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Reconstitution of regulatory properties of adenylate cyclase in *Escherichia coli* extracts

(phospho*enol*pyruvate:glycose phosphotransferase system/phospho*enol*pyruvate/pyruvate/potassium phosphate/catabolite repression)

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ABSTRACT **The inhibition of adenylate cyclase activity of *Escherichia coli* by methyl α-glucoside has been demonstrated in intact or in permeable cells but not in cell-free extracts. In intact or permeable cells, this inhibition is demonstrable only in strains expressing the genes for proteins of the phospho*enol*pyruvate:glycose phosphotransferase system (PTS); in permeable cells, the inhibition also requires potassium phosphate. Using homogeneous proteins of the PTS, we have reconstituted in cell-free extracts many of the features of the regulated form of adenylate cyclase: (*i*) In the absence of K_2HPO_4, permeable cells have lower adenylate cyclase activity than extracts; addition of homogeneous PTS proteins to the extracts brings adenylate cyclase activity close to the level observed in permeable cells. (*ii*) The low activity observed in permeable cells is stimulated by potassium phosphate; this stimulation is also observed in extracts supplemented with PTS proteins and phospho*enol*pyruvate. (*iii*) In permeable cells, potassium phosphate-stimulated adenylate cyclase activity is inhibited by methyl α-glucoside or pyruvate; extracts behaved similarly when supplemented with PTS proteins, K_2HPO_4, and phospho*enol*pyruvate. Thus, the regulated form of adenylate cyclase has been reconstituted in cell-free extracts by addition of homogeneous PTS proteins.**

In *Escherichia coli*, cAMP plays a crucial role in regulating the expression of inducible genes (1). The levels of this nucleotide are controlled primarily by a catabolite-dependent modulation of adenylate cyclase activity (2). Insight into the mechanism of regulation of the activity of this enzyme has come primarily from studies of permeable cells. Current information indicates that the phospho*enol*pyruvate:glycose phosphotransferase system (PTS) (3) is intimately involved in the regulation (4). Additionally, potassium and phosphate ions play key roles in modulating adenylate cyclase activity (5). A model for interaction of adenylate cyclase with PTS proteins and potassium phosphate to form a regulatory complex has been proposed (5). The purpose of the present studies was to test the proposed model for adenylate cyclase regulation using a reconstitution approach. All of the unique features of adenylate cyclase characteristic of the regulatory complex observed in permeable cells were reconstituted in cell-free extracts. The results strongly support the proposal that adenylate cyclase activity is regulated by PTS proteins.

MATERIALS AND METHODS

Materials. Phospho*enol*pyruvate (*P-e*Prv), pyruvate, creatine phosphate, and creatine phosphokinase were obtained from Sigma. Methyl α-D-glucopyranoside was a product of Pfanstiehl Laboratories (Waukegan, IL). Methyl α-glucoside 6-phosphate was prepared by the PTS-mediated phosphorylation of methyl α-glucoside followed by two cycles of purification by Dowex-1 chromatography eluting with a gradient of triethylammonium bicarbonate (pH 7). [α-^{32}P]ATP was from New England Nuclear. All other chemicals were of analytical grade.

Growth of Bacteria and Induction of Adenylate Cyclase. *E. coli* strain MZ1 harboring plasmid pPR57 (6) encoding the adenylate cyclase gene under the control of the λ P_L promoter was grown at 32°C to early logarithmic phase (OD_{650} = 0.3–0.4) in salts medium E (7) supplemented with 0.8% Difco nutrient broth, 0.5% glucose, and ampicillin (25 μg/ml). An equal volume of the medium at 65°C was added, and incubation was continued at 42°C for 2 hr. Under these conditions, the P_L promoter was activated, and adenylate cyclase was overproduced.

Preparation of Permeable Cells. Ten milliliters of cells grown as above were harvested by centrifugation at 8000 × *g*, washed with 25 mM *N,N*-bis(2-hydroxyethyl)glycine (Bicine), pH 8.5, and suspended in 1 ml of this buffer. Cells were permeabilized by treatment with toluene as described (2). Protein concentrations of permeable cell preparations were about 2 mg/ml.

Preparation of Extracts. For comparison of adenylate cyclase activities in extracts and permeable cells, extracts were prepared from the same concentration of cells in suspension in Bicine buffer as was used for permeable cells (5). For reconstitution studies, however, concentrated extracts (about 40 mg/ml) were prepared from 500 ml of cells grown as above. The cells were harvested, washed with 25 mM Bicine, pH 8.5, and suspended in 2 ml of Bicine buffer. The cells were broken by two passes through an Aminco French pressure cell at 11,000 psi (1 psi = 6.89 kPa). The viscous extract was treated with DNase (10 μg/ml) for 10 min at 37°C. Glycerol was added to the extract to a concentration of 10% (vol/vol). The extract was divided into aliquots (200 μl) and stored at −70°C. Adenylate cyclase was stable for at least 5 months under these conditions. The concentration of PTS proteins in the extract was assessed by rocket immunoelectrophoresis and found to be at levels previously reported for *Salmonella typhimurium* (8–10).

PTS Proteins. Enzyme I (8) and HPr (9) were purified to homogeneity from a strain of *E. coli* harboring a plasmid containing genes for Enzyme I and HPr by the procedure described for *S. typhimurium* proteins. The sugar-specific protein III^{Glc}_{Slow} was purified to homogeneity from *E. coli* 1100 by procedure B described for *S. typhimurium* (10) except that HPLC-ion exchange chromatography was used for the final step. This procedure produces III^{Glc}_{Slow} containing no detectable III^{Glc}_{Fast}.

Adenylate Cyclase Assay. The enzyme assays (2, 5) were carried out at 30°C for 20 min in reaction volumes as specified

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Abbreviations: PTS, phospho*enol*pyruvate:glycose phosphotransferase system; *P-e*Prv, phospho*enol*pyruvate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

in the legends for figures. Each reaction mixture contained 25 mM Bicine (pH 8.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 20 mM creatine phosphate, creatine phosphokinase (100 units/ml) and [α-^{32}P]ATP to give 20–40 cpm/pmol. Reactions were started by adding extract or permeable cells. PTS proteins, K_2HPO_4, *P-e*Prv, methyl α-glucoside, methyl α-glucoside 6-phosphate, and pyruvate were added to the reaction mixtures as indicated in the legends to the figures. In experiments not shown, the rates of the reactions were found to be constant over a period of at least 20 min. The data reported here are representative of at least two experiments. Protein concentration was determined by the method of Lowry *et al.* (11).

RESULTS

PTS Proteins Inhibit Adenylate Cyclase Activity in Extracts. Permeable *E. coli* cells have a regulated adenylate cyclase that is potently inhibited by glucose, whereas the enzyme in extracts is not regulated. Our strategy in reconstructing the regulated form of adenylate cyclase in extracts was to reproduce the unique properties of permeable cells. One important feature of permeable cells prepared in Bicine buffer is that they have ≈25% as much adenylate cyclase activity as a similarly prepared extract (Fig. 1*A*). Our working hypothesis is that the adenylate cyclase in permeabilized cells is in a complex with proteins of the PTS whereas adenylate cyclase in the extract is uncoupled from the PTS. Therefore, exogenously added PTS proteins should inhibit the adenylate cyclase in cell extracts. The data shown in Fig. 1*B* describe the effects of added PTS proteins on adenylate cyclase in an extract. Various concentrations of PTS proteins up to the amount corresponding to cellular levels (8–10) were added. The individual proteins produced a variety of effects. Enzyme I addition resulted in a dose-dependent inhibition of adenylate cyclase; at cellular levels (36 μg per 50 μl), the inhibition was about 30%. Addition of III^{Glc}_{Slow} inhibited adenylate cyclase by 10–15% at all levels tested. In contrast to the inhibitory effects of Enzyme I and III^{Glc}_{Slow}, addition of HPr led to a dose-dependent increase in adenylate cyclase activity; a cellular level of HPr (108 μg per 50 μl) produced a 25% stimulation. When all three PTS proteins were added, the most potent inhibition was achieved. At cellular levels, the three PTS proteins produced a 45% inhibition of adenylate cyclase activity. We conclude that the three PTS proteins interact with adenylate cyclase to form an enzyme complex with decreased activity that resembles the adenylate cyclase of permeable cells.

Phosphorylation of PTS Proteins in the Absence of Potassium Phosphate Does Not Affect Adenylate Cyclase Activity. On the basis of data in Fig. 1, we concluded that a partial reconstitution of the adenylate cyclase complex was achieved by addition of cellular levels of Enzyme I, HPr, and III^{Glc}_{Slow} to extracts. Models for regulation of adenylate cyclase have been proposed (4), in which phosphorylation of PTS proteins is accompanied by activation of adenylate cyclase. The inhibition of adenylate cyclase by PTS proteins provided a system for evaluating this hypothesis. The effect of increasing concentrations of *P-e*Prv, which should phosphorylate the PTS proteins, on adenylate cyclase activity was therefore investigated (Fig. 2). Concentrations of *P-e*Prv up to 1.0 mM produced no more than a 15% change in adenylate cyclase activity in either the absence or presence of added PTS proteins. These data suggest that, in the absence of other modulating factors, the state of phosphorylation of PTS proteins does not significantly regulate the level of adenylate cyclase activity.

Potassium Phosphate Stimulates Adenylate Cyclase Activity in Extracts Supplemented with PTS Proteins and *P-e*Prv. An important characteristic of regulated adenylate cyclase in permeable cells is the substantial stimulation of activity by potassium phosphate (5). This effect had not been observed in extracts. When extracts, supplemented with PTS proteins, were tested for the effect of K_2HPO_4 on adenylate cyclase, the activity was found to be stimulated ≈20% (Fig. 3). However, if the PTS proteins were phosphorylated as a result of inclusion of *P-e*Prv in the assay, the stimulation by potassium phosphate was increased to 60%. Thus, another essential feature of regulated adenylate cyclase was restored.

Earlier experiments using *E. coli* 433, which has a 10-fold overproduction of adenylate cyclase, demonstrated a 7-fold stimulation by K_2HPO_4 in permeable cells (5). The strain of *E. coli* used in the present study (MZ1, harboring plasmid pPR57) which has a 60-fold overproduction of adenylate

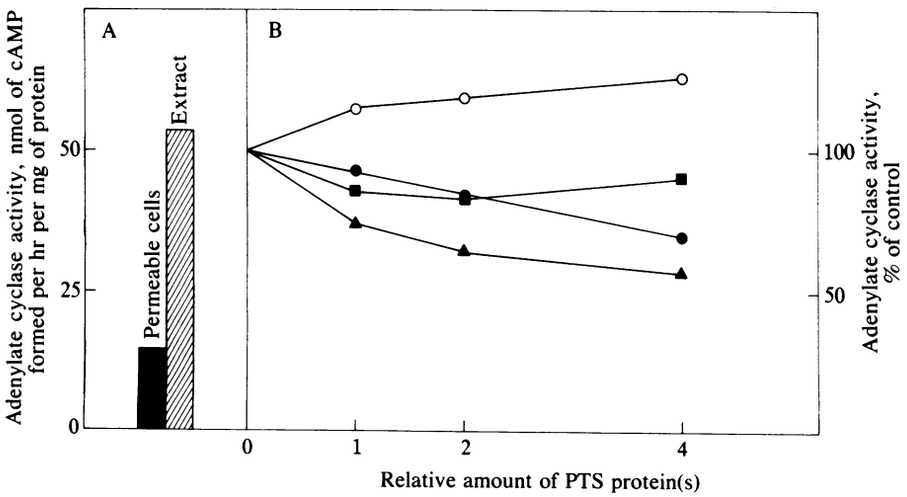

FIG. 1. Inhibition of adenylate cyclase activity in extracts of *E. coli* by PTS proteins. (*A*) Permeable cells and extracts of *E. coli* strain MZ1 harboring plasmid pPR57 (6) were prepared, and adenylate cyclase activity was determined at 30°C using 41 μg of protein from permeable cells or cell extracts in a reaction volume of 0.1 ml (2, 5). (*B*) Concentrated extract (80 μg of protein) from *E. coli* strain MZ1 harboring plasmid pPR57 (6) was assayed for adenylate cyclase activity at 30°C for 20 min in a final volume of 0.05 ml in the absence and presence of added PTS proteins at the indicated concentrations. Enzyme activity represented as 100% corresponds to a specific activity of 22 nmol of cAMP formed per hr per mg of protein. The specific activity of adenylate cyclase in *B* is lower than that in *A* (extract) because of the nonproportionality of the enzyme activity at higher protein concentration. ●, Enzyme I; ○, Hpr; ■, III^{Glc}_{Slow}; ▲, Enzyme I, Hpr, and III^{Glc}_{Slow}. 1× Enzyme I, 9 μg; 1× Hpr, 27 μg; 1× III^{Glc}_{Slow}, 3 μg.

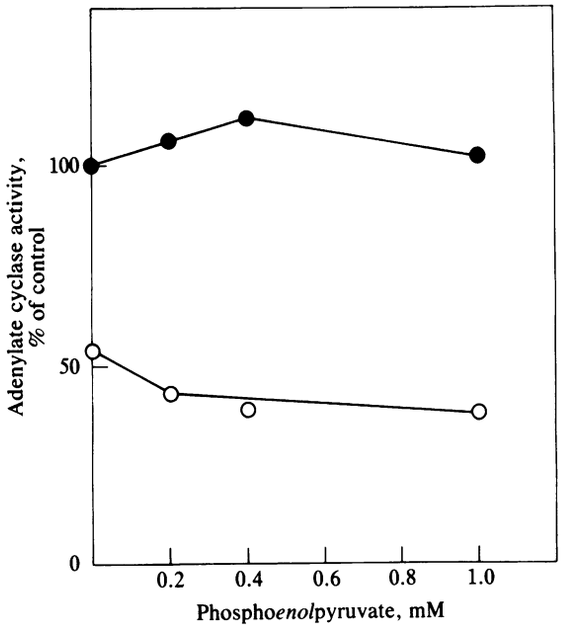

FIG. 2. Effect of *P-e*Prv on adenylate cyclase activity in *E. coli* extract in the absence and presence of PTS proteins. Concentrated extract of *E. coli* MZ1 carrying plasmid pPR57 (6) was prepared, and adenylate cyclase activity in the extract (80 μg of protein) was determined at 30°C for 20 min in a final volume of 0.05 ml in the absence (●) or presence (○) of added PTS proteins (36 μg of Enzyme I, 108 μg of HPr and 12 μg of III^{Glc}_{Slow}) at the indicated concentrations of *P-e*Prv. Enzymatic activity of 100% corresponds to a specific activity of 22 nmol of cAMP formed per hr per mg of protein.

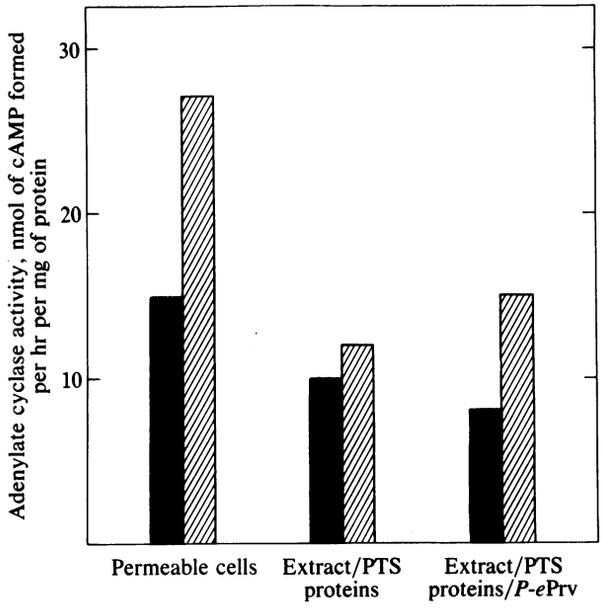

FIG. 3. Effect of potassium phosphate on adenylate cyclase activity in *E. coli* extract reconstituted with PTS proteins. Permeable cells and concentrated extract of *E. coli* strain MZ1 carrying plasmid pPR57 (6) were prepared and the activity of adenylate cyclase in the absence (solid bars) and presence (hatched bars) of 20 mM K_2HPO_4 was measured (5). Permeable cells (41 μg of protein) or an extract (80 μg of protein) reconstituted with PTS proteins (36 μg of Enzyme I, 108 μg of HPr, and 12 μg of III^{Glc}_{Slow}) were tested. Further, the reconstituted extract was also tested in the absence and presence of 1 mM *P-e*Prv as indicated. Assays were carried out at 30°C for 20 min. The reaction volumes were 0.1 ml for permeable cells and 0.05 ml for extracts.

cyclase, showed only a 2-fold stimulation of adenylate cyclase by K_2HPO_4. We established that the difference in magnitude of phosphate stimulation in these two strains was, to some degree, due to the temperature at which the strains were cultured. When strain 433 was grown at 30°C, 37°C, and 42°C, permeable cells showed K_2HPO_4 stimulation of adenylate cyclase activity of 8- to 10-fold, 8- to 10-fold, and 2- to 4-fold, respectively (data not shown).

Methyl α-Glucoside Inhibits Adenylate Cyclase Activity in Extracts Supplemented with PTS Proteins, *P-e*Prv, and Potassium Phosphate. Adenylate cyclase in permeable cells is inhibited by methyl α-glucoside when the incubation mixtures are supplemented with potassium phosphate (Fig. 4). The adenylate cyclase activity in a typical extract is not affected by methyl α-glucoside in either the absence or presence of potassium phosphate (Fig. 4). However, when extracts were supplemented with PTS proteins and *P-e*Prv, the methyl α-glucoside inhibition of adenylate cyclase was clearly restored. Furthermore, as with permeable cells, the adenylate cyclase in the extract supplemented with PTS proteins and *P-e*Prv was inhibited more (55%) in the presence of K_2HPO_4 than in its absence (25%).

Pyruvate Mediates Methyl α-Glucoside-Dependent Inhibi-

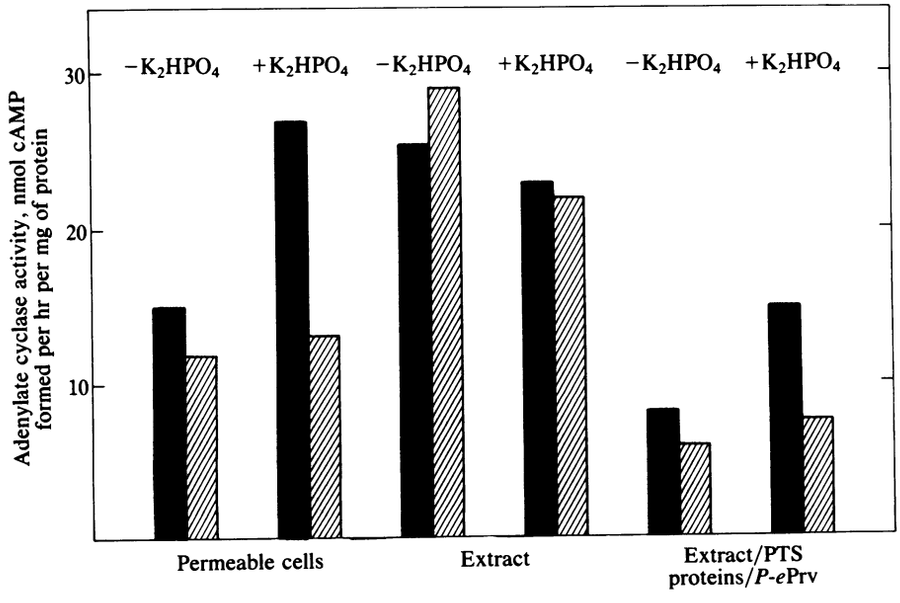

FIG. 4. Effect of methyl α-glucoside on adenylate cyclase activity of *E. coli* permeable cells and extract reconstituted with PTS proteins. Permeable cells and concentrated extract of *E. coli* strain MZ1 harboring plasmid pPR57 (6) were prepared, and permeable cells (41 μg of protein) or concentrated extract (80 μg of protein) were assayed for adenylate cyclase activity in the presence (hatched bars) or absence (solid bars) of 1 mM methyl α-glucoside. As indicated, 20 mM K_2HPO_4, 1 mM *P-e*Prv, and PTS proteins (36 μg of Enzyme I, 108 μg of HPr, and 12 μg of III^{Glc}_{Slow}) were added also. Assays were carried out at 30°C for 20 min. The reaction volumes were 0.1 ml for permeable cells and 0.05 ml for extracts.

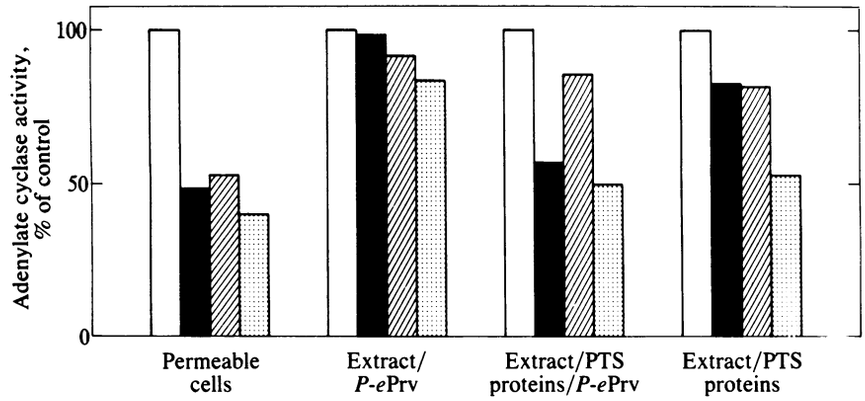

FIG. 5. Effect of methyl α-glucoside, methyl α-glucoside 6-phosphate and pyruvate on adenylate cyclase activity in *E. coli* permeable cells and extract reconstituted with PTS proteins. Permeable cells and concentrated extract of *E. coli* strain MZ1 carrying plasmid pPR57 (6) were prepared. Adenylate cyclase was assayed in permeable cells (41 μg of protein) or concentrated extract (80 μg of protein) in the presence of 20 mM K_2HPO_4. Control samples (open bars); 1 mM methyl α-glucoside (solid bars); 1 mM methyl α-glucoside 6-phosphate (hatched bars); and 1 mM pyruvate (stippled bars). *P-e*Prv (1 mM) or PTS proteins (36 μg of Enzyme I, 108 μg of HPr, and 12 μg of III^{Glc}_{Slow}) were added as indicated. Assays were carried out at 30°C for 20 min. The reaction volumes were 0.1 ml for permeable cells and 0.05 ml for extracts. The values for control activities were: permeable cells, 27; extracts, 22; extracts plus PTS proteins, *P-e*Prv and K_2HPO_4, 15; and extracts plus PTS proteins and K_2HPO_4, 12 nmol of cAMP formed per hr per mg of protein.

tion of Adenylate Cyclase Activity in Reconstituted Extracts. The mechanism of the sugar-dependent inhibition of adenylate cyclase was then explored. Permeable cells supplemented with K_2HPO_4 have adenylate cyclase activity that was inhibited by methyl α-glucoside as well as by both of the PTS-mediated metabolic products of this sugar, methyl α-glucoside 6-phosphate and pyruvate (Fig. 5). The inhibition by methyl α-glucoside, methyl α-glucoside 6-phosphate, or pyruvate was 50–60% (all at final concentrations of 1 mM). The adenylate cyclase in an extract supplemented only with *P-e*Prv and K_2HPO_4 is not sensitive to these compounds. In contrast, the extract reconstituted with PTS proteins and K_2HPO_4 has adenylate cyclase activity that is inhibited about 50% by 1 mM pyruvate. Moreover, if the extract is further supplemented with *P-e*Prv, methyl α-glucoside (1 mM) also inhibits adenylate cyclase activity to a degree similar to that achieved with 1 mM pyruvate. Thus, extracts supplemented with PTS proteins have another feature of the permeable cell system, the pyruvate inhibition of adenylate cyclase. However, the methyl α-glucoside 6-phosphate inhibition of adenylate cyclase has not been restored in the extracts supplemented with PTS proteins.

DISCUSSION

The decrease in cellular cAMP levels accompanying exposure of *E. coli* to a variety of carbon sources to a large measure results from the inhibition of adenylate cyclase (12). Experiments in permeable cells have led to some understanding of the mechanism of this effect. The PTS has been implicated in the control of adenylate cyclase (4), because strains carrying mutations in the PTS have altered activity and/or regulation of adenylate cyclase. It has been suggested that the state of phosphorylation of the PTS proteins modulates the activity of adenylate cyclase (4). Potassium and phosphate ions have been shown to stimulate the activity of adenylate cyclase, possibly mediated through an effect on the PTS (2, 5). An understanding of the precise mechanism by which these factors modulate the activity of adenylate cyclase has been hampered by the unresponsiveness of *E. coli* extracts to these effectors. Our working model has been that the adenylate cyclase in cells exists as a noncovalent complex of the enzyme with PTS proteins and the cytoplasmic membrane (Fig. 6) and that in extracts this complex dissociates by dilution. Therefore, addition of sufficient amounts of PTS proteins and adenylate cyclase to extracts, to approximately cellular levels, should reconstitute the regulated adenylate cyclase system. In the experiments described in this report, the PTS proteins were added as homogeneous components. To experimentally approach cellular levels of adenylate cyclase in extracts, we used a strain of *E. coli* (MZ1) harboring a plasmid encoding the adenylate cyclase gene, which can, under the appropriate growth conditions, overproduce adenylate cyclase approximately 60-fold (6). Using this approach, we were successful in reconstituting the regulated form of adenylate cyclase.

Addition of the PTS proteins (Enzyme I, HPr, and III^{Glc}_{Slow}) to cellular levels leads to a 45% inhibition of adenylate cyclase. This does not completely account for the 70% decrease in adenylate cyclase activity observed when permeable cells are compared to an extract (Fig. 1). Thus, we suspect that there is at least one other factor that is still deficient in the extract that limits the effectiveness of the coupling of PTS proteins to adenylate cyclase. Another reason to believe that other factor(s) are involved in regula-

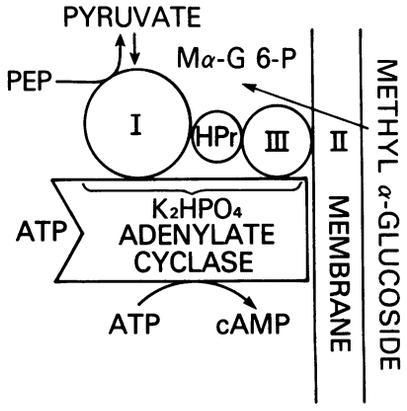

FIG. 6. A model for the regulatory complex of adenylate cyclase. Potassium phosphate in conjunction with PTS proteins in their phosphorylated forms interacts with adenylate cyclase activating it. The transport of methyl α-glucoside into the cell is accompanied by phoshorylation of methyl α-glucoside by a pathway that involves the sequential transfer of a phosphate group from *P-e*Prv to Enzyme I, HPr, III^{Glc}_{Slow}, and finally to a membrane-bound Enzyme II. Pyruvate formed in the PTS reaction interacts with Enzyme I. The pyruvate-dephospho-Enzyme I complex lowers adenylate cyclase activity. PEP, *P-e*Prv; Mα-G 6-P, methyl α-glucoside 6-phosphate.

tion of adenylate cyclase is that stimulation of adenylate cyclase activity by K_2HPO_4 in extracts supplemented with PTS proteins and *P-e*Prv is not observed when extracts are diluted (data not shown). We suspect that a coupling factor necessary for activity stimulation by K_2HPO_4 is limiting in our extracts.

The effects of PTS proteins are specific since supplementation of *E. coli* extracts with another *E. coli* protein, β-galactosidase (140 μg per 50 μl), has no effect on activity. Further, the three PTS proteins differentially regulate adenylate cyclase. The inhibitory effect of Enzyme I is mirrored by the stimulatory effect of HPr. Possibly, the binding of endogenous Enzyme I to adenylate cyclase in the extract inhibits the adenylate cyclase activity, while added HPr reverses this inhibition by formation of a HPr-Enzyme I complex. The demonstration that maximal inhibition of adenylate cyclase is achieved in the presence of the three PTS proteins suggests that the three proteins form yet another functional complex that can interact with adenylate cyclase and decrease its activity.

When the adenylate cyclase activity of permeable cells is fully stimulated by K_2HPO_4, addition of methyl α-glucoside results in 50% inhibition of the enzyme activity (Fig. 4). Similarly, the adenylate cyclase activity of the fully reconstituted extract is inhibited 55% by methyl α-glucoside (Fig. 4). The adenylate cyclase activity in permeable cells is inhibited by a variety of metabolites including methyl α-glucoside, methyl α-glucoside 6-phosphate, and pyruvate (Fig. 5). When extracts were supplemented with PTS proteins, the inhibitory effect of methyl α-glucoside and pyruvate, but not of methyl α-glucoside 6-phosphate was restored. We propose that there is a phosphatase in permeable cells that can dephosphorylate methyl α-glucoside 6-phosphate; this phosphatase may be labile or inactive in extracts. Accordingly, we propose that inhibition of adenylate cyclase activity by methyl α-glucoside 6-phosphate in permeable cells is actually mediated by the methyl α-glucoside generated by the phosphatase.

It has been proposed that the state of PTS protein phosphorylation plays an important role in the regulation of adenylate cyclase (12). The inhibition of adenylate cyclase by methyl α-glucoside in permeable cells may reflect this type of regulation. While it has been shown that adenylate cyclase activity in permeable cells is inhibited by pyruvate, inhibition of adenylate cyclase activity by methyl α-glucoside in the absence of *P-e*Prv in permeable cells is not accompanied by the accumulation of sufficient pyruvate to account for such an inhibition (13). In contrast, the inhibition of adenylate cyclase in extracts reconstituted with PTS proteins and K_2HPO_4 is inhibited by methyl α-glucoside only in the presence of added *P-e*Prv (Fig. 5). We found that the degree of inhibition was proportional to *P-e*Prv concentration and that similar levels of inhibition were observed at various concentrations of either *P-e*Prv and methyl α-glucoside or pyruvate alone. It remains to be established whether the regulation of adenylate cyclase by the state of phosphorylation of PTS proteins is operative in the reconstituted extracts.

Fig. 6 presents a diagram of the proposed adenylate cyclase–PTS complex. According to this model, the PTS proteins interact with each other to produce a functional transport complex. In a reaction promoted by potassium phosphate, this PTS complex binds to adenylate cyclase and stimulates its enzymatic activity. In the presence of methyl α-glucoside, the sugar is metabolized to form the sugar phosphate with a concomitant decrease in the state of phosphorylation of the PTS proteins and a resultant decrease in the activity of adenylate cyclase. As a by-product of this reaction, *P-e*Prv is converted to pyruvate. The pyruvate formed can interact with Enzyme I also promoting inhibition of the adenylate cyclase. Studies using the individual PTS proteins have established (data not shown) that the inhibition of adenylate cyclase activity in extracts by pyruvate requires only Enzyme I. The important ramifications of this model are that adenylate cyclase activity is subject to control at a variety of levels all of which are mediated by the PTS. They are as follows: stimulation of the activity by K_2HPO_4, regulation of the activity by the state of phosphorylation of the PTS proteins, and inhibition of the activity by pyruvate. This multifunctional regulation of adenylate cyclase activity should result in rapid changes in the concentration of cAMP in intact cells.

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