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Cooperative Binding of the Sugar Substrates and Allosteric Regulatory Protein (Enzyme III^{Glc} of the Phosphotransferase System) to the Lactose and Melibiose Permeases in *Escherichia coli* and *Salmonella typhimurium*

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An *Escherichia coli* strain which overproduces the lactose permease was used to investigate the mechanism of allosteric regulation of this permease and those specific for melibiose, glycerol, and maltose by the phosphoenolpyruvate-sugar phosphotransferase system (PTS). Thio-β-digalactoside, a high affinity substrate of the lactose permease, released the glycerol and maltose permeases from inhibition by methyl-α-D-glucoside. Resumption of glycerol uptake occurred immediately upon addition of the galactoside. The effect was not observed in a strain which lacked or contained normal levels of the lactose permease, but growth of wild-type *E. coli* in the presence of isopropyl-β-thiogalactoside plus cyclic AMP resulted in enhanced synthesis of the lactose permease so that galactosides relieved inhibition of glycerol uptake. Thiodigalactoside also relieved the inhibition of glycerol uptake caused by the presence of other PTS substrates such as fructose, mannitol, glucose, 2-deoxyglucose, and 5-thioglucose. Inhibition of adenylate cyclase activity by methyl-α-glucoside was also relieved by thiodigalactoside in *E. coli* T52RT provided that the lactose permease protein was induced to high levels. Cooperative binding of sugar and enzyme III^{Glc} to the melibiose permease in *Salmonella typhimurium* was demonstrated, but no cooperativity was noted with the glycerol and maltose permeases. These results are consistent with a mechanism of PTS-mediated regulation of the lactose and melibiose permeases involving a fixed number of allosteric regulatory proteins (enzyme III^{Glc}) which may be titrated by the increased number of substrate-activated permease proteins. This work suggests that the cooperativity in the binding of sugar substrate and enzyme III^{Glc} to the permease, demonstrated previously in in vitro experiments, has mechanistic significance in vivo. It substantiates the conclusion that PTS-mediated regulation of non-PTS permease activities involves direct allosteric interaction between the permeases and enzyme III^{Glc}, the postulated regulatory protein of the PTS.

It has long been known that many species of bacteria are capable of regulating their transport activities such that preferred carbon sources are selected for uptake when many substrates are present in the environment (4, 10). This ability in part allows them to repress the synthesis of proteins which are unnecessary for growth (8, 15). In a broader physiological sense, this ability enables the bacteria to expend their energy most efficiently while continually adapting to a changing environment (1, 25).

Although it is clear that such regulatory mechanisms confer upon the organism a selective physiological advantage over less well regulated constituents in a natural population, the molecular details of these processes have been difficult to ascertain. Several theories have been proposed to explain this complex set of phenomena, originally termed "the glucose effect" (10) but now more generally referred to as "catabolite repression" and "inducer exclusion" (8, 25). In 1975, our laboratory proposed a unifying model for the repression of carbohydrate catabolic enzyme synthesis by the phosphoenolpyruvate-sugar phosphotransferase system (PTS) in *Escherichia coli* and *Salmonella typhimurium* (1, 6, 17). This proposal relied on the existence of a generalized regulatory protein of the PTS which would bind allosterically to the primary sites of regulation, namely adenylate cyclase and the

permeases for lactose, maltose, melibiose, and glycerol (17). Physiological and genetic evidence indicated that the glucose-specific enzyme III of the PTS (enzyme III^{Glc}) was probably this regulatory species (9, 11, 19–21, 23) and that the state of phosphorylation of this protein as well as the ratio of the amounts of the regulatory protein and the permease determined the degree of sensitivity to regulation (1, 16, 18).

Recent work in this laboratory has provided direct biochemical evidence for this proposal (16). Initial studies showed that partially purified enzyme III^{Glc} inhibited lactose uptake into *E. coli* membrane vesicles when shocked into the intravesicular compartment (2). Recent work has demonstrated the inhibition of lactose permease function by enzyme III^{Glc} in proteoliposomes containing only the lactose permease (M. J. Newman, T. Osumi, and M. H. Saier, Jr., unpublished data; see reference 16). Additionally, isolated *E. coli* membrane fragments were used to establish the direct in vitro binding of enzyme III^{Glc} to the lactose permease, and this interaction was shown to exhibit positive cooperativity with respect to substrate binding to the permease (12, 13). The experiments described in this paper were designed to test the significance of these last results with respect to the mechanism of regulation of the sensitive permeases in vivo and to demonstrate the possible relevance of this cooperativity to other systems regulated by the phosphotransferase system.

MATERIALS AND METHODS

Materials. [^{14}C]thiomethyl-β-galactoside was purchased from New England Nuclear Corp., [^{14}C]lactose and [^{14}C]glycerol were from Amersham Corp., [^{14}C]maltose was from ICN Pharmaceuticals, Inc., and [^{3}H]melibiitol was synthesized by reduction of melibiose with sodium [^{3}H]borohydride, removal of salts, and final purification by paper chromatography (19). All radioactive sugars were used at a specific activity of 5 to 10 mCi/mmol. Other biochemicals were from Sigma Chemical Co.

Bacterial strains. *E. coli* strains T52RT and T28RT were orginally obtained from P. Overath Max-Planck-Institut für Biologie, and have been described elsewhere (24). T52RT contains a hybrid pBR322 plasmid carrying the *lacY* gene. T28RT is the isogenic strain which lacks the plasmid. For these studies, an unmodified pBR322 plasmid was transformed into T28RT, and the resulting strain, designated T28RT/pBR322, was used as the *lacY*$^-$ control for T42RT. Other strains used in this study have been described (17, 18, 21, 22).

Growth of cells for uptake assays. Bacterial cultures of plasmid-carrying strains were grown overnight at 37°C with shaking in medium 63 (21) with the following additions: 0.5% Na^+ DL-lactate (pH 7.0); 100 μg of L-methionine per ml; 20 μg of thiamine per ml; and a carbon-inducer source such as maltose (0.5%) when indicated. These cultures were used to inoculate fresh medium of the same composition supplemented with 0.4% glycerol and 20 μg of tetracycline per ml. After 1 h of growth, isopropyl-β-D-thiogalactoside was added to a final concentration of 600 μM. Two hours later, glucose was added at 0.6%, final concentration. Cells were harvested after 2 h of growth in the presence of glucose, washed twice with ice cold medium 63, and suspended to a density of 0.2 mg dry weight per ml in medium 63 plus 0.4% sodium lactate. Growth of other bacterial strains was conducted as described in the figure and table legends.

Uptake assays. Uptake activities with plasmid-carrying strains were assayed as follows: in each tube 0.5 ml of water containing the [^{14}C]sugar and nonradioactive thiodigalactoside (TDG) (or thiomethylgalactoside) was allowed to preincubate for 5 min at 37°C. Cell suspensions were also preincubated for temperature equilibration. The assay was initiated by mixing 2 ml of the cell suspension with the radioactive sugar solution, and uptake was followed for 10 min, a time which had been determined to be within the range of linear uptake. Uptake was terminated by the addition of 3 ml of ice cold 0.1 M LiCl, and cells were immediately filtered on HA Millipore filters (0.45-μm pore diameter). Filters were washed with a total of 6 ml of ice cold 0.1 M LiCl and dried for liquid scintillation counting. Uptake assays with other bacterial strains were conducted as described previously (17, 19, 21) and in the figure legends. The degree of inhibition observed depended on a variety of factors including the cell preparation. Comparisons should only be made for experiments employing a single cell suspension. The variable degree of inhibition observed with different cell suspensions has been studied and discussed in a previous publication (18).

Adenylate cyclase assays. Cyclic AMP was determined by the cyclic AMP binding protein procedure in bacterial cultures lacking cyclic AMP phosphodiesterase (17, 18). Adenylate cyclase activity was estimated from the increase in cyclic AMP produced during a 30-min incubation period at 37°C as described previously (17, 18).

RESULTS

Relief of PTS-mediated inhibition of glycerol and maltose uptakes by TDG in an *E. coli* strain which overproduces the lactose permease. Glycerol uptake in many *E. coli* strains is strongly inhibited by the presence of methyl-α-glucoside (1, 16). When TDG was added to an inhibited culture of *E. coli* T52RT, the inhibitory effect of the glucoside was overcome. The concentration of TDG necessary to achieve half maximal release from inhibition was independent of the methyl-α-glucoside concentration in the range investigated (Fig. 1A). Maltose uptake also exhibited release from inhibition upon addition of TDG, but a less dramatic effect was observed (Fig. 1B). The apparent decrease in sensitivity of the maltose permease to inhibition in the absence of TDG may suggest that enzyme III^{Glc} binds to some extent to the lactose permease even in the absence of a galactoside substrate of the permease (see discussion).

Release of glycerol transport from inhibition by methyl-α-glucoside could also be demonstrated as a function of the concentration of thio-

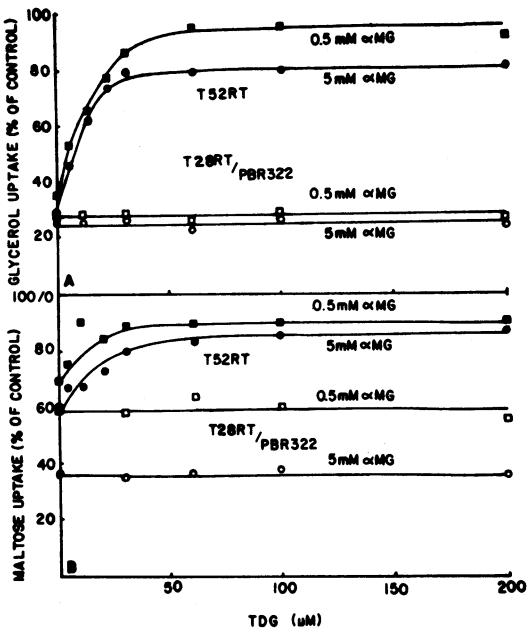

FIG. 1. Relief of PTS-mediated inhibition of glycerol and maltose uptake by TDG in T52RT or T28RT. Cells were grown and assayed as described in the text. The concentrations of glycerol and maltose were 1 and 0.1 mM, respectively.

methylgalactoside, with higher concentrations required to achieve the maximal effect (data not shown). A time course of glycerol uptake demonstrated that the release of inhibition occurred immediately upon addition of TDG with a lag period of less than 15 s (Fig. 2).

Relief of PTS-mediated inhibition of glycerol uptake in plasmid-free *lac* constitutive and inducible strains of *E. coli*. When *E. coli* strains were grown in medium containing lactose (0.5%) or lactate (1%) plus isopropyl-β-thiogalactoside (0.5 mM) little or no relief of glycerol uptake from inhibition was observed. However, when these strains were grown in the presence of both isopropyl-β-thiogalactoside (for inducible strains) and cyclic AMP, conditions which result in enhanced expression of the lactose operon, relief from inhibition was observed. Figure 3 shows the effects of TDG and thiomethylgalactoside on the inhibitory effects of methyl-α-glucoside in *E. coli* ML308. Both analogs partially relieved the inhibition of the glucoside, although the concentration of thiomethylgalactoside which caused this effect was over 10-fold higher than that required for a comparable effect with TDG. This result was expected, since TDG is a high affinity substrate of the lactose permease, whereas thiomethylgalactoside is a low affinity substrate. Comparable results were observed when Crooke's strain (*cpd*$^-$) was grown in medium containing glucose, isopropylthiogalactoside, and cyclic AMP (data not shown).

Several other sugar substrates of the PTS-inhibited glycerol uptake after *E. coli* ML308 was grown in medium containing glucose plus cyclic AMP. These sugars included mannose, fructose, 2-deoxyglucose, and 5-thioglucose. The addition of TDG caused partial relief of inhibition regardless of the sugar present (Table 1). Mannitol was not inhibitory unless expression of the mannitol operon was induced by growth in the presence of mannitol. After induction, however, TDG partially relieved mannitol-promoted inhibition of glycerol uptake as was observed for the hexoses. It can therefore be concluded that the relief of inhibition which results from the addition of TDG to cell suspensions is general for all PTS sugars.

Very low concentrations of TDG (<1 μM) were found to cause slight stimulation of glycerol uptake in *E. coli* ML308 in the absence of a substrate of the PTS. This stimulatory effect was reproducible and was observed with Crooke's strain. The effect depended on the presence of high levels of the lactose permease and was abolished by the *crrA* mutation (17, 20) (data not shown). Such stimulation could result from the inhibition of glycerol uptake by free enzyme III^{Glc}, present in the bacterial cell in the absence of a sugar substrate of the phosphotransferase system.

Cooperative binding of substrates and enzyme III^{Glc} to the melibiose permease in *S. typhimurium*. When *S. typhimurium* strain *cpd-401* or

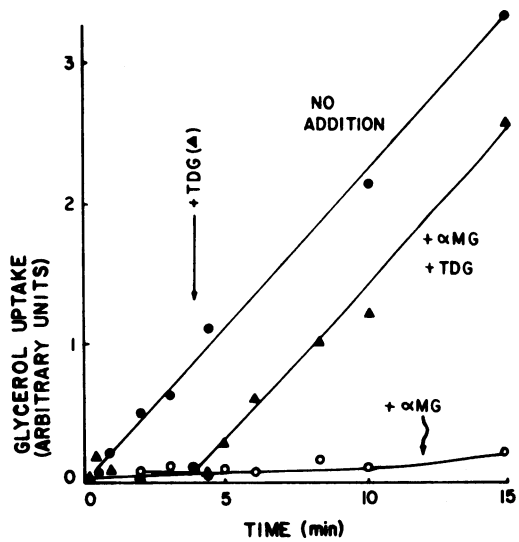

FIG. 2. Time course for relief of PTS-mediated inhibition of glycerol uptake by TDG in T52RT. The protocol for the experiment was as described in the legend to Fig. 1 and in the text. The concentrations of methyl-α-glucoside and TDG were 1 mM and 100 μM, respectively.

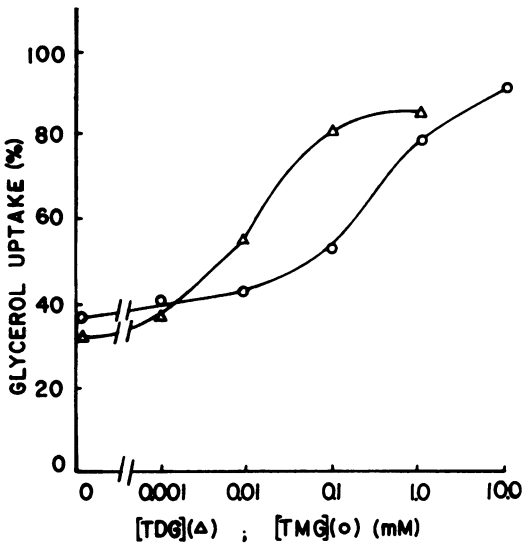

FIG. 3. Concentration curve for relief of PTS-mediated inhibition of glycerol uptake by TDG and thiomethylgalactoside in *E. coli* ML308. Cells were grown in medium 63 containing 1% sodium DL-lactate diluted 20-fold into fresh medium of the same composition. After 1 h, isopropylthiogalactoside and cyclic AMP were added to final concentrations of 0.5 and 5 mM, respectively. After one additional hour, glucose was added to a final concentration of 0.5%, and cells were allowed to grow for an additional 2 h. Cells were harvested and washed three times with medium 63 containing 1% lactate and suspended to a cell density of 0.3 mg (dry cells) per ml in the same medium. For uptake assays, cells were preequilibrated for 5 min at 37°C with or without 5 mM methyl-α-glucoside and TDG at the concentration indicated. To initiate the uptake assay [^{14}C]glycerol was added to a final concentration of 0.5 mM. At 5, 10, and 20 min, 1-ml samples were removed and filtered on 0.45-μm (pore size) HA Millipore filters and washed 2 times with 3 ml of medium 63 containing 1% lactate. Filters were dried, and cellular radioactivity was determined in a scintillation counter.

cpd-401 ptsI17 was grown in medium containing melibiose and cyclic AMP, the melibiose permease was induced to high levels, and cooperative interactions analogous to those observed with the lactose permease in *E. coli* could be demonstrated. The addition of thiomethylgalactoside to a methyl-α-glucoside-inhibited culture which was transporting glycerol relieved the inhibitory effect of the glucoside in a concentration-dependent fashion (Fig. 4). Thiomethylgalactoside had no effect on the rate of glycerol uptake in an isogenic *crrA* mutant. From these results it was concluded that the melibiose permease in *Salmonella* sp., like the lactose permease in *E. coli*, exhibits cooperative substrate-enzyme III^{Glc} interactions.

Comparable experiments were conducted with *S. typhimurium* strains to ascertain whether cooperative enzyme III^{Glc} substrate binding could be demonstrated for the glycerol and maltose permeases. Two strains were examined. One strain, LJ14 (*ptsI17 glp-252*) (22), possesses an "up promoter" mutation in the glycerol operon and synthesizes the glycerol operon gene products in about fourfold enhanced amounts under all growth conditions. This strain was grown in glycerol minimal medium containing cyclic AMP (5 mM) so that glycerol uptake activity was induced to high levels. The uptake of [^{3}H]melibiitol or [^{14}C]maltose was studied as a function of the methyl-α-glucoside and glycerol concentrations. Glycerol did not relieve inhibition of [^{3}H]melibiitol or [^{14}C]maltose uptake by methyl-α-glucoside. A second strain, SB1667 (*malQ62*) (21), was grown in medium containing lactate (1%), maltose (0.2%), and cyclic AMP (5 mM). Maltose did not relieve inhibition of [^{3}H]melibiitol or [^{14}C]glycerol uptake by methyl-α-glucoside. Thus, cooperative binding of enzyme III^{Glc} and the substrates of the maltose and glycerol permeases were not demonstrable in these strains.

Effect of TDG on PTS-mediated inhibition of adneylate cyclase activity. The phosphotransferase system regulates the activity of adenylate cyclase as well as those of the various perme-

TABLE 1. Relief of PTS-mediated inhibition of glycerol uptake by TDG in *E. coli* ML308[a]

Inhibiting sugar	TDG	% Inhibition of glycerol uptake
Mannitol	−	0
	+	3
Glucose	−	65
	+	34
Fructose	−	53
	+	17
Mannose	−	64
	+	27
Glucosamine	−	73
	+	50
5-Thioglucose	−	66
	+	29
2-Deoxyglucose	−	43
	+	32
6-Deoxyglucose	−	1
	+	5
Methyl-α-glucoside	−	73
	+	27

[a] ML308 cells were grown and assayed for glycerol uptake as described in the legend to Fig. 3. The concentration of the PTS sugar was 5 mM, whereas that of TDG was 0.5 mM. When the cells were grown in the presence of mannitol, mannitol inhibited glycerol uptake by 70%. In the presence of 0.5 mM TDG, this value decreased to 43%.

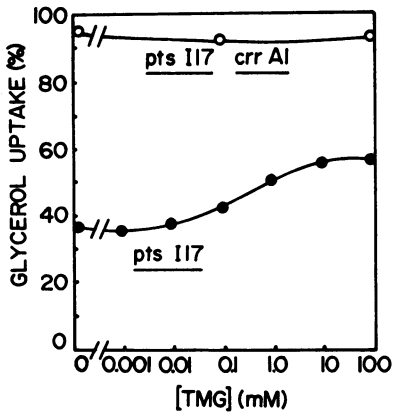

FIG. 4. Relief of PTS-mediated inhibition of glycerol uptake by thiomethylgalactoside in *S. typhimurium*. Strains LJ101 or LJ102 were grown in medium 63 containing 1% lactate for 1 h; melibiose (0.5%) and cyclic AMP (5 mM) were added, and cells were grown for an additional 2 h. Glucose (0.2%) was then added, and cells were harvested after two additional hours during exponential growth, washed three times with medium 63 containing 1% sodium DL-lactate, and suspended in the same medium to 0.2 mg (dry cells) per ml. Uptake assays were performed as described in the legend to Fig. 3. Values represent the average of triplicate determinations.

ases in *E. coli* and *S. typhimurium* (17, 18). Therefore, the effect of TDG on the inhibition of this enzyme by methyl-α-glucoside was studied in *E. coli* T52RT (Fig. 5). Increasing concentrations of the glucoside caused increasingly intense inhibition of cyclic AMP production. Regardless of the concentration of methyl-α-glucoside employed, however, the inhibitory effect could be reversed if a sufficient concentration of TDG was added. TDG did not stimulate adenylate cyclase in the absence of methyl-α-glucoside, and no effect of the galactoside was observed in strains which lacked or contained low levels of the lactose permease (data not shown).

DISCUSSION

Enzyme III^{Glc} is produced constitutively in *E. coli* and *S. typhimurium* (16), and the binding of this protein to the lactose permease shows cooperativity with substrate binding in vitro (12, 13). We predicted that if the permease exhibited similar behavior in vivo, then it should be possible to shift the equilibrium of bound enzyme III^{Glc} within the cell by adding a substrate of the lactose permease to the medium. In other words, the addition of substrate should enhance the binding affinity of the lactose permease for enzyme III^{Glc}, and the enzyme III^{Glc} should then preferentially bind to the lactose permease. This shift in binding could be measured as a decrease in sensitivity to PTS-mediated inhibition, due to a reduction in the amount of enzyme III^{Glc} bound to other PTS-regulated permeases.

To observe this predicted phenomenon, we first chose a strain of *E. coli* with greatly enhanced levels of the lactose permease. Employing this strain, it was possible to titrate the enzyme III^{Glc} by the addition of a lactose substrate analog and to observe the release of the glycerol and maltose permeases from inhibition by methyl-α-glucoside. Relief of inhibition was quantitative for the glycerol permease and very substantial for the maltose permease.

Release from inhibition was shown to occur immediately and completely upon the addition of TDG with a lag period of less than 15 s. This observation suggests that a very fast process, such as direct protein-protein interaction, is involved. Since positively cooperative binding of enzyme III^{Glc} to the lactose permease has been demonstrated in vitro (12, 13), this result strongly suggests a mechanism for PTS-mediated regulation of the lactose, melibiose, glycerol, and maltose permeases which involves direct allosteric interaction between these permeases and enzyme III^{Glc}, the generalized regulatory protein of the PTS. This suggestion has recently been substantiated by studies with purified lactose permease and purified proteins of the PTS reconstituted in a proteoliposome system (M. J. Newman, T. Osumi, and M. H. Saier, Jr., unpublished data).

By growing *E. coli* and *S. typhimurium* cells in the presence of inducer plus cyclic AMP, enhanced levels of the permease proteins are synthesized (14). Physiological desensitization of the permeases to PTS-mediated regulation occurs under these conditions (18), and, as shown here, relief of the glycerol or maltose permeases from PTS-mediated regulation occurs when a substrate of the lactose or melibiose permease is present. The fact that inhibition by any sugar substrate of the PTS is relieved by the lactose analogs, confirms the suggestion that a single allosteric mechanism, involving the enzyme III^{Glc}, is responsible for the inhibition of all of the non-PTS permeases by all PTS sugars. The demonstration of galactoside-promoted relief of inhibition of the glycerol and maltose permeases only when the lactose or melibiose permease is synthesized in large amounts suggests that the permease must be present in amounts equivalent to or in excess of the enzyme III^{Glc}. Even under these conditions a high affinity site for the enzyme III^{Glc} must be generated on the cytoplasmic surface of the permease by galactoside binding. Since the enzyme III^{Glc} is synthesized constitutively (20, 23), the molar ratio of permease to enzyme III^{Glc} is of primary importance

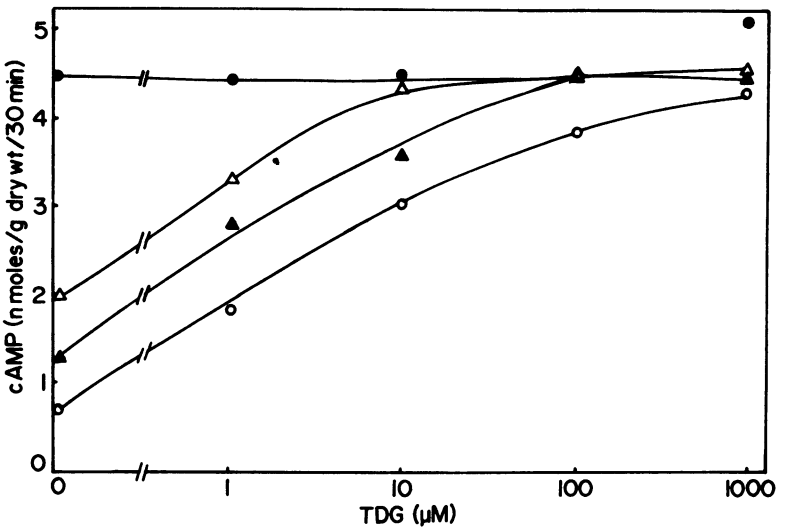

FIG. 5. Effect of methyl-α-glucoside and TDG on the activity of adenylate cyclase in *E. coli* T52RT. Cells were grown in medium 63 containing isopropyl-β-thiogalactoside (0.5 mM), glucose (0.4%), L-threonine (20 μg/ml), L-methionine (20 μg/ml), and thiamine (10 μg/ml). They were harvested during exponential growth, washed three times with medium 63 by centrifugation at 0°C, and suspended to a cell density of 0.4 mg/ml (dry weight). The levels of cyclic AMP determined at time zero and after 30 min at 37°C during exposure to the indicated concentrations of TDG in the presence of methyl-α-glucoside at the following concentrations: 0 μM (●); 50 μM (△); 500 μM (▲); and 5000 μM (○).

in determining whether desensitization (18) and relief from inhibition (as demonstrated in this communication) can occur. These results strongly suggest that physiological desensitization to PTS-mediated inhibition results directly from the production of an excess of permease proteins relative to enzyme III^{Glc} molecules in the cell. Thus, the results reported here are fully consistent with our earlier mechanistic predictions (17) and the subsequent suggestions of others (14, 23).

Although positive cooperativity appears established for sugar and enzyme III^{Glc} binding to the lactose and melibiose permeases, the same could not be demonstrated for the glycerol and maltose permeases. This result was not unexpected. Glycerol passes through the *E. coli* membrane via a nonstereospecific pore which apparently possesses no binding site for glycerol (5), and the maltose permease functions by a mechanism quite different from that of the melibiose and lactose permeases (1). The latter two permeases function by Na^+ and H^+ symport, respectively, and a common evolutionary origin for these two proteins seems likely (1). The similarities of the *mel* and *lac* operons, the similar transport mechanisms, the comparable ease of solubilization and reconstitution of the two transport systems, and the positive cooperativity demonstrated here all point to a common ancestry. Further work will be required to establish this relationship.

The positive cooperativity observed in the binding of enzyme III^{Glc} and substrate to the lactose and melibiose permeases may be viewed as an efficient mechanism for utilizing a limited number of allosteric regulatory proteins for the control of multiple targets. The enzyme III^{Glc} binds preferentially to those galactoside permease proteins which are "substrate activated." Substrate activation of the glycerol and maltose permeases was not demonstrated and, at least in the former case, was not anticipated (5). It should be noted that although enzyme III^{Glc} binding may control non-PTS permease function by promoting formation of an inactive conformation of the permease, inhibition of glycerol or maltose transport might involve physical blocking of the pore through which the solutes pass. Thus, cooperativity may be a characteristic of carrier-mediated transport, and not of channel-mediated transport (7).

TDG was found to exert an effect on adenylate cyclase comparable to that on the permeases. The galactoside relieved inhibition by the glucoside (Fig. 5). This result was not expected. Our model (17) predicted that although the permeases are negatively controlled by enzyme III^{Glc}, adenylate cyclase is positively controlled for the phospho form of the enzyme. If this were the

case, TDG would be expected either to exert no effect or to inhibit adenylate cyclase, depending on the rate-limiting step. The previous suggestion that adenylate cyclase is controlled by a positive mechanism was based on the low activity of adenylate cyclase in *crrA* mutants of *S. typhimurium* and *E. coli* (17). More recent results have generalized this observation but have also shown that in some genetic backgrounds of *E. coli*, *crrA* mutants exhibit appreciable adenylate cyclase activity (3). It should be noted that the *crrA* mutation might reduce adenylate cyclase activity in ways which are not directly related to PTS-mediated inhibition of its activity. Further experimentation will be required to resolve this problem.

ACKNOWLEDGMENTS

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