The EMBO Journal Vol.2 No.5 pp.715-720, 1983

The mechanism of inducer exclusion. Direct interaction between purified III^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system and the lactose carrier of *Escherichia coli*

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Communicated by P. Overath
Received on 11 February 1983

A hypothesis for the regulation of some sugar transport systems by the bacterial phosphoenolpyruvate:sugar transport system postulates an interaction between III^{Glc} of this system and the carrier whose activity is regulated. We have studied this interaction in more detail, employing one of these transport systems, the lactose carrier of *Escherichia coli*. Purified III^{Glc} of the phosphotransferase system interacted directly with the lactose carrier. The binding of III^{Glc} to lactose carrier required the presence of the non-phosphorylated form of III^{Glc} and substrates of the carrier and exhibited a stoichiometry of 1.2 ± 0.2 mol III^{Glc}/mol lactose carrier. The K_d of lactose carrier for III^{Glc} was 10 ± 5 μM. III^{Glc} is apparently unable to interact with a mutant lactose carrier which still binds but does not transport galactosides. The binding of III^{Glc} to the lactose carrier results in a 3.5-fold increase in the apparent affinity of galactosides for the carrier. Significantly, the binding of III^{Glc} to the lactose carrier results in an inhibition of galactoside translocation both in membrane vesicles and liposomes reconstituted with the purified lactose carrier. This inhibition may thus be the basis for the well-documented phenomenon of inducer exclusion.
Key words: inducer exclusion/lactose carrier/phosphotransferase system/reconstitution/regulation

Introduction

The preference of microorganisms for certain sugars above others is well known. Diauxic growth and the phenomenon of catabolite repression has been studied for many years, but no clear-cut answer as to the mechanism(s) is possible (Magan-asik, 1970; Botsford, 1981). One regulatory mechanism involves the phosphoenolpyruvate:sugar phosphotransferase system which is thought to be involved in the modulation of sugar tansport and adenylate cyclase (Postma and Roseman, 1976; Saier, 1977).

Transport and concomitant phosphorylation of many sugars in *Escherichia coli* and *Salmonella typhimurium* is catalysed by the phosphotransferase system (PTS). A phosphoryl group is transferred from phosphoenolpyruvate (PEP) to the various PTS sugars *via* the soluble PTS proteins: Enzyme I, HPr, and the membrane-bound sugar-specific Enzymes II (Postma and Roseman, 1976). Genetic analysis of mutants defective in components of the PTS has, however, revealed a more profound effect of *pts* mutations on cell physiology than would be expected from the description above of the PTS as a sugar transport system. Mutants lacking Enzyme I (*ptsI*) or HPr (*ptsH*) are not only defective in growth on all PTS sugars (as expected) but, in addition, are unable to grow on many non-PTS compounds such as maltose, melibiose, glycerol and lactose [Class I compounds (Postma, 1982)] or Krebs-cycle intermediates and xylose [Class II compounds (Postma, 1982)]. The pleiotropic nature of *ptsH,I* mutations has been attributed in general to two effects (Postma and Roseman, 1976; Saier, 1977): (i) inability of the mutant cells to accumulate certain non-PTS compounds (Class I) which are required inside the cell as inducers (inducer exclusion) and (ii) lowering of adenylate cyclase activity in such *pts* strains, resulting in intracellular cAMP levels too low for expression of certain cAMP-dependent operons (Class I and II compounds).

Isolation of secondary mutations allowing *pts* mutants to grow on all or some of these non-PTS compounds (Saier and Roseman, 1976a) and analysis of the phenomenon of inducer exclusion (Saier and Roseman, 1976b) has been the basis for the hypothesis that regulation by the PTS is mediated through one of the soluble, sugar-specific PTS proteins, III^{Glc}, which is involved in glucose (Glc) transport *via* the PTS. It has been postulated that III^{Glc} in its non-phosphorylated form (i.e., PTS sugar present) directly binds to the various Class I non-PTS transport systems and thus inhibits transport activity. Secondly, phosphorylated III^{Glc} would activate adenylate cyclase.

Osumi and Saier (1982) have demonstrated that partially purified III^{Glc} binds to *E. coli* membranes from the lactose carrier overproducing strain T206 and that this binding is dependent on the presence of non-phosphorylated III^{Glc} and galactoside. Here, we present direct evidence that III^{Glc} indeed binds stoichiometrically to one such Class I transport system, the (purified) lactose carrier of *E. coli*, and inhibits transport activity both in membrane vesicles and in liposomes reconstituted with the purified lactose carrier. Thus, one of the postulates of the model of PTS-mediated regulation, described above, has been verified experimentally with the purified components.

Results

Binding of III^{Glc} to E. coli membranes

Since III^{Glc} cannot penetrate the cytoplasmic membrane, external III^{Glc} can only interact with lactose carrier whose cytoplasmic face is directed outward (i.e., inside-out). One might expect the inside-out topology to be relatively high in Ribi vesicles (high pressure lysis) and cytoplasmic membrane vesicles (sonication) and low in reconstituted liposomes.

Purified III^{Glc} from *S. typhimurium* binds to cytoplasmic membrane vesicles from the *lacY* plasmid harboring *E. coli* strain T206 which contains elevated levels of lactose carrier. After incubation of III^{Glc} with membranes and centrifugation in an airfuge, the fraction of III^{Glc} remaining in the supernatant and that bound to membranes was determined quantitatively by rocket immunoelectrophoresis. At III^{Glc} concentrations varying between 0 and 0.44 mg/ml, T206 cytoplasmic membrane vesicles, containing the lactose carrier at the elevated level of 3-4 nmol/mg protein, bind between 0 and 5 μg III^{Glc} per mg membrane protein (Figure 1). T184 cytoplasmic membrane vesicles containing no lactose carrier

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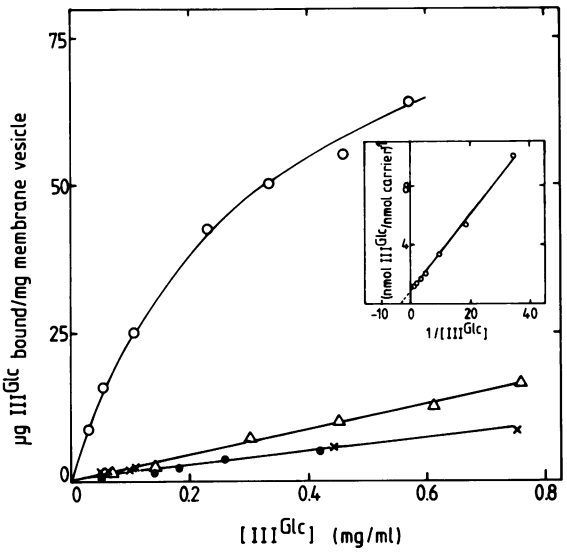

Fig. 1. Binding of III^{Glc} to cytoplasmic membrane vesicles. Suspensions were prepared of 0.1 mg cytoplasmic membrane vesicles made from strain T206 (3.8 nmol lactose carrier/mg vesicle protein) or T184 (no lactose carrier) in 50 μl 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM dithiothreitol 2.5 mM KF, 5 mM $MgCl_2$, 0.0015 IU of Enzyme I and HPr each, 20 mM TDG (if present), and varying amounts (0.03 – 0.8 mg/ml) of III^{Glc}. PEP, when added, was present at a concentration of 10 mM. After incubation at room temperature for 30 – 60 min, the membranes were separated and the bound and free III^{Glc} were determined as described in Materials and methods. To generate the reciprocal plot (**insert**), the binding data for strain T206 were corrected for non-specific binding by subtracting the amount of III^{Glc} bound to T184 membranes (no lactose carrier) at the corresponding free III^{Glc} concentration. x—x, III^{Glc} binding to T184 in the presence or absence of TDG; ●—●, III^{Glc} binding to T206 in the presence of enzyme I/HPr and absence of TDG; △—△, III^{Glc} binding to T206 in the presence of Enzyme I/HPr, PEP and TDG; ○—○, III^{Glc} binding to T206 in the presence of Enzyme I/HPr and TDG (no PEP).

bind the same amount of III^{Glc} per mg protein (Figure 1). Addition of substrates of the lactose carrier such as thiodigalactoside (TDG) or *p*-nitrophenyl α-D-galactopyranoside (αPNPG) considerably increased the binding of III^{Glc} to T206 membranes. No increase was observed with the control T184 membranes. The amount of III^{Glc} bound to T206 membranes is a function of the III^{Glc} added (Figure 1). From the double reciprocal plot (see insert, Figure 1) a K_d of 12 μM can be calcuated for III^{Glc}, assuming that III^{Glc} exists as a monomer of mol. wt. 20 000 (Scholte *et al.*, 1981). The number of III^{Glc} molecules (monomer) bound to a lactose carrier (monomer) was calculated to be 1 in this experiment, assuming the binding of 1 mol of αPNPG per mol of lactose carrier monomer (Teather *et al.*, 1980; Overath *et al.*, 1979).

The experiment reported in Figure 1 represents binding of III^{Glc} to lactose carrier at pH 7.5 in the presence of the soluble purified PTS proteins Enzyme I and HPr. Table I also shows results obtained at pH 6.3, the optimal pH for galactoside binding, and results obtained in the absence of the soluble PTS proteins. The K_d for III^{Glc} varies between 16 and 5 μM and the number of III^{Glc} molecules bound to a molecule of lactose carrier between 1 and 1.3, independent of pH, galactoside used, membrane preparation and the presence of the soluble PTS components. The values of III^{Glc} bound to the lactose carrier in T206 membranes have been corrected for the amount of III^{Glc} bound to T184 membranes containing no lactose carrier. Figure 1 shows that this amount increased linearly with increasing amounts of III^{Glc}.

Table I. III^{Glc} binding to membrane vesicles, K_d and stoichiometry

Exp.	Type of vesicles	pH	Substrate	III^{Glc}		Enzyme I + HPr
				K_d (μM)	Stoichiometry (III^{Glc}/lactose carrier)	
1	Cytoplasmic membrane	6.3	αPNPG	16	1.0	–
2	Cytoplasmic membrane	6.3	TDG	5	1.3	+
3	Cytoplasmic membrane	7.5	TDG	12	1.0	+
4	Ribi	7.5	TDG	5	1.3	+

Binding of varying amounts (0.01 – 1.3 mg/ml) of III^{Glc} to a fixed amount of membrane vesicles (0.1 or 0.6 mg/ml) was determined as described in Materials and methods. The K_d and maximum amount of III^{Glc} bound was determined as shown in Figure 1. Background binding, as determined by measuring III^{Glc} binding to T184 vesicles (Figure 1) which contained no lactose carrier, was subtracted in each case. Cytoplasmic membrane vesicles contained 3.2 (Exp. 1) or 3.8 (Exps. 2 and 3) nmol lactose carrier/mg protein, respectively. The Ribi vesicles (Exp. 4) contained 1.4 nmol lactose carrier/mg protein. Lactose carrier substrates were added to a final concentration of 0.2 mM (αPNPG) or 20 mM (TDG). Where indicated, Enzyme I and HPr were added.

The lactose carrier is sensitive to thiol reagents which inhibit binding of galactosides (Kennedy *et al.*, 1974; Overath *et al.*, 1979). *p*-Chloromercuribenzenesulphonate (PCMBS) inhibits the stimulation of III^{Glc} by the addition of galactosides.

Determination of the number of III^{Glc} molecules bound to the lactose carrier in the absence of galactose yields results that are similar to those with T184 membranes. Within the range of III^{Glc} concentrations used (0.2 – 0.7 mg/ml) the amount of III^{Glc} bound increased linearly. Most likely this represents non-specific binding and suggests that the lactose carrier has a much lower or no affinity for III^{Glc} in the absence of its substrate.

We have also studied the binding of III^{Glc} to a mutated lactose carrier. Membranes made from cells containing the *lacY*^{59} mutation on a plasmid still bind galactosides but are unable to catalyse translocation of the galactoside (P. Overath *et al.*, in preparation). In fact, the affinity of the mutated carrier for galactosides was increased. The mutated lactose carrier is unable to bind any III^{Glc} in the presence of αPNPG.

Effect of III^{Glc} on galactose binding to lactose carrier

We have described above the enhancement of III^{Glc} binding to the lactose carrier by its substrates TDG or αPNPG. On thermodynamic grounds one would also expect the reverse, i.e., an apparent increased affinity of lactose carrier for galactosides in the presence of III^{Glc}. This is indeed the case. Wright *et al.* (1981) reported a K_d of 24 μM for αPNPG from flow dialysis experiments. Using Ribi vesicles from strain T206, we found a K_d of 27 μM (Figure 2.) the K_d decreased to 6.5 μM in the presence of III^{Glc} (0.75 mg/ml, saturating 74% of lactose carriers, assuming a K_d for III^{Glc} of 10 μM) (Figure 2). The amount of galactoside bound per mg protein remained almost the same, 1.1 *versus* 0.9 nmol αPNPG bound per mg vesicle protein. The small difference might be explained by the inaccessibility of some lactose carrier to III^{Glc}; whereas αPNPG is able to reach all lactose carriers (Overath *et al.*, 1979), III^{Glc} is unable to cross the membrane. Under conditions where 0.78 nmol αPNPG is bound

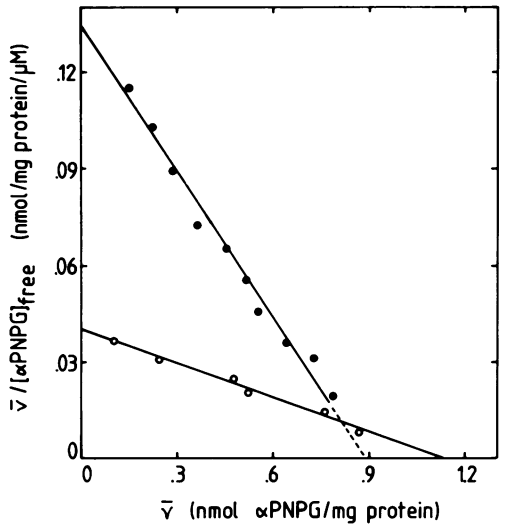

Fig. 2. Binding of αPNPG to Ribi vesicles from T206 in the presence and absence of III^{Glc}. Samples containing 11 mg membrane protein/ml were titrated with [^{3}H]αPNPG in the range 2.7–43 μM. The III^{Glc} concentration, when present, was 0.7 mg/ml. ●——●, plus III^{Glc}; ○——○, in the absence of III^{Glc}.

Table II. Binding of III^{Glc} and αPNPG to lactose carrier reconstituted in liposomes

	III^{Glc} bound μg/ml phospholipid)	K_d αPNPG (μM)	
		+ III^{Glc}	− III^{Glc}
Liposomes	6	ND	ND
Liposomes + lactose carrier	7	7	22
Liposomes + lactose carrier + Enzyme I, HPr, PEP	ND	20	ND

Binding of III^{Glc} to liposomes with or without lactose carrier was measured as described in Materials and methods. Binding of αPNPG to these liposomes was measured with flow dialysis in 30 mM MES, 2 mM $MgSO_4$, 10 mg/ml BSA, pH 6.3, after a freeze-thaw cycle. If present, purified Enzyme I, HPr and 10 mM PEP were added. III^{Glc} was added to a final concentration of 0.7 mg/ml. Reconstituted liposomes contained 0.13 nmol lactose carrier/mg phospholipids. ND, not determined.

per mg protein, the amount of III^{Glc} bound is 0.58 nmol per mg protein. In a complementary experiment, titration of Ribi vesicles of T206 in the presence of sub-saturating levels of αPNPG (10 μM) with increasing III^{Glc} (up to 0.7 mg/ml) resulted in a steady increase of bound galactoside to about three times the original level (data not shown).

Binding of phosphorylated III^{Glc} to the lactose carrier

According to the hypothesis outlined in the Introduction, non-phosphorylated III^{Glc} is the inhibiting agent. Whether phosphorylated III^{Glc} was unable to bind or binds in a non-inhibitory way is not known. Phosphorylated III^{Glc} ($P \sim III^{Glc}$), generated *in situ* by added Enzyme I, HPr and PEP, binds less well to the lactose carrier (Figure 1). In contrast to results with non-phosphorylated III^{Glc}, no saturation of $P \sim III^{Glc}$ binding is seen in the range 0–0.7 mg III^{Glc}/ml. A similar binding of III^{Glc} to T184 membranes containing no lactose carrier is also detected (Figure 1). This linearly increased binding might represent non-specific binding of III^{Glc} to

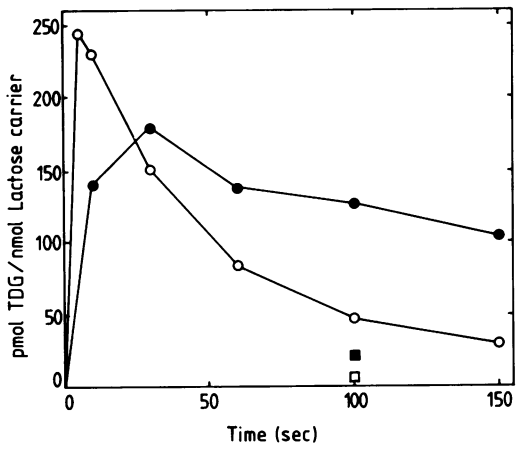

Fig.3. Effect of III^{Glc} on lactose-driven TDG countertransport in cytoplasmic membrane vesicles. Transport was measured in T206 membrane vesicles as described in Materials and methods in the presence (●——●) and absence (○——○) of III^{Glc} (0.7 mg/ml). Pretreatment of the vesicles with 20 mM N-ethylmaleimide for 1 h at room temperature suppresses counterflow in the presence (■——■) or absence (□——□) of III^{Glc}.

membranes and entrapment in the pellet. Binding of phosphorylated III^{Glc} is not affected by the absence or presence of a galactoside (data not shown).

Interaction of III^{Glc} and liposomes reconstituted with lactose carrier

Since direct interaction between III^{Glc} and the lactose carrier is required in the regulatory model proposed, a reconstituted system consisting of the purified components provides an ideal way to test whether other components are involved. In the preceding sections we have demonstrated the interaction of purified III^{Glc} and lactose carrier in membrane vesicles. Similar studies were attempted with III^{Glc} and purified lactose carrier reconstituted in liposomes.

Table II shows the binding of III^{Glc} to liposomes, with or without a lactose carrier, and the effect of galactosides. Only a small increase is seen, due to the high level of non-specific binding of III^{Glc} to phospholipids. The partly hydrophobic character of III^{Glc} has previously been documented (Scholte *et al.*, 1981). At a ratio of 0.13 nmol lactose carrier per mg phospholipids, the maximal increase above the high background that can be expected in the presence of galactoside is calculated as 22% (assuming that half of the lactose carriers are oriented inside-out; see Discussion), which is within the limits of experimental error. Thus, quantitative measurements are difficult, and 10 mg/ml bovine serum albumin (BSA) does not effectively suppress the non-specific binding.

The complementary effect, a decreased K_d of lactose carrier for αPNPG in the presence of III^{Glc}, is easier to observe. Table II shows that the K_d for αPNPG is 22 μM in the absence of III^{Glc} and 7 μM in its presence for 57% of the available sites. When III^{Glc} was phosphorylated by PEP in the presence of Enzyme I and HPr, the K_d characteristic for the binding in the absence of any III^{Glc} was found: 20 μM (Table II). The biphasic nature of αPNPG binding to the reconstituted lactose carrier in the presence of III^{Glc} indicates that not all III^{Glc} sites are accessible.

Effect of III^{Glc} on galactoside transport in membrane vesicles

Lactose-driven TDG countertransport and lactose-lactose equilibrium exchange are two energy-independent transport

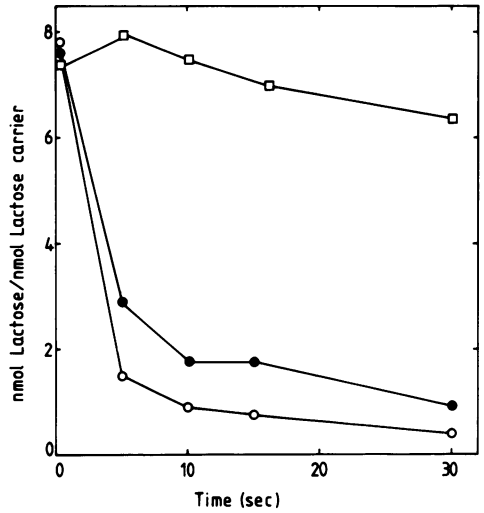

Fig. 4. Effect of III^{Glc} on outward-directed tracer flux during lactose-lactose equilibrium exchange in cytoplasmic membrane vesicles. Transport was measured as described in Materials and methods in the presence (●——●) or absence (○——○) of III^{Glc} (0.7 mg/ml). Pretreatment of the vesicles with 0.4 mM PCMBS inhibits exchange (□——□).

processes specifically catalysed by the lactose carrier. A number of experiments have been performed to investigate whether a direct effect of III^{Glc} on transport *via* the lactose carrier could be observed. Cytoplasmic membrane vesicles, preloaded with 40 mM lactose, transiently accumulated ^3H-labelled TDG (20 μM) (Figure 3). After a certain time, labelled TDG left the vesicles again and equilibrium was approached. When this experiment was repeated in the presence of 0.7 mg/ml III^{Glc} (Figure 3), several features of the counter-transport curve were altered: (i) the initial rate decreased to ~30% of the control value without III^{Glc}; (ii) the height of the overshoot was decreased by 27%; (iii) the maximum is attained less rapidly (~30 s compared with <5 s without III^{Glc}); and (iv) the efflux of the accumulated TDG was much slower ($t_{1/2}$ >120 s compared with ~40 s without III^{Glc}). These four effects are consonant with a reduction in the transport activity of the lactose carrier in the presence of III^{Glc} (c.f., Maloney and Wilson, 1973, for a discussion of countertransport and carrier levels).

In a second experiment, the effect of III^{Glc} on equilibrium exchange was investigated. Cytoplasmic membrane vesicles from strain T206 were loaded with 40 mM [^3H]lactose and subsequently diluted into buffer containing 40 mM unlabelled lactose. The internal labelled lactose was rapidly exchanged for unlabelled lactose (Figure 4). However, in the presence of 0.7 mg/ml III^{Glc} (Figure 4), the apparent initial rate decreased from at least 1.3 nmol lactose/s/nmol lactose carrier to 0.9, and the time course of the exchange was slowed down. The 30% inhibition is most likely an underestimate since even at the first sampling point (5 s) most of the label had already left the vesicles.

Effect of III^{Glc} on active transport in liposomes reconstituted with lactose carrier

Purified lactose carrier was reconstituted into valinomycin-containing liposomes (Wright *et al.*, 1983). Active transport was supported by artificial electrical ($\Delta\Psi$ = −120 mV) and pH ($\frac{RT}{F}\Delta pH$ = −60 mV) gradients. Whilst the rate of TDG active transport varies from preparation to preparation (Wright *et al.*, 1983), 0.6 mg/ml III^{Glc} inhibited the initial rate of the active transport of 200 μM TDG by an average of 18% (Table III). This modest decrease in the rate of TDG uptake is nevertheless close to the maximum expected for the reconstituted system under these conditions (see Discussion).

Table III. Effect of III^{Glc} on active transport of TDG in liposomes reconstituted with purified lactose carrier

Preparation	TDG transport		Inhibition (%)
	$-III^{Glc}$	$+III^{Glc}$	
1	0.21	0.15	28
2	0.58	0.52	11
3	0.63	0.53	16

Uptake of 0.2 mM [^3H]TDG was measured as described in Materials and methods in the absence or presence (0.6 mg/ml) of III^{Glc}. The rate of TDG uptake is expressed as nmol TDG/s/nmol lactose carrier.

Discussion

We have shown by a number of criteria that a purified PTS protein, III^{Glc}, can interact with the lactose carrier and that this interaction requires no additional components. The following results were obtained. (i) In the presence of galactosides, III^{Glc} bound to *E. coli* membranes containing a high content of lactose carrier, with a K_d varying between 5 and 16 μM. (ii) 1−1.3 mol of III^{Glc} was bound per mol of lactose carrier, assuming a mol. wt. of 20 000 for III^{Glc} (Scholte *et al.*, 1981) and 1 substrate-binding site per molecule of lactose carrier (Teather *et al.*, 1980; Overath *et al.*, 1979). (iii) Phosphorylation of III^{Glc} prevented binding of III^{Glc} to lactose carrier. (iv) The apparent dissociation constant of lactose carrier for αPNPG decreased in the presence of III^{Glc} from 27 μM to 6.5 μM in membrane vesicles and from 22 μM to 7 μM in liposomes reconstituted with purified lactose carrier. (v) III^{Glc} does not bind to a mutant lactose carrier which is still able to bind galactosides but unable to translocate its substrates (Overath *et al.*, in preparation). This mutant carries three alterations in the first 24 amino acids. This observation may indicate that III^{Glc} binds to the N terminus of the lactose carrier and that the N terminus may be exposed on the cytoplasmic face of the membrane. (vi) III^{Glc} inhibited the transport activity of the lactose carrier. Both transient accumulation of labelled TDG during counter-transport and outward-directed tracer flux during lactose equilibrium exchange were inhibited by III^{Glc} in membrane vesicles. (vii) Finally, active transport of galactoside was also inhibited by III^{Glc} in liposomes reconstituted with purified lactose carrier. The effects described under (i) and (iii) have previously been characterized by Osumi and Saier (1982).

From these results, we draw the following conclusions. Non-phosphoryated III^{Glc} can bind to the lactose carrier and modify its activity. The apparent increase in the affinity of the lactose carrier for galactosides can be attributed to the binding sequence

$$C + G \rightleftharpoons C.G$$

$$C.G + III^{Glc} \rightleftharpoons C.G.III^{Glc}$$

where C represents the lactose carrier and G galactoside. The apparent increase in galactoside affinity is a consequence of the displacement of these equilibria to the right by III^{Glc} and forms a convenient assay for carrier-III^{Glc} interaction. The physiological consequence of this interaction is, however, that

the transport (i.e., not binding) activity of the III^{Glc}.C.G complex is lower than that of the C.G complex, perhaps zero. Inducers of non-PTS systems, therefore, cannot enter the cell as easily. No components other than purified III^{Glc} and lactose carrier are required for this interaction. Since the K_d for III^{Glc} lies between 5 and 16 μM and the intracellular III^{Glc} concentration has been calculated to be ~50 μM (Scholte *et al.*, 1981, 1982), regulation of transport systems such as the lactose carrier by III^{Glc} seems feasible. The data presented in Table I suggest that 1.2 ± 0.2 mol of III^{Glc} bind to 1 mol of lactose carrier. We have shown earlier than III^{Glc} monomers may associate to form oligomers consisting of up to six subunits under physiological conditions (Scholte *et al.*, 1982). The quaternary structure of the lactose carrier is unknown (Overath and Wright, 1982). Both the binding of a III^{Glc} trimer to a putative lactose dimer and monomer-to-monomer binding is possible. At present, the data do not allow us to make a distinction. Interestingly, Hengstenberg and coworkers (Deutscher *et al.*, 1982) have recently proposed that in *Staphylococcus aureus* the soluble lactose PTS protein III^{Lac} exists as a trimer which binds to the membrane-bound Enzyme II^{Lac} as a monomer (in *S. aureus* lactose is transported *via* the PTS, in contrast to *E. coli* which contains an H^+-lactose symport system).

It is clear from Figures 3 and 4 and Table III that inhibition of transport in all cases is <100%. There are several reasons for this. First, it is in practice very difficult to saturate all carriers with III^{Glc}. Substantial amounts of III^{Glc} (>2.5 mg/ml) would be required in all buffers. Most experiments were done at III^{Glc} concentrations which could result in maximally 60–80% of the lactose carrier being complexed with III^{Glc}. Consequently, this is the maximal percentage inhibition that can be attained. From Figures 3 and 4 we can calculate that the transport parameters are altered by III^{Glc} to values between 30 and 70% of those in its absence. Secondly, III^{Glc} must be able to reach its binding site on the lactose carrier, but cannot transverse the membrane. This problem arises both in membrane vesicles and with reconstituted lactose carrier in liposomes. At present, we have no definite way to determine the orientation of these lactose carriers. The reconstituted carrier is probably randomly inserted into the liposomes so that only half might be accessible to III^{Glc}. Since the lactose carrier is a symmetrical protein with respect to transport activity (Teather *et al.*, 1977; Lancaster and Hinkle, 1977), all lactose carriers will catalyse TDG or lactose transport but only half will be able to bind III^{Glc}, resulting in maximally 50% inactive carrier. At the concentrations of III^{Glc} used, one can at best expect 30–40% inhibition. Thirdly, non-specific binding of III^{Glc} to the liposomes (Table II) can reduce the amount available for interaction with the lactose carrier. Finally, even in intact cells inhibition of lactose carrier activity by PTS sugars, is <100% (Saier and Roseman, 1976b). Thus, the percentage inhibition found for active transport of TDG in reconstituted liposomes, 18 ± 5%, approaches the maximal value that might be obtained.

In a strict sense we have shown only interaction between III^{Glc} and one non-PTS transport system that is regulated by the PTS. But these results most likely also apply to the other non-PTS transport systems, such as those for maltose, melibiose or glycerol. These may be studied in the same way as soon as they can be overproduced in cells in a way similar to the lactose carrier. Depending on the complexity of the transport system (consider the maltose transport system, consisting of at least four components), even studies with the reconstituted systems may be possible. If we extrapolate our findings with the lactose carrier to other transport systems that are regulated by the PTS, the following interesting picture emerges. III^{Glc} can bind to the various (inducible) transport systems such as the lactose, maltose, melibiose and glycerol transport systems [Class I compounds (Postma, 1982)]. Since the amount of III^{Glc} is constant under the various growth conditions (Scholte *et al.*, 1981), one can imagine that the number of transport systems that can bind III^{Glc} can exceed the number of III^{Glc} molecules with the following consequences. (i) If the number of copies of any transport system exceeds the number of III^{Glc} molecules its substrate will escape inducer exclusion. This has been demonstrated experimentally (Nelson *et al.*, 1982). Alternatively, inducer exclusion, and thus the inability to synthesize certain inducible metabolic systems, is most effective under conditions in which the inducible transport system is present at a low level (Nelson *et al.*, 1982). (ii) Since III^{Glc} is limiting, one predicts that one non-PTS compound can relieve inducer exclusion of other non-PTS compounds. Preliminary experiments show this to be the case. If both the maltose and glycerol transport systems are induced, the inhibition of maltose tansport by a PTS sugar can be relieved by addition of glycerol (S.O. Nelson, unpublished results). Compounds such as galactose which are not regulated by the PTS, have no effect. (ii) The model proposed above also predicts that non-PTS compounds could affect adenylate cyclase *via* the PTS. If the free concentration of III^{Glc} is lowered by binding to certain non-PTS transport systems, the amount of phosphorylated III^{Glc} is also reduced, resulting in an apparent inhibition of adenylate cyclase. This effect has indeed been observed but interpreted in a different way (Peterkofsky and Gazdar, 1981). This does not imply that this is the only way adenylate cyclase can be regulated. It is known that compounds not regulated by the PTS at all, for instance glucose-6-phosphate, can also inhibit adenylate cyclase.

We have described a complex regulatory system which is present in bacteria such as *E. coli* and *S. typhimurium* and consists of a cascade of phosphoproteins. III^{Glc}, a PTS protein involved in glucose transport, plays a central role in this regulation. III^{Glc} is able to bind to a number of transport systems and (most likely) adenylate cyclase. Substrates of the respective transport systems seem to be required for III^{Glc} binding. In this way III^{Glc} can be distributed between the various transport systems as needed. III^{Glc} binds only to those transport systems whose substrate/inducer is present. III^{Glc} will thus not be made available for use elsewhere by binding to transport systems whose synthesis is not increasing anyway, because the inducer/substrate is absent. As we have discussed above, one non-PTS transport system can in this way affect another or maybe even adenylate cyclase *via* III^{Glc}. One can only speculate how such a small protein is able to bind to so many different proteins, including all Class I transport systems, adenylate cyclase, HPr and Enzyme III^{Glc}. In this respect it may resemble calmodulin in eukaryotic systems. A more detailed investigation of the various bacterial transport systems may serve to define the nature of a common recognition site.

Materials and methods

Strains

E. coli strains T184 and T206 have been described by Teather *et al.* (1980). T213 harboring the *lacY*^{59} mutation on a multicopy plasmid was supplied by P. Overath (Overath *et al.*, in preparation). The multicopy plasmid pBCP2,

containing the structural gene of III^{Glc} from *S. typhimurium* was present in *S. typhimurium* PP642 (Nelson *et al.*, in preparation).

Purification of III^{Glc}

III^{Glc} was purified as described previously (Scholte *et al.*, 1981) from PP642/pBCP2. Enzyme I and HPr were isolated from *S. typhimurium* SB3507 (Scholte *et al.*, 1981; Robillard *et al.*, 1979).

Preparation of membranes

Cytoplasmic membrane vesicles were prepared according to the method of Osborn (Osborn *et al.*, 1972) with slight modifications (Teather *et al.*, 1980; Wright *et al.*, 1983). These vesicles are referred to in the text as cytoplasmic membrane vesicles. Vesicles were also prepared by passage of cells through a Ribi press at ~ 10 000 – 12 000 psi. These vesicles are referred to in the text as Ribi vesicles.

Binding studies

Binding of III^{Glc} to membrane vesicles was measured by incubating vesicles (0.1 – 0.6 mg protein/ml) and III^{Glc} (0.01 – 1.3 mg/ml) in a total volume of 50 μl, including an appropriate buffer (30 mM morpholine-ethanesulfonate (MES), 2 mM $MgSO_4$, pH 6.3, or 30 mM Tris, 5 mM $MgSO_4$, pH 7.5), lactose carrier substrates (0.4 mM αPNPG or 20 mM TDG) and, if needed, purified PTS proteins, Enzyme I and HPr (0.03 IU/ml each) and 10 mM PEP. After 30 – 60 min at room temperature, the membrane vesicles were separated by centrifugation for 10 min at 25 psi (100 000 *g*) in a Beckman airfuge. The supernatant was removed and the volume carefully measured (44 – 49 μl). The pellet surface was rinsed once with 50 μl buffer and the wall of the tube dried with absorbent paper. The pellet was dissolved in sufficient (44 – 49 μl) rocket immunoelectrophoresis buffer (38 mM Tris, 100 mM glycine, pH 8.6) containing 1% Triton X-100, to give a total volume of 50 μl. The III^{Glc} content of both pellet and supernatant fractions were determined by rocket electrophoresis as previously described (Scholte *et al.*, 1981).

Galactoside binding was measured by flow dialysis (Overath *et al.*, 1979; Wright *et al.*, 1981).

Transport in vesicles

Countertransport. Freeze-thawed cytoplasmic membrane vesicles of T206 (3.2 nmol lactose carrier/mg protein) were equilibrated with 40 mM lactose, 30 mM MES, 2 mM $MgSO_4$, pH 6.3, and, if present, 0.7 mg/ml III^{Glc}. At the times indicated, 10 μl (0.59 nmol lactose carrier) was diluted into 250 μl 20 μM [^{3}H]TDG (270 c.p.m./pmol), 30 mM MES, 2 mM $MgSO_4$, 20 mM NaCl, and, if present, 0.7 mg/ml III^{Glc}. At the times indicated countertransport was stopped by adding 4.5 ml stop buffer (30 mM MES, 2 mM $HgCl_2$, 40 mM glycerol, pH 6.3); the vesicles were collected on 6VG filters (Schleicher and Schüll, Assel, FRG) and washed a second time with 4.5 ml stop buffer.

Lactose-lactose exchange. Freeze-thawed cytoplasmic membrane vesicles from T206 (20 μl, 0.8 nmol lactose carrier) equilibrated with 40 mM [^{3}H]lactose (497 c.p.m./nmol), 30 mM MES, 2 mM $MgSO_4$, pH 6.3, and, if present, 0.7 mg/ml III^{Glc} were diluted into 250 μl 40 mM lactose, 2 mM $MgSO_4$, 30 mM MES, pH 6.3, and, if present, 0.7 mg/ml III^{Glc}. At the times indicated, exchange was halted with stop buffer as described above.

Purified lactose carrier was reconstituted into valinomcyin-containing liposomes (Wright *et al.*, 1983) and washed in 0.2 M potassium phosphate, 10 mg/ml BSA, pH 7.0, to discourage non-specific binding of III^{Glc} to the proteoliposomes. Samples were made 0.6 mg/ml in III^{Glc} where indicated.

Active transport. This was initiated by diluting 5 μl (15 – 35 pmol lactose carrier, 0.125 mg *E. coli* lipid) and 0.5 ml 0.2 M sodium phosphate buffer, 200 μM TDG (1000 c.p.m./nmol), 10 mg/ml BSA, pH 6.0 and 0.6 mg/ml III^{Glc} where indicated. Transport was measured up to 30 s and halted by the addition of stop buffer (0.3 M KCl, 5 mM MES, 2 mM $HgCl_2$, 1 mg/ml protamine sulphate, pH 6.5). Vesicles were collected on filters and washed. All samples were counted by placing the filters in 5 ml Quickzint 212 (Zinsser, Frankfurt, FRG).

Chemicals

Tritiated αPNPG, TDG and lactose were synthesized according to the method of Kennedy *et al.* (1974).

Acknowledgements

We thank P. Overath for his support and for making strains carrying the *lacY*^{S9} mutation available. Part of this work was supported by a fellowship from the European Molecular Biology Organisation (EMBO) to one of us (S.O. Nelson).

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