type and leaky Enzyme I mutant were incubated under conditions for the adenylate cyclase reaction, and the concentration of ATP was estimated over a period of 40 min. It can be seen that, in the absence of added PEP, the rate of disappearance of ATP was not significantly faster in toluene-treated cells of the leaky Enzyme I mutant than in those of the wild-type strain. During the course of this experiment, the rate of cAMP formation was linear, indicating that ATP concentration was not becoming a rate-limiting factor.

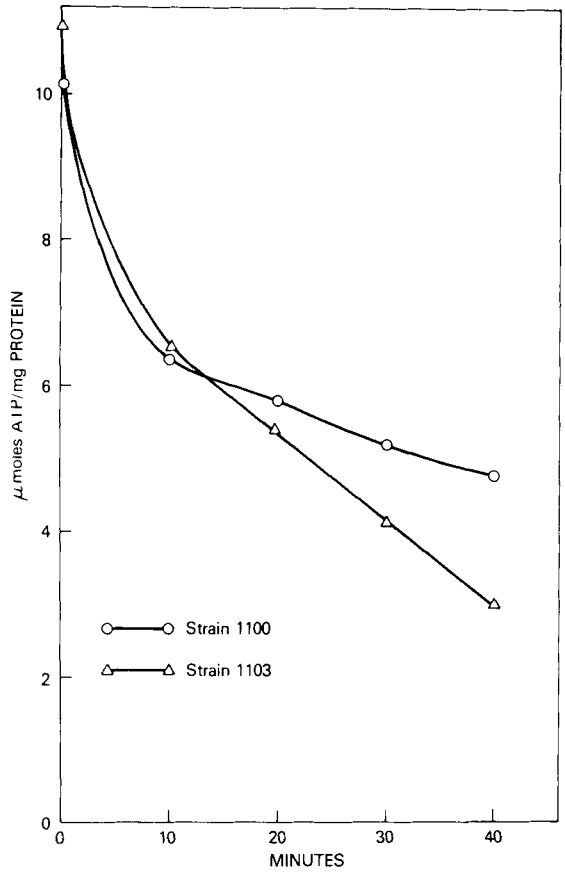

Fig 6. The rate of ATP utilization in toluene-treated cells. E. coli strains 1100 (wild-type) and 1103 (leaky Enzyme I mutant) were grown in salts medium [7] supplemented with 1% nutrient broth. Logarithmic phase cells were processed for the assay of adenylate cyclase, as described. Incubation mixtures containing all the additions for adenylate cyclase assay in toluene-treated cells (except that the ATP was not radioactive) were prepared. After incubation at 30° for the indicated times, aliquots (0.1 ml) were removed into tubes containing 0.1 ml HCOOH (0.5 M). The samples were then diluted to 2 ml with pH 7.4 buffer and assayed for ATP by the luciferase procedure as described elsewhere [11].

The model described in Figure 5 is consistent with the prediction that there is an opposing effect of PEP and glucose on adenylate cyclase activity, where PEP activates the enzyme and glucose inhibits it. The data in Figure 7A (wild-type) and 7B (leaky Enzyme I mutant) provide support for this hypothesis. With toluene-treated cells of either strain, sequential additions of either glucose or PEP produce the predicted results.

Another prediction of the model of Figure 5 is that there is a competitive effect of PEP and pyruvate on adenylate cyclase activity. The experiment shown in Figure 8, in which toluene-treated cells of the wild-type strain were used, supports this idea. The substantial inhibition of adenylate cyclase by 0.5 mM pyruvate is increasingly counteracted by graded concentrations of PEP up to 2 mM. The reciprocal plot shown is compatible with a competitive relationship between pyruvate (the inhibitor) and PEP (the activator).

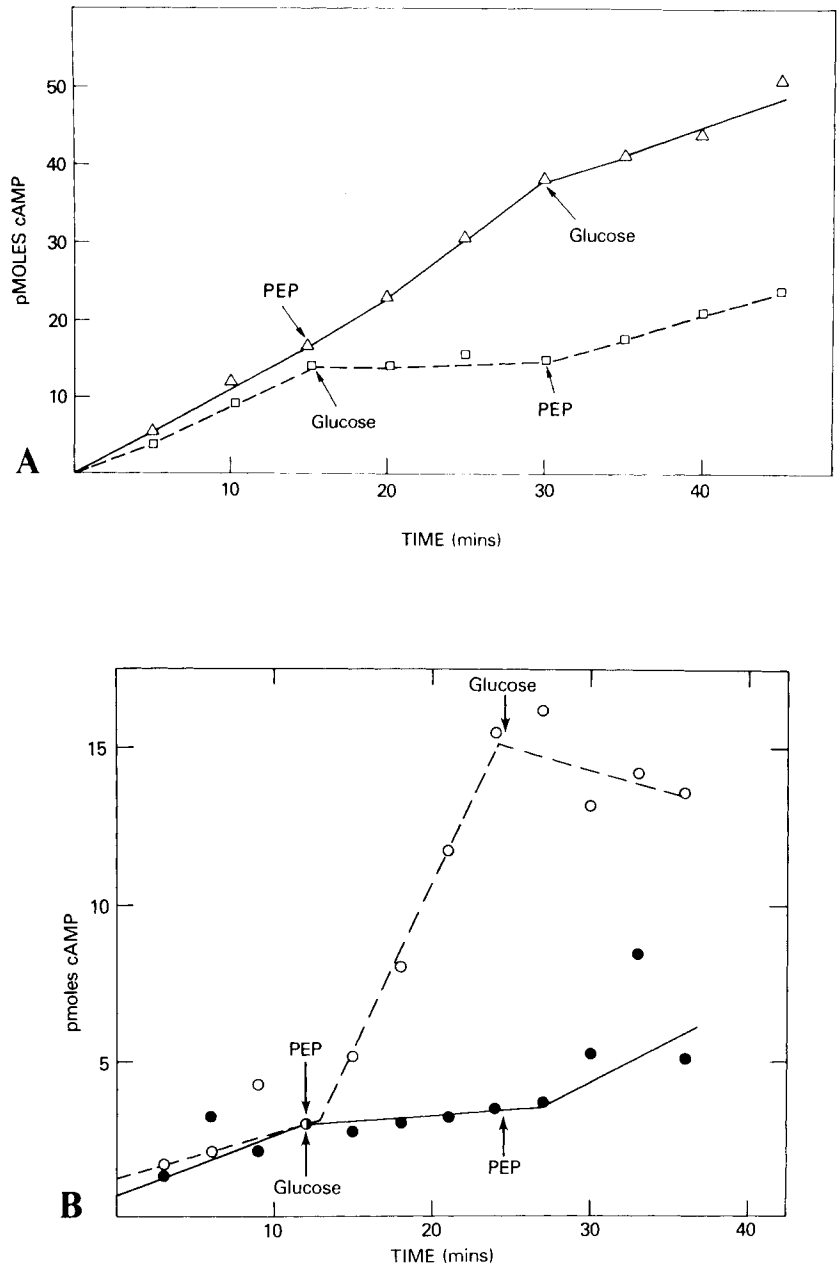

Fig 7. Opposing effects of phosphoenolpyruvate and glucose on adenylate cyclase activity. E. coli strains 1100 (wild-type), panel A, and 1103 (leaky Enzyme I mutant), panel B, were grown and processed for adenylate cyclase activity in toluene-treated cells as in Figure 3. Adenylate cyclase incubation mixtures (1 ml) were prepared and incubated at 30°. At the indicated times, aliquots (0.1 ml) were removed for assay of cAMP [1]. At the times indicated by arrows, PEP or glucose (final concentration = 1 mM) were added to the incubation mixtures.

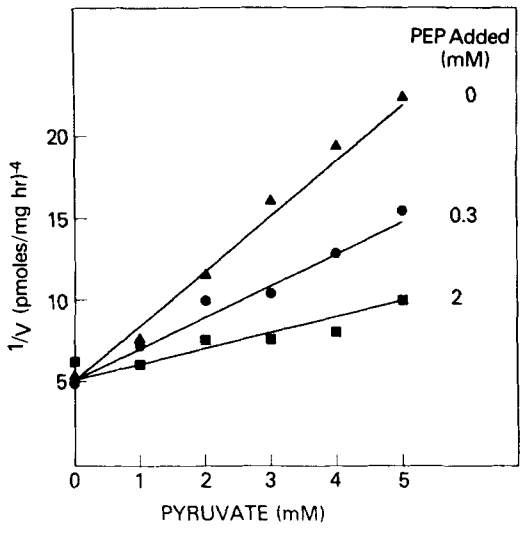

Fig 8. Opposing effects of phosphoenolpyruvate and pyruvate on adenylate cyclase activity. Toluene-treated cells of E. coli strain 1100 (wild-type), A_{650} = 0.35, were assayed for adenylate cyclase activity in the absence or presence of the indicated concentrations of pyruvate. Where indicated, incubation mixtures also contained the designated concentrations of PEP. Each incubation mixture (0.1 ml) contained 0.054 mg protein of permeabilized cells.

Toluene-treated cells of the wild-type strain generally show no increase in adenylate cyclase activity by assay in the presence of PEP (see Figure 7A). The notion that the phosphorylated state of Enzyme I is necessary for maximum adenylate cyclase activity (see Figure 5).presupposes that these cells may have a pool of PEP to maintain Enzyme I in the phospho-form. The experiment in Table III explores this question. When cells are cultured in minimal medium supplemented with glucose, there is a higher basal activity that is seen when cells are cultured in nutrient broth medium (see Figure 3). In addition, the activity is increased approximately 50% by assay in the presence of PEP. Most noteworthy is the substantial inhibition of the activity by assay in the presence of a combination of ADP and pyruvate kinase. A likely interpretation of this inhibition is that the pyruvate kinase–ADP system reacts with endogenous PEP to produce pyruvate. The pyruvate produce can react with phospho-Enzyme I (see Figure 5) to form dephospho-Enzyme I and convert adenylate cyclase to the low-activity form. This interpretation is further supported by the observation (Table III) that the combination of PEP, ADP, and pyruvate kinase results in even greater inhibition, presumably by forming even higher concentrations of pyruvate.

The previous experiments have indicated that adenylate cyclase in toluene-treated cells of the leaky Enzyme I mutant has the unique property of being activated markedly by PEP. The experiment shown in Figure 9 provides an explanation for this property of strain 1103. A study of the effect of PEP concentration on PTS activity in toluene-treated cells of the wild-type strain (1100) and the leaky Enzyme I mutant (1103) was carried out. It is clear that maximum PTS activity requires much higher PEP concentrations in strain 1103 than in strain 1100. Half maximal activity is achieved at approximately 10 μM PEP for strain 1100, and at about 450 μM for strain 1103. It is therefore apparent that the mutation in Enzyme I in strain 1103 has resulted in an approximately 50-fold increase in the Km for PEP, as well as perhaps a change in the maximum velocity of the PTS.

TABLE III. Inhibition of Adenylate Cyclase by a Phosphoenolpyruvate Utilization System

Additions	Adenylate cyclase activity (pmoles/mg hr)
None	6000
PEP	9523
ADP	5714
ADP + pyruvate kinase	2380
ADP + ammonium sulfate	6095
PEP + ADP	5333
PEP + ADP + pyruvate kinase	1333

E. coli strain 1100 (wild-type) was grown to A_{650} = 0.6 in salts medium [7] supplemented with glucose (0.1%) and thiamine (5 μg/ml) and processed for preparation of toluene-treated cells. Toluene-treated cells (0.073 mg protein/0.1 ml of incubation mixture) were assayed for adenylate cyclase activity [1]. Where indicated, PEP (0.1 mM), ADP (1 mM) and pyruvate kinase (320 units/mg, 1 unit/0.1 ml) were added to the incubation mixtures. The pyruvate kinase was a suspension in 2.2 M ammonium sulfate (Sigma). Where indicated, a concentration of ammonium sulfate equivalent to that added with pyruvate kinase was added to one incubation mixture.

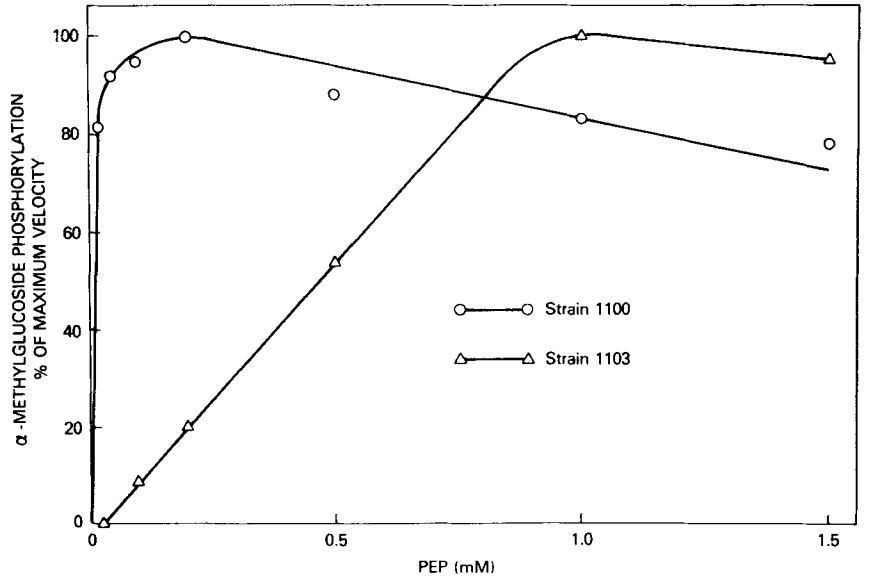

Fig 9. The effect of phosphoenolpyruvate concentration on α-methylglucoside phosphotransferase activity. Toluene-treated cells of E. coli strains 1100 (wild type) and 1103 (leaky Enzyme I mutant) were assayed under standard adenylate cyclase assay conditions [1] for their capacity to phosphorylate [^{14}C]-α-methylglucoside. Master incubations (0.25 ml) contained Tris buffer, pH 8.5, 25 mM; ATP, 1 mM; $MgCl_2$, 20 mM; dithiothreitol, 1 mM; [^{14}C]-α-methylglucoside, 6540 cpm/nmole, 0.1 mM; and the indicated concentrations of PEP. The reactions were initiated at 30° by the addition of permeabilized cells of strain 1100 (3.05 μg of protein) or strain 1103 (220 μg of protein). Aliquots (0.05 ml) were withdrawn after 5, 10, 15, and 20 min of incubation and assayed for the formation of α-methylglucoside-6-phosphate as previously described [12]. The rate of phosphorylation of α-methylglucoside was calculated by drawing the best line through the four time points. The activities presented are corrected for the blank value seen in the absence of added PEP. The maximum specific activity (expressed as pmoles of α-methylglucoside-6-phosphate formed/h/mg protein) observed with strain 1100 was 1,313,000 and with strain 1103 was 56,600.

DISCUSSION

The model presented in Figure 5 provides an attractive working hypothesis for describing the interrelationship of E. coli adenylate cyclase and the PTS. Many of the data presented here as well as in previous studies fits this description of the system. The observation that the leaky Enzyme I mutant, strain 1103, has a high Km for PEP for catalysis of sugar phosphorylation does much to explain the unique behavior of leaky Enzyme I mutants; they have low intracellular cAMP and the adenylate cyclase activity in toluene-treated cells is substantially stimulated by PEP. The capacity of adenylate cyclase to be inhibited by a PEP trapping system (ADP plus pyruvate kinase) suggests that toluene-treated cells have a pool of endogenous PEP that maintains adenylate cyclase in an active form. It appears that, in the high Km leaky Enzyme I mutant, the concentration of the endogenous pool of PEP is insufficiently high to saturate the Enzyme I; this explains the further stimulation of adenylate cyclase by high concentrations of PEP.

The demonstration that the phosphorylatable compounds pyruvate or glucose both inhibit adenylate cyclase indicates the dynamic nature of the regulation of adenylate cyclase by multiple effectors. The common feature of this regulatory system may be the fraction of Enzyme I that is in the phospho-form [9]. It is hoped that it will be possible, using purified components, to reconstruct an adenylate cyclase system that shows the appropriate regulatory responses to sugar, PEP, and pyruvate. Until that time, this model for regulation of adenylate cyclase by PTS must be evaluated as an attractive speculation.

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