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 PROTEOLIPSOMES*

(Received for publication, August 6, 1990)

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Maltose transport in *Escherichia coli* is regulated at the protein level by the glucose-specific enzyme III (III"') 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"', and thus may define a III"'-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"' 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"' does not affect the coupling of ATP hydrolysis to maltose transport, since the ratio of ATP hydrolyzed/maltose transported remains constant in the presence and absence of III"'. Finally, the K_M for III"' was 40 μM, roughly the same as the in vivo concentration of III"'.

The phosphoenolpyruvate-sugar phosphotransferase system (PTS) 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"'). In the phosphorylated state, III"' 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"', of the target permeases and catabolic enzymes that generate endogenous inducers of non-PTS operons. Thus, when III"' 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"' is phosphorylated, as is observed in the wild-type, energy-proficient cell when a PTS sugar is lacking from the extracellular medium, III"' 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"' to the lactose permease and inhibition of transport activity in membrane vesicles has 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"' interacts with glycol 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., 1996). These investigators have identified a gene encoding a *LuxI*-like repressor protein, MalL, which acts as a repressor of at least some components of the regulon (Reidt 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-
tain ATP-binding sites. Several of these proteins have been shown to bind both ATP and ADP analogues (Ilobon et al., 1984). 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., 1988; 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). In this report we describe a strain mapping to the malK gene that rendered the maltose transport system resistant to inducer exclusion (Saier, 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 gain information about the mechanism of regulation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Bacterial strains are listed in Table I. To isolate malK' mutants, LJ143 was spread onto maltose (0.2%) minimal plates containing 0.1% methyl-a-glucoside (M6Glc) and grown at 37°C for 3 days. Colonies were re-streaked 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 fruK mutants (Chin et al., 1989) were isolated on EMB mannitol (1%) plates, and err mutants were isolated on EMB lactose (1%) plates. The resulting isolates were supplemented to maintain the maltose permease. We also utilized the recently developed technique of maltose transport reconstitution (Davidson and Nikaido, 1990) to demonstrate the regulation of the maltose transport system by purified III' and to gain information about the mechanism of regulation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN597</td>
<td>malT' araD lacI rpsL1 ΔuncBC lux::Tn10 F' lacI Tn5</td>
<td>Davidson and Nikaido, 1990</td>
</tr>
<tr>
<td>AD121</td>
<td>F' lacI Tn3 thr ilev lacY ΔmalB214</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>LJ143</td>
<td>thi ptsH315</td>
<td>H. Shuman</td>
</tr>
<tr>
<td>LJ143malK</td>
<td>thi ptsH315 ΔmalK::Tn10</td>
<td>This study</td>
</tr>
<tr>
<td>LJ370</td>
<td>thi ptsH315 malK'</td>
<td>This study</td>
</tr>
<tr>
<td>LJ371</td>
<td>thi ptsH315 malK''</td>
<td>This study</td>
</tr>
<tr>
<td>LJ372</td>
<td>thi ptsH315 malK'''</td>
<td>This study</td>
</tr>
<tr>
<td>LJ373</td>
<td>thi ptsH315 malK''''</td>
<td>This study</td>
</tr>
<tr>
<td>L298 (L167)</td>
<td>F plp galR ptsH&amp;erm cya153 strA</td>
<td>W. Epstein</td>
</tr>
<tr>
<td>JLV86</td>
<td>thi arg66 metB1 hisC1 lacY1 galT6 xyl7 rpsL104 ΔphaO8 supE44 galA50 pmi ptsM162 nagE1 cre</td>
<td>J. W. Lengeler</td>
</tr>
</tbody>
</table>

**Regulation of Maltose Transport by III'**

**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).

**Preparation of Proteoliposomes**—Membranes containing overexpressed maltose transport proteins were prepared from HN597 containing pFG23 and pMR11 grown in 21006 M dithiothreitol, 5 mM MgCl₂, and 20 mM KPi, 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 MgCl₂, and 20 mM KPi, 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 KPi, pH 6.2, containing 5 mM ATP, 1 mM dithiothreitol, and 5 mM MgCl₂. Proteoliposomes were collected by centrifugation for 30 min at 100,000 × g, washed to remove extravesicular ATP, and resuspended in 20 mM KPi, pH 6.2, containing 5 mM MgCl₂, for assays.

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

**Phosphorylation of III'**—III' was phosphorylated by incubation with equimolar HPr, 1 μM Enzyme I, and 5 mM phosphoenolpyruvate in 50 mM KPi, pH 6.2, containing 1 mM dithiothreitol and 5 mM MgCl₂ for 1 h at 37°C.

**Determination of the Intravesicular III' Concentration**—Proteoliposomes containing increasing amounts of III' were prepared by varying the amount of III' added to solubilized membrane protein prior to the dilution step. The amounts of III' added were between 5 and 165 μg/0.1 mg of total membrane protein. Approximately 2% of the III' added was trapped within the vesicles. Several dilutions of the washed proteoliposomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, native gel electrophoresis and NH₄-terminal amino acid sequence determination. Enzyme I and II'p, also from B. subtilis, were purified as previously described (Reizer et al., 1989).

**Determination of the Intravesicular ATP Concentration**—Proteoliposomes containing increasing amounts of III' were prepared by varying the amount of III' added to solubilized membrane protein prior to the dilution step. The amounts of III' added were between 5 and 165 μg/0.1 mg of total membrane protein. Approximately 2% of the III' added was trapped within the vesicles. Several dilutions of the washed proteoliposomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, native gel electrophoresis and NH₄-terminal amino acid sequence determination. Enzyme I and II'p, also from B. subtilis, were purified as previously described (Reizer et al., 1989).

**Cloning and Sequencing of the malK' Mutations**—Cloning and sequencing of the malK' mutations was performed as previously described. **Determination of the Intravesicular III' Concentration**—Proteoliposomes containing increasing amounts of III' were prepared by varying the amount of III' added to solubilized membrane protein prior to the dilution step. The amounts of III' added were between 5 and 165 μg/0.1 mg of total membrane protein. Approximately 2% of the III' added was trapped within the vesicles. Several dilutions of the washed proteoliposomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, native gel electrophoresis and NH₄-terminal amino acid sequence determination. Enzyme I and II'p, also from B. subtilis, were purified as previously described (Reizer et al., 1989).

2 H. Nikaido, unpublished results.

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

Isolation and Characterization of malK’ Mutants—To facilitate demonstration of the regulatory interaction between the maltose permease and III\textsuperscript{E} 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, while the malK mutants showed essentially the same transport rate. We attempted to reverse the inhibition by phosphorylating III\textsuperscript{E} with Enzyme I, HPr, and phosphoenolpyruvate, as has been previously demonstrated for the lactose permease (Dills et al., 1982). We were able to reconstitute inducer exclusion by adding purified III\textsuperscript{E} during the solubilization step and trapping it in the vesicles upon dilution (Fig. 1). The concentration of III\textsuperscript{E} typically used in the solubilization step was 15 \mu M, and the protein was then diluted to 0.6 \mu M upon formation of proteoliposome vesicles. Control samples containing either no added protein or soybean trypsin inhibitor (at the same concentration as III\textsuperscript{E}) showed essentially the same transport rate. We attempted to reverse the inhibition by phosphorylating III\textsuperscript{E} 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\textsuperscript{E}-mediated inhibition of maltose uptake by phosphorylation of III\textsuperscript{E} (data not shown). Under the conditions of this experiment, III\textsuperscript{E} 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\textsuperscript{E} on the Kinetics of Maltose Transport**—Using equimolar concentrations of maltose and MBP, we followed

TABLE II

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose*</th>
<th>Maltose uptake*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control +1 mM aMeGlc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>LJ143</td>
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<td>7.0</td>
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<tr>
<td></td>
<td>+</td>
<td>4.6</td>
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<tr>
<td>LJ370</td>
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<td>LJ371</td>
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<td>LJ373</td>
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<td>6.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*To measure catabolite repression, cultures were split in two, 0.25% glucose was added to one subculture, and the cells were allowed to grow for 1 h.

*To measure inducer exclusion, 1 mM \textit{a}-methylglucoside was added to the transport assay immediately before [\textsuperscript{14}C]maltose.

**Properties**

Maltozyme 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\textsuperscript{E} during the solubilization step and trapping it in the vesicles upon dilution (Fig. 1). The concentration of III\textsuperscript{E} typically used in the solubilization step was 15 \mu M, and the protein was then diluted to 0.6 \mu M upon formation of proteoliposome vesicles. Control samples containing either no added protein or soybean trypsin inhibitor (at the same concentration as III\textsuperscript{E}) showed essentially the same transport rate. We attempted to reverse the inhibition by phosphorylating III\textsuperscript{E} 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\textsuperscript{E}-mediated inhibition of maltose uptake by phosphorylation of III\textsuperscript{E} (data not shown). Under the conditions of this experiment, III\textsuperscript{E} 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.

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**TABLE III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Summary of MalK’ mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJ370 malK’1</td>
<td>844 T → C</td>
</tr>
<tr>
<td>LJ371 malK’2</td>
<td>370 G → A</td>
</tr>
<tr>
<td>LJ372 malK’3</td>
<td>850 G → A</td>
</tr>
<tr>
<td>LJ373 malK’4</td>
<td>721 T → A</td>
</tr>
</tbody>
</table>

* M. Schwartz, personal communication.
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 IIIp was included, 0.25 μg of IIIp was added to 0.5 μg of membrane protein in the cold 20 mM IIIp. Proteoliposomes made in the presence of IIIp were measured in triplicate assays as described. Symbols: O, proteoliposomes made in the absence of IIIp; Δ, proteoliposomes made in the presence of IIIp.

Effect of IIIp 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 IIIp (15 μM during solubilization). The concentration of ATP added was 5 mM. MBP and [14C]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: Δ, net ATP hydrolyzed; O, maltose accumulated.

Stoichiometry of IIIp-mediated inhibition of maltose uptake—Proteoliposomes containing increasing amounts of IIIp were prepared by varying the amount of IIIp added to solubilized membrane protein. The amount of IIIp trapped within the washed proteoliposomes was determined and correlated with the amount of maltose transported (Fig. 4). Using an intravesicular volume of 15 pl/mg protein (Davidson and Nikaido, 1990), the internal concentration of IIIp was determined. The maximal inhibition that we could achieve was 65%, with half-maximal inhibition at 40 μM IIIp (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, the inhibition by IIIp appears to be stoichiometric: the ratio of IIIp to maltose permease at 40 μM IIIp is 0.4:1, not far from the theoretical value of 0.5:1.
uptake. Thus, we appear to have faithfully reconstituted in-
transport in E. coli both in vivo and in vitro suggests that the 
target for the control of the maltose transport system 
of the IIP"-like domain from B. subtilis to regulate maltose 
in E. coli by UP" is the product of the malK gene. The ability 
regulatory properties of this protein have been conserved in 
these two organisms over 2 billion years of evolutionary time.

were able to achieve 65% inhibition of maltose transport by 
glycerol concentration, as was that of lactose transport by the 
maltose permease in our system. 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" and MalFGK, 
would be between 1 and 2 at an external III" concentration 
of 350 μM. This is in accordance with our results. Although 
we attempted to demonstrate binding between III" and 
MalFGK2 by cross-linking using diithiobis(succinimidyl 
propionato) 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 mutations of Lacy and MelB is from Yazyu et al. (1984). 
The alignment 

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"- is the product of the malK gene. The ability 
of the III"-like domain from B. subtilis to regulate maltose 
transport in E. coli both in vivo and in vitro suggests that the 
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Depression of the V, of transport resembles the inhibition 
of glyceral kinase and lactose permease by III". The maximal 
velocity of glyceral phosphorylation was depressed approxi-
mately 50% with respect to both ATP concentration and 
glyceral 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" 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 in-

**FIG. 5.** Amino acid sequence similarities between portions 
of several proteins regulated by inducer exclusion. The deduced 
amino acid sequences from glyceral kinase (GlpK), malK, the 
lactose permease (lacY), and the melibiose permease (melB) are aligned 
around the region containing two of the malK mutations (in boldface). 
The bold residues, Ala-198 and Ser-209, in the lactose permease 
are also mutations which render lactose uptake independent 
of propionate) and formaldehyde, we were unable to detect an 
interaction (data not shown).

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The bold residues, Ala-198 and Ser-209, in the lactose permease 
are also mutations which render lactose uptake independent 
of inducer exclusion. Identical residues are marked with a colon 
and conservative replacements are marked with a period. The alignment 
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**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"- is the product of the malK gene. The ability 
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**REFERENCES**


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and H. Shuman for strains used in this study. We also thank A. 
Davidson for helpful discussions.
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.

D A Dean, J Reizer, H Nikaido and M H Saier, Jr