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Regulation of the Maltose Transport System of *Escherichia coli* by the Glucose-specific Enzyme III of the Phosphoenolpyruvate-Sugar Phosphotransferase System

CHARACTERIZATION OF INDUCER EXCLUSION-RESISTANT MUTANTS AND RECONSTITUTION OF INDUCER EXCLUSION IN PROTEOLIPOSOMES*

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Maltose transport in *Escherichia coli* is regulated at the protein level by the glucose-specific enzyme III (III^{glc}) of the phosphoenolpyruvate-sugar phosphotransferase system, by a mechanism known as inducer exclusion. We have isolated and characterized four mutants in the maltose transport system, all of which are in *malK*, which are resistant to inducer exclusion. The mutations in three of these mutants fall within the COOH-terminal domain of MalK and suggest the first reported function for this domain. Two of these are in a region which shows sequence similarity to *lacY* and *melB*, both of which are also regulated by III^{glc}, and thus may define a III^{glc}-binding domain. We have also reconstituted inducer exclusion in proteoliposomes made from membranes overexpressing the maltose permease. Maltose transport is inhibited by 50–60% when III^{glc} is included in the intravesicular space. The inhibition is due to a decrease in the V_{max} of transport by a factor of 2. III^{glc} does not affect the coupling of ATP hydrolysis to maltose transport, since the ratio of ATP hydrolyzed/maltose transported remained constant in the presence and absence of III^{glc}. Finally, the K_i for III^{glc} was 40 μM, roughly the same as the *in vivo* concentration of III^{glc}.

The phosphoenolpyruvate-sugar phosphotransferase system (PTS)[1] of *Escherichia coli* regulates the uptake of a number of non-PTS sugars, including maltose, by both transcriptional and post-transcriptional mechanisms (for a recent review, see Saier, 1989). Transcriptional regulation of target operons involves both catabolite repression and inducer exclusion (Magasanik, 1970). Catabolite repression is largely mediated by regulatory interactions believed to involve the cyclic AMP biosynthetic enzyme, adenylate cyclase, and the central regulatory protein of the PTS, the glucose-specific enzyme III (III^{glc}). In the phosphorylated state, III^{glc} is believed to function as an allosteric activator of adenylate cyclase. On the other hand, inducer exclusion involves direct allosteric inhibition, by the free (dephosphorylated) form of III^{glc}, of the target permeases and catabolic enzymes that generate endogenous inducers of non-PTS operons. Thus, when III^{glc} is not phosphorylated, as is true in the wild-type, energy-proficient cell supplied with a PTS sugar in the extracellular medium, this regulatory protein binds to and inhibits the various target permeases and catabolic enzymes which generate cytoplasmic inducers. Under these same conditions, adenylate cyclase is in its inactive (or less active) form. Conversely, when III^{glc} is phosphorylated, as is observed in the wild-type, energy-proficient cell when a PTS sugar is lacking from the extracellular medium, III^{glc} does not bind to the permeases and catabolic enzymes, and the inhibition of their activities is relieved. Under these conditions, adenylate cyclase is activated (Saier, 1989). Hence, cyclic AMP synthesis and the cytoplasmic accumulation of non-PTS inducers are coordinately regulated (Saier and Feucht, 1975).

Demonstration of direct binding of III^{glc} to the lactose permease and inhibition of transport activity in membrane vesicles have led to general acceptance of the model described above (Dills *et al.*, 1982; Misko *et al.*, 1987; Nelson *et al.*, 1983; Osumi and Saier, 1982; Saier *et al.*, 1983). The demonstration that III^{glc} interacts with glycerol kinase to inhibit its activity has also provided confirmation of this model (de Boer *et al.*, 1986; Novotny *et al.*, 1985; Postma *et al.*, 1984).

Maltose and maltooligosaccharides are transported into *E. coli* by a binding protein-dependent transport system, consisting of a periplasmic maltose binding-protein (MBP or MalE), two hydrophobic, integral inner membrane proteins, MalF and MalG, and a peripheral inner membrane protein, MalK (for a review see Schwartz, 1987). The maltose regulon, composed of several different operons, is positively regulated by the product of the malT gene. To date, maltotriose is the only known inducer of the maltose regulon, and it binds to MalT as a coactivator. Several of the *mal* operons, including the two which encode the proteins of the transport system, are also subject to control by the cAMP/cAMP receptor protein (CAP) transcriptional activator complex. Recent work by Boos and colleagues further suggests that the *mal* regulon is osmotically regulated (Bukau *et al.*, 1986). These investigators have identified a gene encoding a *LacI*-like repressor protein, MalI, which acts as a repressor of at least some components of the regulon (Reidl *et al.*, 1989). MalK has also been implicated in the regulation of maltose regulon expression (Schwartz, 1987), but the mechanism by which it functions in regulation is as yet unknown.

MalK is believed to be the energy-transducing protein of the maltose transport system. As initially suggested by sequence analyses, MalK and the MalK homologs in other binding protein-dependent transport systems appear to con-

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[1] The abbreviations used are: PTS, phosphoenolpyruvate-sugar phosphotransferase system; III^{glc}, glucose-specific Enzyme III; MBP, maltose-binding protein; αMeGlc, methyl-α-glucoside.

TABLE I
Bacterial strains

Strain	Genotype	Source
HN597	*malT^c araD lac rpsL1 ΔuncBC ilv*::Tn*10 F′ lacI^q* Tn*5*	Davidson and Nikaido, 1990
AD121	*F′ lacI^q Tn5 thr leu lacY ΔmalB214*	Laboratory collection
LJ143	*thi ptsH315*	
LJ143ΔmalK	*thi ptsH315 ΔmalK*::Tn*10*	H. Shuman
LJ370	*thi ptsH315 malK′1*	This study
LJ371	*thi ptsH315 malK′2*	This study
LJ372	*thi ptsH315 malK′3*	This study
LJ373	*thi ptsH315 malK′4*	This study
LJ288 (LU167)	*F$^-$ glpR galR ΔptsHlcrr cysA153 strA*	W. Epstein
JLV86	*thi arg66 metB1 hisG1 lacY1 galT6 nyl-7 rpsL104 ΔphoA8 supE44 galA50 pmi ptsM162 nagE1 crr*	J. W. Lengeler

tain ATP-binding sites. Several of these proteins have been shown to bind both ATP and ATP analogues (Hobson *et al.*, 1984).[2] Recently, using well-defined cell-free systems, ATP has been shown to be the energy source driving transport via two of these permease systems, those specific for maltose and histidine (Ames, 1990; Ames *et al.*, 1989; Bishop *et al.*, 1989; Davidson and Nikaido, 1990; Dean *et al.*, 1989; Dean *et al.*, 1990; Higgins, 1990).

The consequences of PTS-mediated control of maltose transport in whole cells were first observed by Monod in the 1940s (Monod, 1942). However, due to the complexity of the maltose transport system, this regulation has not been studied extensively. Several years ago we isolated mutations which mapped to the *malK* gene that rendered the maltose transport system resistant to inducer exclusion (Saier, 1985; Saier *et al.*, 1978). Similar mutants have been obtained in the lactose permease, the melibiose permease and glycerol kinase (Novotny *et al.*, 1985; Saier *et al.*, 1978). In this report we describe the isolation and molecular characterization of such mutants in the maltose permease. We also utilize the recently developed technique of maltose transport reconstitution (Davidson and Nikaido, 1990) to demonstrate the regulation of the maltose transport system by purified III^{glc} and to gain information about the mechanism of regulation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains are listed in Table I. To isolate *malK^r* mutants, LJ143 was spread onto maltose (0.2%) minimal plates containing 0.1% methyl-α-glucoside (αMeGlc) and grown at 37 °C for 2 days. Colonies were restreaked on the same plates and subsequently streaked on a variety of plates to ensure that the mutations were specific for the maltose transport system; *ptsH* revertants and *fruR* mutants (Chin *et al.*, 1987) fermented mannitol on EMB mannitol (1%) plates, and *crr* mutants fermented lactose on EMB lactose (1%) plates containing 0.1% αMeGlc. The remaining mutations were mapped to *malK* and confirmed to be in *malK* by sequence analysis. *malK^r* mutants were grown in medium 63 (Miller, 1972) containing 0.4% maltose and 0.1 μg/ml thiamine at 37 °C with aeration by shaking for whole cell experiments.

Preparation of Proteoliposomes—Membranes containing overexpressed maltose transport proteins were prepared from HN597 containing pFG23 and pMR11 grown in 2 × LB (20 g of tryptone, 20 g of yeast extract, 5 g of NaCl/liter) containing the appropriate antibiotics and induced for 3 h with 0.1 mM isopropyl-β-D-thiogalactoside. Cells from 2 liters were washed in 0.1 M potassium phosphate buffer (KP_i), pH 7.0, resuspended in 10 ml of the same, and passed twice through a French pressure cell at 10,000 psi. Whole cells were removed, and membranes were collected by centrifugation at 100,000 × *g* and stored in portions at −70 °C. Proteoliposomes were prepared as described (Davidson and Nikaido, 1990) using an octyl glucoside dilution procedure. Membrane proteins (0.5 mg) were solubilized by treating with 1.1% octyl glucoside (Calbiochem) in the presence of 20% glycerol, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 20 mM KP_i, pH 6.2, in a total volume of 500 μl, for 30 min at 0 °C. The octyl glucoside-soluble supernatant was removed after centrifugation for 30 min at 100,000 × *g* and added to a solution of sonicated *E. coli* phospholipids (5 mg; Avanti Polar Lipids, Inc.) in 1.1% octyl glucoside in the presence of 5 mM ATP. After 15 min on ice, the mixture was rapidly diluted 25-fold into 20 mM KP_i, pH 6.2, containing 5 mM ATP, 1 mM dithiothreitol, and 5 mM $MgCl_2$. Proteoliposomes were collected by centrifugation for 30 min at 100,000 × *g*, washed to remove extravesicular ATP, and resuspended in 20 mM KP_i, pH 6.2, containing 5 mM $MgCl_2$ for assays.

Transport Assays—Maltose transport activity in whole cells and proteoliposomes was estimated by measuring the accumulation of [^{14}C]maltose (Amersham Corp., specific activity 130 μCi/μmol) in a filtration assay as described previously (Davidson and Nikaido, 1990; Dean *et al.*, 1989).

ATP Assays—ATP concentrations within the proteoliposomes were determined in duplicate assays with the Boehringer Mannheim Biochemicals ATP Bioluminescence CLS kit as previously described (Dean *et al.*, 1989).

Purification of PTS Proteins—The glucose-specific Enzyme III, derived from the cloned and overproduced III^{glc} domain of the *Bacillus subtilis* Enzyme II^{glc},[3] was purified as previously described.[4] The effectiveness of this protein in the regulation of the lactose, maltose, and melibiose permeases as well as glycerol kinase and adenylate cyclase *in vivo* has been established.[3] III^{glc} was greater than 95% pure as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, native gel electrophoresis and NH_2-terminal amino acyl sequence determination. Enzyme I and HPr, also from *B. subtilis*, were purified as previously described (Reizer *et al.*, 1989).[3]

Phosphorylation of III^{glc}—III^{glc} (78 μM) was phosphorylated by incubation with equimolar HPr, 1 μM Enzyme I, and 5 mM phosphoenolpyruvate in 50 mM KP_i, pH 6.2, containing 1 mM dithiothreitol and 5 mM $MgCl_2$ for 1 h at 37 °C.

Determination of the Intravesicular III^{glc} Concentration—Proteoliposomes containing increasing amounts of III^{glc} were prepared by varying the amount of III^{glc} added to solubilized membrane protein prior to the dilution step. The amounts of III^{glc} added were between 5 and 165 μg/0.11 mg of total membrane protein. Approximately 2% of the III^{glc} was trapped within the vesicles. To quantitate the amount of III^{glc} trapped within the vesicles, several dilutions of the washed proteoliposomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed with antisera directed against III^{glc}. The intensities of the developed bands were compared with known amounts of III^{glc} which were also transferred. MalF, -G, and -K represented approximately 35% of the proteoliposome protein as determined by densitometry of the sodium dodecyl sulfate-polyacrylamide gels.

Cloning and Sequencing of the malK^r Mutations—Oligonucleotide primers were made complementary to sites approximately 150 base pairs upstream from the *malK* +1 site (5′-ATATAAGCT TCCATGGTTTAGTTCACAGAAGC-3′) and 70 bp downstream from the end of *malK* (5′-ATATAAGCTTCTATCTCCTGAGTCAT-3′). These were used to clone the wild-type and mutant genes using the polymerase chain reaction (Perkin Elmer Cetus Instruments) as described by the manufacturer. Restriction enzyme sites were constructed at the ends of the primers, and the polymerase chain reaction

[2] H. Nikaido, unpublished results.

[3] J. Reizer and M. H. Saier, manuscript in preparation.

[4] Sutrina, S. L., Reddy, P., Gonzy-Tréboul, G., and Reizer, J. (1990) *J. Biol. Chem.* **265**, 18581–18589.

TABLE II

Inducer exclusion and catabolite repression of maltose transport in LJ143 and malK^r mutants

Cells were grown in M63 mineral medium containing 0.4% maltose (LJ143 was grown in the presence of 0.4% glycerol as well as maltose), and prepared for transport assays by washing the cells twice in M63 mineral medium without maltose, and resuspending in the same. Uptake was measured by a filtration assay measuring the amount of [^{14}C]maltose retained by the cells on the filters.

Strain	Glucose[a]	Maltose uptake[b]	
		Control	+1 mM αMeGlc
		nmol/min/mg protein	
LJ143	−	7.0	3.8
	+	4.6	1.5
LJ370	−	7.6	7.7
	+	4.6	4.6
LJ371	−	6.2	6.2
	+	5.8	5.7
LJ372	−	10.2	10.4
	+	4.6	4.6
LJ373	−	6.5	6.6
	+	3.5	3.5

[a] To measure catabolite repression, cultures were split in two, 0.2% glucose was added to one subculture, and the cells were allowed to grow for 1 h.

[b] To measure inducer exclusion, 1 mM α-methylglucoside was added to the transport assay immediately before [^{14}C]maltose.

fragments were digested with restriction enzymes and inserted into pKK223-2 behind the *trc* promoter (Amann and Brosius, 1985). We used Sequenase 2.0 (USB) to sequence double-stranded plasmid DNA with primers complementary to sequences within the *malK* gene, kindly provided by H. Shuman of Columbia University.

RESULTS

Isolation and Characterization of malK^r Mutants—To facilitate demonstration of the regulatory interaction between the maltose permease and III^{glc} of the PTS, we isolated mutants in the maltose transport system that rendered it resistant to inducer exclusion. This was accomplished by selecting mutants from an *E. coli ptsH* mutant which fermented maltose in the presence of αMeGlc, a non-metabolizable glucose analog. Since III^{glc} cannot be phosphorylated in the absence of HPr, only mutants in the maltose permease which are no longer sensitive to inhibition by unphosphorylated III^{glc} will be able to grow. Since the *ptsH315* mutation is slightly leaky, the addition of αMeGlc ensures that any phosphorylated III^{glc} will be dephosphorylated. We isolated four independent mutants all of which mapped to the *malK* gene.[5] Whole cell transport assays designed to measure the extent of catabolite repression and inducer exclusion confirmed that the mutants we isolated were no longer sensitive to PTS-mediated inducer exclusion (Table II). Maltose transport was inhibited 46% by the presence of αMeGlc in strain LJ143, wild type for the maltose transport system, while the *malK^r* mutants showed no inhibition by αMeGlc. Addition of glucose to the cultures for several generations decreased the maltose uptake rate relative to controls by approximately 50% in each strain, indicating that catabolite repression was still operative.

We cloned the *malK^r* mutant genes by polymerase chain reaction using primers directed against sequences both upstream and downstream of the *malK* gene. The polymerase chain reaction inserts were cloned into pKK223-2 under the control of the *trc* promoter. Transformants containing inserts were isolated in AD121 by selecting for growth on minimal maltose plates containing the appropriate antibiotic. To ensure that we had cloned the *malK^r* genes, we transformed LJ143Δ*malK* with plasmids isolated in AD121 and screened for growth on minimal maltose plates containing 1 mM αMeGlc. 25 of 25 transformants carrying each of the cloned, mutant *malK^r* alleles grew in the presence of αMeGlc while none of the 25 transformants carrying the wild-type *malK* gene was capable of growth. The mutations were identified as described under "Experimental Procedures" and are summarized in Table III. We also cloned and sequenced the wild-type *malK* gene from LJ143 and found that the sequence was identical to that published (Dahl *et al.*, 1989).

Properties of the B. subtilis III^{glc}-like Domain Expressed in E. coli—We have found that the III^{glc}-like carboxyl terminus of the *B. subtilis* enzyme II^{glc} can function as an independent III^{glc}-like domain, during both glucose and sucrose uptake in *B. subtilis* and in the regulation of non-PTS permeases when transferred to *E. coli*.[3,4] In order to characterize the regulatory interaction between III^{glc} and the maltose permease, we transformed *E. coli* strains LJ288 and JLV86 (Δ*ptsHIcrr* and *crr*, respectively) with a plasmid carrying the III^{glc}-like domain, pBS33.[3] JLV86 alone does not grow on glucose, but when pBS33 is present, the cells can utilize this sugar. LJ288 ferments maltose, lactose, and melibiose as expected for a Δ*ptsHI* strain lacking the entire *pts* operon including part of the *crr* gene encoding III^{glc}. However, when the cells are transformed with pBS33, they no longer ferment any of these sugars.[3] These results show that the *B. subtilis* III^{glc}-like domain is able to complement an *E. coli crr* mutant with respect to both glucose transport and regulation of other permeases in *E. coli*. Consequently, we could use the *B. subtilis* III^{glc} for the biochemical experiments described below.

Maltose Uptake in Reconstituted Proteoliposomes—Proteoliposomes were prepared as described by Davidson and Nikaido (1990) from membranes isolated from a strain which overproduces the maltose transport proteins MalF, MalG, and MalK 10–20-fold. Maltose transport was dependent on intravesicular ATP and extravesicular MBP. We were able to reconstitute inducer exclusion by adding purified III^{glc} during the solubilization step and trapping it in the vesicles upon dilution (Fig. 1). The concentration of III^{glc} typically used in the solubilization step was 15 μM, and the protein was then diluted to 0.6 μM upon formation of proteoliposome vesicles. Control samples containing either no added protein or soybean trypsin inhibitor (at the same concentration as III^{glc}) showed essentially the same transport rate. We attempted to reverse the inhibition by phosphorylating III^{glc} with Enzyme I, HPr, and phosphoenolpyruvate, as has been previously demonstrated for the lactose permease (Dills *et al.*, 1982). We were able to relieve 40% of the III^{glc}-mediated inhibition of maltose uptake by phosphorylation of III^{glc} (data not shown). Under the conditions of this experiment, III^{glc} appeared to be largely phosphorylated as determined spectrophotometrically by the method of Meadow and Roseman (1982). Elimination of HPr and Enzyme I from the phosphorylation reaction prevented relief of inhibition, and the presence of phosphoenolpyruvate alone did not stimulate maltose transport.

Effect of III^{glc} on the Kinetics of Maltose Transport—Using equimolar concentrations of maltose and MBP, we followed

TABLE III

Summary of MalK^r mutations

Strain	Nucleotide change	Amino acid change
LJ370 malK^{r}1	844 T → C	G278 → P
LJ371 malK^{r}2	370 G → A	A124 → T
LJ372 malK^{r}3	850 G → A	G284 → S
	370 G → A	A124 → T
LJ373 malK^{r}4	721 T → A	F241 → I

[5] M. Schwartz, personal communication.

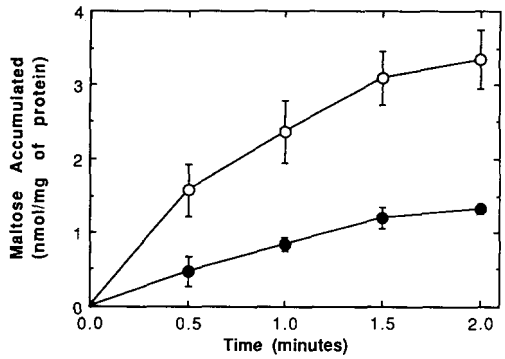

FIG. 1. **Reconstitution of maltose uptake and inducer exclusion in proteoliposomes.** Membranes prepared from HN597 (pMR11, pFG23) with isopropyl-β-D-thiogalactoside were thawed on ice, and proteoliposomes were prepared as described under "Experimental Procedures." 5 mM ATP was added to the sonicated phospholipid and was present in the dilution buffer. When III^{glc} was included, 0.25 mg of III^{glc} was added to 0.5 mg of membrane protein in the solubilization step. The proteoliposomes were washed once with ice-cold 20 mM KP_i, pH 6.2, containing 5 mM $MgCl_2$, and resuspended in the same. MBP was added to 10 μM. Accumulation of [^{14}C]maltose was measured in triplicate assays as described. Symbols: ○, proteoliposomes containing no III^{glc}; ●, proteoliposomes made in the presence of III^{glc}.

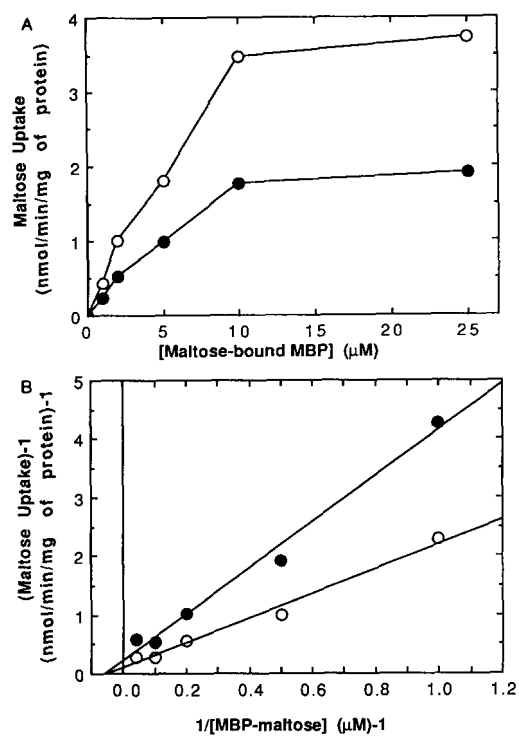

FIG. 2. **Effect of III^{glc} on the relative affinity of the maltose permease for maltose-bound MBP.** Proteoliposomes were prepared from HN597 (pMR11, pFG23) membranes as described under "Experimental Procedures" in the presence (●) and absence (○) of III^{glc} (15 μM during solubilization). 5 mM ATP was included in the dilution buffer and with the phospholipid. *A*, [^{14}C]maltose and MBP were premixed and added at equimolar concentrations to initiate the transport assay. *B*, double-reciprocal plot (1/v *versus* 1/maltose-MBP) of the results from A.

the kinetics of maltose uptake into proteoliposomes made with or without III^{glc} (Fig. 2). When transport activity was studied as a function of the maltose-MBP concentration, the K_m for liganded MBP was 9 μM both in the presence and absence of III^{glc}. It can be seen (Fig. 2) that the inhibition by III^{glc} is due to depression of the V_{max} value, in this case by a factor of 2, from 5.4 nmol of maltose accumulated/min/mg

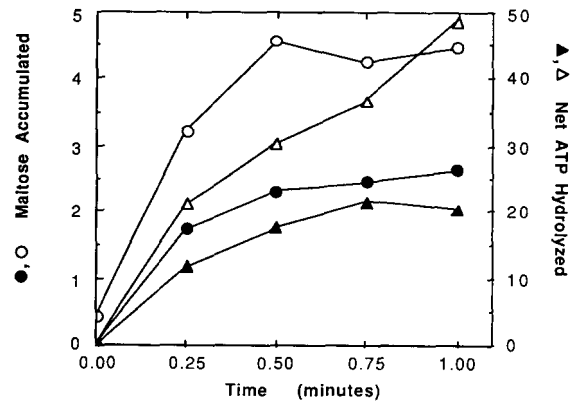

FIG. 3. **Effect of III^{glc} on the ratio of ATP hydrolyzed/maltose transported.** Washed proteoliposomes were prepared from HN597 (pMR11, pFG23) membranes as described in the presence (*closed symbols*) and absence (*open symbols*) of III^{glc} (15 μM during solubilization). The concentration of ATP added was 5 mM. MBP and [^{14}C]maltose were both added to 10 μM to measure maltose transport. To determine the amount of ATP remaining in the proteoliposomes, proteoliposomes were diluted into buffer containing unlabeled maltose (10 μM), samples were withdrawn, and ATP levels were determined as described under "Experimental Procedures." The ATP hydrolysis assay was initiated by either the addition of buffer or MBP (10 μM), and the net transport-dependent hydrolysis is the difference between these two conditions. All assays and ATP determinations were performed in duplicate. Symbols: △, ▲, net ATP hydrolyzed; ○, ●, maltose accumulated.

protein in the absence of III^{glc} to 2.8 nmol/min/mg protein in its presence.

Effect of III^{glc} on the Ratio of ATP Hydrolyzed/Maltose Transported—Since the MalK protein is thought to act as the energy-coupling protein of the maltose transport system, and since the mutations that we isolated mapped to within *malK*, we decided to look at the effect of III^{glc} on the energetics of maltose transport. We had demonstrated previously that ATP is hydrolyzed concomitantly with maltose transport in both membrane vesicles and proteoliposomes (Davidson and Nikaido, 1990; Dean *et al.*, 1989). In both systems, the stoichiometry of ATP hydrolyzed/maltose transported ranges from approximately 1:1 to 10:1, but it remains constant for a given vesicle or proteoliposome preparation. Fig. 3 shows the effect of the inclusion of III^{glc} in proteoliposomes on the ratios of ATP hydrolysis to maltose transport. The inclusion of III^{glc} decreased the amount of ATP hydrolyzed in parallel with the amount of maltose transported. Thus, the ratio of ATP hydrolyzed to maltose transported remained constant with or without III^{glc}, in this experiment at a ratio of about 15.

Stoichiometry of III^{glc}-mediated Inhibition of Maltose Uptake—Proteoliposomes containing increasing amounts of III^{glc} were prepared by varying the amount of III^{glc} added to solubilized membrane protein. The amount of III^{glc} trapped within the washed proteoliposomes was determined and correlated with the amount of transport inhibition (Fig. 4). Using an intravesicular volume of 15 μl/mg protein (Davidson and Nikaido, 1990), the internal concentration of III^{glc} was determined. The maximal inhibition that we could achieve was 65%, with half-maximal inhibition at 40 μM III^{glc} (12 μg/mg membrane protein). Since the maltose permease constitutes approximately 35% of the membrane protein in these proteoliposomes and the molecular weight of the complex is 171,000 daltons,[6] the inhibition by III^{glc} appears to be stoichiometric: the ratio of III^{glc} to maltose permease at 40 μM III^{glc} is ~0.4:1, not far from the theoretical value of 0.5:1.

[6] A. Davidson, and H. Nikaido, manuscript in preparation.

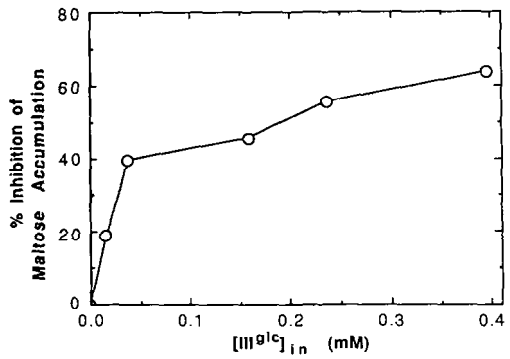

FIG. 4. **Inhibition of maltose transport in proteoliposomes at different concentrations of III^{glc}.** Proteoliposomes containing increasing amounts of III^{glc} were prepared by varying the amount of III^{glc} added to solubilized membrane protein prior to the dilution step as described under "Experimental Procedures." The proteoliposomes were washed to remove extravesicular III^{glc}, and the amount of intravesicular III^{glc} was determined as described. A volume of 15 μl/mg of membrane protein was used as the internal volume of the proteoliposomes (Davidson and Nikaido, 1990) to determine the concentration of III^{glc}. The control proteoliposomes made in the absence of III^{glc} transported maltose at a rate of 4.62 nmol/min/mg membrane protein.

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GlpK  370                    Gly Val Asn Ala Asn His --- Ile Ile
                             :   .   :                       :
MalK  275  Val Gln --- Val Gly Ala Asn Met Ser Leu --- Gly Ile
           .   .       :   :   :   :       :   .           .
LacY  198  Ala Asn Ala Val Gly Ala Asn His Ser Ala Phe Ser Leu
                             :               :               :
MelB  211                    Gly Ser --- His Leu --- --- Thr Leu

GlpK       Arg Ala Thr Leu Glu Ser Ile Ala     385
           :               :       .   .
MalK       Arg Pro --- --- Glu His Leu Leu     291
           .               :       :
LacY       Lys Leu Ala Leu Glu --- Leu Phe     217
           :   :   .       :
MelB       Lys --- Ala Ile Val Ala Leu Ile     223
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FIG. 5. **Amino acid sequence similarities between portions of several proteins regulated by inducer exclusion.** The deduced amino acid sequences from glycerol kinase (*glpK*), *malK*, the lactose permease (*lacY*), and the melibiose permease (*melB*) are aligned around the region containing two of the *malK*^{r} mutations (in *boldface*). The bold residues, Ala-198 and Ser-209, in the lactose permease sequence are also mutations which render lactose uptake independent of inducer exclusion.[7] Identical residues are marked with a *colon* and conservative replacements are marked with a *period*. The alignment of LacY and MelB is from Yazyu *et al.* (1984).

DISCUSSION

The genetic results presented in this paper clearly suggest that the target for the control of the maltose transport system in *E. coli* by III^{glc} is the product of the *malK* gene. The ability of the III^{glc}-like domain from *B. subtilis* to regulate maltose transport in *E. coli* both *in vivo* and *in vitro* suggests that the regulatory properties of this protein have been conserved in these two organisms over 2 billion years of evolutionary time.

Depression of the V_{max} of transport resembles the inhibition of glycerol kinase and lactose permease by III^{glc}. The maximal velocity of glycerol phosphorylation was depressed approximately 50% with respect to both ATP concentration and glycerol concentration, as was that of lactose transport by the lactose permease (Dills *et al.*, 1982; Novotny *et al.*, 1985). We were able to achieve 65% inhibition of maltose transport by the inclusion of III^{glc} in the proteoliposomes. This is very similar to the inhibition seen in whole cells where the addition of αMeGlc to LJ143 causes a 46% inhibition of maltose uptake. Thus, we appear to have faithfully reconstituted inducer exclusion in proteoliposomes.

The intracellular concentration of III^{glc} in *E. coli* is around 50 μM (Scholte *et al.*, 1981), a value similar to that which gave half-maximal inhibition in our *in vitro* experiments. Since the subunit stoichiometry of the maltose permease is FGK_2,[6] the concentration of III^{glc} that gave half-maximal inhibition (40 μM) would correspond to a ratio of 0.4 molecules III^{glc}/maltose permease in our system. In the wild-type *E. coli* cell, the ratio would be expected to be much higher since the maltose permease is expressed maximally at about 1000 copies/cell (Schwartz, 1987). Postma and colleagues (1988) have demonstrated binding, by cosedimentation, of III^{glc} to membranes containing overexpressed amounts of the maltose permease. While the degree of overexpression is not reported, if we assume that it is the same as in our system (5–10% of total membrane protein), the ratio between III^{glc} and $MalFGK_2$ would be between 1 and 2 at an external III^{glc} concentration of 350 μM. This is in accordance with our results. Although we attempted to demonstrate binding between III^{glc} and $MalFGK_2$ by cross-linking using dithio*bis*(succinimidyl propionate) and formaldehyde, we were unable to detect an interaction (data not shown).

The mutations in *malK* that we isolated and mapped by sequencing occurred within two domains of the MalK protein. The Ala to Thr change at residue 124 is between the two putative ATP-binding domains. While the region around the two ATP-binding domains shows significant homology to other energy coupling proteins of the binding protein-dependent permease systems, the region between the two sites shows very little similarity among the homologous proteins.

The remaining three mutations fall within the carboxyl terminus of MalK. MalK is approximately 100 amino acids larger than most of the other MalK homologs. These hundred residues are in the carboxyl terminus and are thought to comprise a separate domain, perhaps involved in regulation of maltose transport. The fact that three of the mutations lie within this region provides the first evidence that this domain may serve as the III^{glc}-binding domain.

The region around residues 278 and 284 (mutated in two of the mutants, Table III) was compared with the sequences of other proteins known to be subject to inducer exclusion (Fig. 5). Striking similarity was found between a region of LacY and the intervening sequence of MalK, and some similarity was also found with GlpK and MelB, the genes encoding glycerol kinase and the melibiose transporter. Noteworthy is the fact that the region of LacY showing this sequence similarity is the central, putative cytoplasmically localized loop in which two independent mutations have been isolated that abolish inducer exclusion of lactose transport.[7] The region of similarity in MelB is also within the central loop of this permease. Sequence similarity between very different proteins, all of which bind III^{glc}, suggests that these regions of the three permeases function as enzyme III^{glc}-binding sites. It is anticipated that this will prove to provide an example of convergent evolution to accommodate a common regulatory mechanism, superimposed on evolutionarily divergent types of sugar transport proteins (Saier, 1990).

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