Transport of *p*-Nitrophenyl- α -Maltoside by the Maltose Transport System of *Escherichia coli* and Its Subsequent Hydrolysis by a Cytoplasmic α -Maltosidase

MORAIMA REYES, NANCY A. TREPTOW, AND HOWARD A. SHUMAN*

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received 20 November 1985/Accepted 6 December 1985

In wild-type *Escherichia coli* the activity of the maltose transport system is dependent on a periplasmic maltose-binding protein. It has been possible, however, to isolate mutants in which transport activity is mediated by the membrane components of the system and is no longer dependent on the periplasmic binding protein. In this manuscript we show that in these binding protein-independent strains, *p*-nitrophenyl- α -maltoside is a potent inhibitor of maltose transport. In contrast, *p*-nitrophenyl- α -maltoside is only a weak inhibitor of maltose transport in wild-type bacteria. In addition, we show that *p*-nitrophenyl- α -maltoside is transported by the binding protein-independent strains but not by wild-type bacteria. We were able to detect transport of this compound because there is a cytoplasmic enzyme that cleaves *p*-nitrophenyl- α -maltoside. This enzyme has not previously been described. We show that although the synthesis of this enzyme is subject to the same regulation as the components of the maltose regulon, and is MalT dependent, it is not coded for by a known *mal* gene. We refer to this enzyme as α -maltosidase. These results strengthen our proposal that the membrane components of the maltose transport system comprise a recognition site for maltose and related substrates.

We are interested in the mechanism of active transport. The periplasmic binding protein-dependent transport systems of gram-negative bacteria are attractive model systems because they can be dissected genetically as well as biochemically and physiologically. We have focused on the maltose transport system of *Escherichia coli* K-12 because this system is genetically well characterized and there is considerable biochemical information about some of its components (2, 3, 11; M. Schwartz, in F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger, ed., *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*, in press). Despite this wealth of background information, there is very little insight into the molecular details of how substrate molecules are transported and accumulated in the cytoplasm and how this process is coupled to energy.

The most outstanding characteristic of this and other similar systems is the participation of an abundant substrate-binding protein with a very high affinity for substrate, maltose-binding protein (MBP) (12). It is clear from a variety of experimental results that the periplasmic binding protein is the primary recognition site for substrates (2).

We have previously shown that MBP is essential for detectable substrate transport across the cytoplasmic membrane (9). This implies that MBP interacts with the membrane components during transport. We have reported that it is possible to isolate mutants in which MBP is no longer required for the active accumulation of maltose (13). In these mutants the MalF or MalG proteins have been altered so that the membrane components of the system transport maltose without the participation of MBP. The fact that these mutants transport maltose actively without the participation of MBP implies that the membrane components also contain a substrate recognition site.

We now provide additional evidence for this membrane

recognition site. We found that *p*-nitrophenyl- α -maltoside (pNPG2) is a potent inhibitor of maltose transport in the MBP-independent mutants but not in wild-type bacteria. In addition, this compound is transported by the MBP-independent mutants, but not by wild-type bacteria. We were able to detect the transport of pNPG2 by its intracellular hydrolysis and the release of *p*-nitrophenol. The cytoplasmic enzyme which hydrolyzes pNPG2 is part of the maltose regulon but is not encoded by a known *mal* gene. We think that this enzyme may function physiologically to remove a phosphate from malto-oligosaccharide-1-phosphates which could arise during the normal metabolism of maltose and maltodextrins.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains are listed in Table 1.

Media and chemicals. Rich medium and minimal salts M63 medium have been described previously (7). pNPG2 and *p*-nitrophenyl- α -maltoheptaoside were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Assays. α -Maltosidase activity was measured by following the release of *p*-nitrophenol from pNPG2. One unit of α -maltosidase is that amount of enzyme which liberates 1 nmol of *p*-nitrophenol per min at 30°C from pNPG2. We used the same buffer conditions as are used for measuring β -galactosidase (7). To quench the reaction we added 0.5 volume of 1 M Na₂CO₃ to raise the pH of the reaction mixture. Preliminary tests showed that this effectively stopped the enzymatic reaction. The assays were initiated by the addition of pNPG2 to a final concentration of 10 mM. *p*-Nitrophenol was detected by measuring the absorbance of the reaction mixture at 405 nm. The molar extinction coefficient of *p*-nitrophenol is 14,000 M⁻¹ liter⁻¹ (14). We verified that the assays were linear with respect to the amount of extract added and to the time of incubation. Typically, reactions were carried out in a total volume of 0.5 ml which

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Genotype	Origin or reference
MC4100	F ⁻ <i>araD139 lacU169 rpsL150 thi rel-1 fbbB5301 ptsF25 deoC7</i>	M. Casadaban
HS3166	MC4100, <i>malQ20(Am)</i>	Laboratory stock
HS3134	MC4100, ϕ (<i>malP-lacZ</i> ⁺)	(1)
HS2513	F' <i>lacI-3 lacY mal-1 pro</i> ⁺ MC4100, Δ <i>malB101</i>	(10)
HS3366	F' <i>lacI-3 lacY mal-1 pro</i> ⁺ MC4100, <i>malT::Tn10</i>	Laboratory stock
NT205	MC4100, <i>malE444 malF500</i>	(13)
NT206	MC4100, <i>malE444 malF502</i>	(13)
NT209	MC4100, <i>malE444 malF512</i>	(13)
HS3025	MC4100, <i>malE444 malG510</i>	(9, 13)

contained between 50 and 500 μ g of protein. All assays were performed in triplicate, and individual values did not differ by more than 5%.

Protein concentrations were determined with the Folin-Ciocalteu reagent by the method of Lowry et al. (6).

Preparation of crude extracts and assay of *p*-nitrophenol release. To measure the intracellular amounts of α -maltosidase, we prepared crude lysates of the strains and measured the rates of *p*-nitrophenol release from pNPG2. Overnight cultures of bacteria grown in M63 medium containing 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.) and the indicated carbon sources were centrifuged at 10,000 rpm in the SS-34 rotor of a Sorvall RC5B centrifuge and suspended in 0.1 volume of 50 mM Tris hydrochloride (pH 8.0) containing 5 mM EDTA and 100 μ g of lysozyme per ml. After 10 min on ice, the bacteria were sonicated with a Heat Systems sonicator equipped with a microprobe and an ice bath. Sonication was carried out three times for 30 s each at 70% output. After sonication, the lysate was clarified by centrifugation at 100,000 rpm in the Ti70 rotor at 4°C. The supernatant contained all of the α -maltosidase activity.

Transport assays. Transport assays were performed as described previously (13).

RESULTS

pNPG2 is a competitive inhibitor of maltose transport. In the course of examining the substrate specificity of the maltose transport system in MBP-independent mutants, we discovered that pNPG2 is a potent inhibitor of maltose transport in these strains (Fig. 1). Inhibition appears to be competitive on the basis of double-reciprocal plots (Fig. 1). The apparent K_i for pNPG2 in the different mutant strains is about 0.1 mM; this is at least 20-fold lower than the apparent K_m for maltose transport. When we examined the ability of pNPG2 to inhibit maltose transport in wild-type cells we found that to inhibit [¹⁴C]maltose uptake at concentrations near the K_m of transport (1 μ M), it was necessary to add 0.5 mM pNPG2 (data not shown). Since it is possible that the pNPG2 contains small amounts of maltose which could merely lower the specific activity of the radiolabel, we can not tell whether pNPG2 is a weak inhibitor of the wild-type system or whether it does not inhibit at all.

pNPG2 is hydrolyzed by whole cells. When pNPG2 was incubated with whole cells of the MBP-independent mutants we noticed that *p*-nitrophenol was produced. This was not the case when wild-type bacteria were used. The time course of *p*-nitrophenol release is depicted in Fig. 2. Crude extracts

of all strains, however, were equally able to cleave pNPG2 and release *p*-nitrophenol (Table 2). We interpret these results to mean that pNPG2 is transported by the MBP-independent mutants but not by the wild-type bacteria.

The ability of other *p*-nitrophenyl-glucosides to interact with the maltose system was then examined. *p*-Nitrophenyl- α -glucoside and *p*-nitrophenyl- β -glucoside did not inhibit maltose transport via the wild-type or the MBP-independent transport systems. In addition, neither one of these compounds was cleaved by cells or crude extracts to yield *p*-nitrophenol (data not shown). For this reason we were unable to directly determine whether they were transported. The highest-molecular-weight *p*-nitrophenyl- α -glucoside to be tested was pNPG7. This compound did not inhibit maltose transport in whole cells of the wild type or the MBP-independent mutant strains. One reason could be its inability to penetrate the outer membrane. To examine the ability of pNPG7 to inhibit the MBP-independent mutant transport system in the absence of the outer membrane permeability barrier, we measured inhibition of transport in spheroplasts rather than whole cells. Under these conditions as well, pNPG7 did not inhibit maltose transport in any of the MBP-independent mutants. In addition, we were unable to detect transport of pNPG7 by spheroplasts, even though this compound is efficiently cleaved by extracts made from cells (see below). To conclude that, in fact, the pNPG2 is being transported inside the cells we began to characterize the enzymatic activity that hydrolyzes pNPG2.

α -Maltosidase is maltose inducible and is not the product of a known *mal* gene. Since the products of the *mal* genes have been studied for some time, we were interested to know whether this activity was due to one of the known *mal* gene products. We first determined whether the activity was maltose inducible by measuring the ability of extracts from bacteria grown on different carbon sources to liberate *p*-nitrophenol from pNPG2. The activity of this enzyme was maltose inducible (Table 2). To determine whether this activity is controlled by the *malT* gene, we prepared extracts from an *malT* mutant strain (1). This strain, HS3366, contains a copy of the *lacY mal* gene that is constitutively expressed. This altered *lac* permease enables the strain to transport maltose into the cell independently of the normal MalT-controlled transport system (10). There was much less enzymatic activity in the extract of the *malT* mutant than in the extract from uninduced wild-type cells (Table 2). In addition, growth of the cells in the presence of maltose did not result in a high level of α -maltosidase activity even though maltose was able to enter these cells. This indicates that the structural gene for the α -maltosidase activity is subject to positive control by the *malT* gene product.

Next, we wanted to know whether the enzyme was coded for by any of the known *mal* genes. The most obvious choice was the *malQ* gene which codes for the enzyme amyloamylase. An extract that was prepared from a *malQ(Am)* strain contained at least the same amount of α -maltosidase activity as the wild-type strain (Table 2). The *malQ* gene therefore does not encode α -maltosidase. Extracts from a *malP lacZ* gene fusion strain also contain this enzyme so that the *malP* gene is not the structural gene. In addition, a strain which contains a large deletion of the *malB* region of the chromosome, strain HS 2513, contains this enzyme. We conclude therefore that the structural gene for this enzyme is not one of the known *mal* genes. We do not understand the reason for the higher specific activities of the enzyme in the extracts of the *malP* and *malQ* mutant strains.

Kinetics of *p*-nitrophenol release. To characterize the prop-

FIG. 1. Inhibition of [^{14}C]maltose uptake by pNPG2. Uptake of [^{14}C]maltose into four MBP-independent mutant strains was measured as previously described (13) at different external concentrations of maltose, in the absence of inhibitor, and in the presence of 0.1 and 0.2 mM pNPG2. The data are represented as double-reciprocal plots of the initial rates of maltose uptake and external maltose concentration. Symbols: $\bullet$, no inhibitor; $\blacksquare$, 0.1 mM pNPG2; $\blacktriangle$, 0.2 mM pNPG2. Panels: A, strain NT205; B, NT206; C, NT209; D, HS3025.

erties of the α -maltosidase, we measured the saturation kinetics of *p*-nitrophenol release from pNPG2 and from *p*-nitrophenyl- α -maltoheptaoside. We performed these experiments with crude extracts that were prepared from strains which lack amylomaltase and maltodextrin phosphorylase. This precaution was taken because the *p*-nitrophenyl compounds could be substrates for either enzyme. If this were the case, the kinetic results might be complicated. We found linear double-reciprocal plots with apparent K_m s of 4 mM for pNPG2 and 1 mM for pNPG7 (Fig. 3). The maximum velocity of *p*-nitrophenol release from the two compounds was different; release from pNPG2 was about seven times more rapid than from pNPG7 (28.6 versus 3.9 nmol/min). In addition, saturation kinetics of *p*-nitrophenol release from pNPG2 done in the presence of 100 mM maltose yielded an apparent K_i of 30 mM. Last, none of the extracts was able to liberate *p*-nitrophenol from *p*-nitrophenyl- α -glucoside or from *p*-nitrophenyl- β -glucoside. These results indicate that this enzymatic activity is probably physiologically more relevant for the catabolism of malto-oligosaccharides than for maltose itself.

Cellular location of α -maltosidase. To determine the cellular location of α -maltosidase, we fractionated wild-type cells which had been grown on maltose-containing medium. The cells were first converted to spheroplasts by the method of Witholt, et al. (15). The spheroplasts were centrifuged, and the resulting supernatant contained the contents of the periplasmic space. The spheroplasts were resuspended in Tris buffer containing 20 mM MgSO_4 and briefly sonicated. The lysed spheroplasts were then centrifuged at $130,000 \times g$ for 60 min. The clarified supernatant corresponds to the cytoplasm of the cell, and the pellet contains the inner and outer membranes. Each fraction was assayed for α -maltosidase activity. There was no detectable activity in the periplasmic fraction (<0.1 nmol of *p*-nitrophenol released

per min per ml; protein concentration of 0.19 mg/ml), and essentially all of the activity can be recovered from the cytoplasmic fraction (59.0 nmol of *p*-nitrophenol released per min per ml; protein concentration of 2.4 mg/ml). These results indicate that for pNPG2 to be hydrolyzed by intact cells it must reach the cytoplasm.

DISCUSSION

We have presented data that demonstrate that the cytoplasmic membrane components of the maltose transport system of *E. coli* are capable of recognizing and transporting pNPG2 in a saturable manner. This capacity is apparent only in certain mutant strains that are able to transport independently of the periplasmic maltose binding protein. In wild-type cells, transport is completely dependent on the periplasmic MBP and is not inhibited by pNPG2, nor is pNPG2 transported. These results lend support to the view that the cytoplasmic membrane components of the periplasmic protein-dependent systems do contain a substrate recognition site, but that in wild-type cells this site is available only to ligands carried on the periplasmic protein. This site becomes available to free ligands in the MBP-independent mutant strains.

Another reason that pNPG2 is interesting is that *p*-nitrophenyl ethers have been used as photoactivated affinity-labeling agents (4, 5). We hope to use pNPG2 in this way to localize the site(s) within the membrane components of the maltose transport system which interacts with substrates.

We have identified an enzymatic activity which cleaves the *p*-nitrophenyl group from the *p*-nitrophenyl ethers of malto-oligosaccharides. This enzymatic activity is maltose inducible; induction is dependent on the *malT* gene product, but is not coded for by any of the known *mal* genes. We refer to this enzyme as α -maltosidase. It has apparent K_m s of 4

FIG. 2. Release of *p*-nitrophenol by whole cells. Cells were washed and incubated at 30°C in the presence of 1 mM pNPG2 at an absorbance at 600 nm of 10. At the indicated times samples were removed, and the extent of *p*-nitrophenol production was measured by stopping the reaction by the addition of 0.5 volume of 1 M Na₂CO₃ and pelleting the cells by low-speed centrifugation for 5 min. Essentially all of the *p*-nitrophenol was recovered in the external medium. The amount of *p*-nitrophenol was quantitated by measuring the absorbance of the supernatant at 405 nm (*A*₄₀₅). Panels: A, strain MC4100; B, NT205; C, NT206; D, NT209; E, HS3025.

TABLE 2. α -Maltosidase activity in various strains^a

Strain	Carbon Source	α -maltosidase (nmol/min per mg of protein)
MC4100	Glycerol	1.8
	Glycerol-maltose	24.5
NT205	Glycerol-maltose	43.2
NT206	Glycerol-maltose	52.8
NT209	Glycerol-maltose	17.3
HS3025	Glycerol-maltose	22.5
HS3366	Glycerol	0.8
	Glycerol-maltose	1.5
HS3166	Glycerol	4.6
	Glycerol-maltose	124.3
HS3134	Glycerol	4.6
	Glycerol-maltose	94.0
HS2513	Glycerol	3.0
	Glycerol-maltose	31.0

^a Extracts were prepared and *p*-nitrophenol release was assayed as described in Materials and Methods.

FIG. 3. Saturation kinetics of *p*-nitrophenol release from pNPG2 (●) and *p*-nitrophenyl- α -maltoheptaoside (■). A crude extract was prepared from strain HS3134 as described in Materials and Methods. The rate of *p*-nitrophenol production was measured at different concentrations of pNPG2 and *p*-nitrophenyl- α -maltoheptaoside as described in Materials and Methods; each reaction contained 340 μ g of protein. The data are represented as double-reciprocal plots of the rate of *p*-nitrophenol production as a function of the substrate concentration.

mM for the pNPG2 substrate and 1 mM for the pNPG7 substrate. The enzyme is located in the cytoplasm of the cell. At the present time the function of this enzyme is unclear; we intend to isolate mutants which no longer contain this activity and to examine their phenotype. One possible role for this enzyme could be to protect the cell against the accumulation of malto-oligosaccharide-1-phosphates. These could arise during maltose catabolism in two ways. Either amylomaltase could use glucose 1-phosphate as an acceptor and transfer additional malto-oligosaccharide units to it, or maltodextrin phosphorylase may liberate a malto-oligosaccharide-1-phosphate rather than glucose 1-phosphate (8). Even if either of these possible reactions were occurring at a low rate, the accumulation of such compounds would probably be toxic since there would be no efficient way to metabolize them. The physiological reaction catalyzed by the enzyme which we describe may be to remove the phosphate from any malto-oligosaccharide-1-phosphate which is produced during maltose or maltodextrin catabolism. To evaluate this hypothesis we will need to purify the enzyme and isolate mutants which are defective for the production of this enzyme.

ACKNOWLEDGMENTS

This research was supported by grant AI 19276 from the National Institutes of Health. N.A.T. was supported by Public Health Service training grant T32 AI 017161 from the National Institutes of Health and by a Faculty Fellowship. H.A.S. is a Career Scientist of the Irma T. Hirsch Trust.

We thank Ron Kaback for pointing out the utility of the *p*-nitrophenyl ether compounds. We acknowledge the excellent technical assistance of Carmen Rodriguez.

LITERATURE CITED

1. Debarbouillé, M., H. A. Shuman, T. J. Silhavy, and M. Schwartz. 1978. Dominant constitutive mutations in *malT*, the

- positive regulator gene of the maltose regulon in *Escherichia coli*. J. Mol. Biol. **124**:359–371.
2. Hengge, R., and W. Boos. 1983. Maltose and lactose transport in *Escherichia coli*. Examples of two different types of concentrative transport systems. Biochim. Biophys. Acta **737**:443–478.
  3. Hofnung, M. (ed.). 1982. The maltose system as a tool in molecular biology. Ann. Microbiol. Paris **133A**:5–273.
  4. Jelenc, P. C., C. R. Cantor, and S. R. Simon. 1978. High yield photoreagents for protein crosslinking and affinity labelling. Proc. Natl. Acad. Sci. USA **75**:3564–3568.
  5. Kaczorowski, G. J., G. LeBlanc, and H. R. Kaback. 1980. Specific labelling of the *lac* carrier protein in membrane vesicles of *Escherichia coli* by a photoaffinity reagent. Proc. Natl. Acad. Sci. USA **77**:6319–6323.
  6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
  7. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  8. Monod, J., and A. M. Torriani. 1948. Synthèse d'un polysaccharide de type amidon aux dépens du maltose, en présence d'un extrait enzymatique d'origine bactérienne. C. R. Acad. Sci. **227**:240–242.
  9. Shuman, H. A. 1982. Active transport of maltose in *Escherichia coli* K-12. Role of the periplasmic maltose binding protein and evidence for a substrate recognition site in the cytoplasmic membrane. J. Biol. Chem. **257**:5455–5461.
  10. Shuman, H. A., and J. Beckwith. 1979. *Escherichia coli* mutants that allow transport of maltose via the β -galactoside transport system. J. Bacteriol. **137**:365–373.
  11. Shuman, H. A., and N. A. Treptow. 1984. The maltose/maltodextrin transport system of *Escherichia coli* K-12, p. 561–575. In A. N. Martonosi (ed.), The enzymes of biological membranes, 2nd ed. Plenum Publishing Corp., New York.
  12. Szmelcman, S., M. Schwartz, T. J. Silhavy, and W. Boos. 1976. Maltose transport in *Escherichia coli* K-12. A comparison of transport kinetics in wild-type and lambda-resistant mutants with the dissociation constants of the maltose binding protein as measured by fluorescence quenching. Eur. J. Biochem. **65**:13–19.
  13. Treptow, N. A., and H. A. Shuman. 1985. Genetic evidence for substrate and binding protein recognition by the MalF and MalG proteins, cytoplasmic membrane components of the *Escherichia coli* maltose transport system. J. Bacteriol. **163**:654–660.
  14. Walsh, C. 1979. Enzymatic reaction mechanisms, p. 68. W. H. Freeman and Co. San Francisco.
  15. Witholt, B., M. Boekhout, M. Brock, J. Kingma, H. van Heerikhuizen, and L. de Leij. 1976. An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. Anal. Biochem. **74**:160–170.