Biochimica et Biophysica Acta, 551 (1979) 157—168

BBA 78268

UNMASKING OF AN ESSENTIAL THIOL DURING FUNCTION OF THE MEMBRANE-BOUND ENZYME II OF THE PHOSPHO*ENOL*PYRUVATE β-GLUCOSIDE PHOSPHOTRANSFERASE SYSTEM OF *ESCHERICHIA COLI*

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(Received September 28th, 1978)

Key words: Phosphoenolpyruvate; Hexosephosphotransferase; β-Glucoside transport; Thiol reagent; (Escherichia coli)

Summary

β-Glucoside transport by phospho*enol*pyruvate-hexose phosphotransferase system in *Escherichia coli* is inactivated in vivo by thiol reagents. This inactivation is strongly enhanced by the presence of transported substrates. In a system reconstituted from soluble and membrane-bound components, only the particulate component, the membrane-bound enzyme II^{bgl} appeared as the target of *N*-ethylmaleimide inactivation. The same feature was found in the case of methyl-α-D-glucoside uptake via enzyme II^{glc}.

It is shown that the sensitizing effect of substrates is specific and not generalized, methyl-α-D-glucoside only sensitizes enzyme II^{glc} and *p*-nitrophenyl-β-D-glucoside only sensitizes enzyme II^{bgl} towards *N*-ethylmaleimide inactivation.

The inactivation of enzyme II^{bgl} by thiol reagents is also promoted in vivo by fluoride inhibition of phospho*enol*pyruvate synthesis. In toluene-treated bacteria, the presence of phospho*enol*pyruvate protects against inactivation by thiol reagents of *p*-nitrophenyl-β-D-glucoside phosphorylation. Both results suggest that the inactivator resistent form of enzyme II^{bgl} is an energized form of the enzyme.

Abbreviations: enzyme I, phospho*enol*pyruvate protein phosphotransferase (EC 2.7.3.9); HPr, histidine containing phosphate carrier protein of the phospho*enol*pyruvate-dependent phosphotransferase system; HPr-*P*, phospho histidine protein; enzyme II, phosphohistidino-protein-hexose phosphotransferase (EC 2.7.1.69); enzyme II^{bgl}, enzyme II specific for β-glucosides; enzyme II^{glc}, enzyme II specific for glucose and methyl-α-D-glucoside.

Introduction

We have reported previously that methyl-α-D-glucoside uptake via the phospho*enol*pyruvate hexose phosphotransferase system [1] is inactivated in vivo by thiol reagents. This inactivation is strongly enhanced by the presence of the transported substrate or by inhibition of the HPr-*P* generating system, either by in vivo inhibition of phospho*enol*pyruvate synthesis by fluoride, or by heat inactivation of enzyme I in a thermosensitive enzyme I mutant [2—4]. It has been demonstrated that the target of this inactivation by thiol reagents in vivo is the membrane-bound component enzyme II^{glc} of the phospho*enol*pyruvate glucose phosphotransferase system.

Data reported here show that β-glucoside transport via the phospho*enol*pyruvate hexose phosphotransferase system presents the same features, and that the sensitizing effect of the presence of substrate on inactivation by thiol reagents is specific: methyl-α-D-glucoside sensitizes only its own transport and has no effect on β-glucoside transport, and reciprocally with the *p*-nitrophenyl-β-D-glucoside, which only makes available to the inactivating thiol reagent the essential -SH group of the β-glucoside phosphotransferase. Wild type *Escherichia coli* does not ferment β-glucosides, but mutants have been isolated which take up and metabolize these sugars [5,6]. In such mutants, alkyl- and aryl-β-glucosides enter the bacteria by phosphorylative uptake involving a specific membrane-bound enzyme II^{bgl} from the phospho*enol*pyruvate hexose phosphotransferase system [7]. The phosphorylated sugars formed during this first step are hydrolysed by two phosphoglucosidases (phosphoglucosidase A and phosphoglucosidase B) which results in the formation of glucose 6-phosphate and aglycone [8]. Enzyme II^{bgl} and phosphoglucosidase B are coinducible while phosphoglucosidase A is constitutive [8]. Phosphorylation of β-glucosides via enzyme II^{bgl} is the limiting step in uptake and metabolism of β-glucosides by intact bacteria [8].

In the following experiments, bacteria were induced by growth in the presence of arbutin. Phosphorylative uptake was measured by in vivo formation of *p*-nitrophenol after incubation of bacteria in the presence of *p*-nitrophenyl-β-D-glucoside. This aromatic β-glucoside only enters *E. coli* via the specific enzyme II^{bgl} unlike aliphatic-β-D-glucosides such as methyl-β-D-glucoside, which are also phosphorylated by enzyme II^{glc} [18].

Materials and Methods

Bacterial strains

Bacterial strains inducible for β-glucoside uptake and metabolism were constructed by transduction. Phage P1 grown on *E. coli* K12 strain 1101 (*thi*-1, *rel*-1, *bgl*-11, *HPr*-1, *his*-62, λ^-) [7] was used to infect strains W 1895 dl (*Met*$^-$, enzyme II *glc*$^-$) [9], and AJ 19 (*Met*$^-$) constitutive for glucuronate permease and deficient in glucuronic acid metabolism [10,11]. Transducing lysates and transductions were carried out as described in ref. 12.

bgl$^+$ tranductants were selected on minimal arbutin plates and further tested for growth on minimal methyl-β-D-glucoside plates and for in vivo formation of *p*-nitrophenol after incubation of induced cells in the presence of *p*-nitro-

phenyl-β-D-glucoside. One transductant from each recipient strain: W 1895 dl R *bgl*$^+$ and AJ R *bgl*$^+$ positive for all three tests was used throughout this study.

Culture conditions

Cells were grown at 37° C in medium 63 [13] with 4 g/l glycerol as carbon source, 50 μg/ml thiamine, and 100 μg/ml methionine in aerated Erlenmeyer flasks. They were induced for three generation times by 2 g/l arbutin. Cultures were harvested during exponential growth and resuspended for further experiments at 275 μg/ml (dry weight) in the same medium without the amino acid supplement and inducer.

In vivo uptake of p-nitrophenyl-β-D-glucoside was measured as described by Schaefler [5]. Bacteria were incubated at 37° C in the presence of 1 mM *p*-nitrophenyl-β-D-glucoside. The reaction was stopped by addition of 0.2 M NaOH. The lysed culture was centrifuged to eliminate the residual turbidity. The amount of *p*-nitrophenol formed was measured by reading the absorbance at 410 nm of the supernatant.

In vivo uptake of methyl-α-D-glucoside. The bacterial suspension was equilibrated at 25° C with aeration. Methyl-α-D-[U-^{14}C]glucoside was then added at a final specific activity of 1 Ci/M and a final concentration of 0.05 mM. Accumulation of total sugar (free and phosphorylated) was monitored by filtration of 1-ml samples on millipore HA 0.45 μm pore size, followed by wash with medium 63 at 25° C. The filters were dried and counted in a toluene-based scintillant mixture in a Mark 1 scintillation spectrometer (Nuclear Chicago).

Toluene treatment was performed according to Gachelin [14]. 10 μl toluene were added to 10-ml samples of a bacterial suspension. The mixture was shaken vigorously for 1 min, then incubated 30 min at 37° C with constant agitation. *p*-Nitrophenol formation in toluene-treated bacteria was measured after addition of 1 mM phospho*enol*pyruvate and 1 mM *p*-nitrophenyl-β-D-glucoside. The reaction was then followed as described for in vivo uptake.

Inactivation by *N*-ethylmaleimide was performed in medium 63 at 25° C at a bacterial concentration of 275 μg/ml (dry weight) unless otherwise stated. Inactivation was stopped by addition of an excess of 2-mercaptoethanol. Cells were then filtered, washed with cold distilled water, and resuspended in medium 63. This treatment was used in order to eliminate external *N*-ethylmaleimide and intracellular solutes used during preincubation [15]. In vivo uptake of methyl-α-D-glucoside and *p*-nitrophenyl-β-D-glucoside was subsequently measured as described above.

Preparation of cell-free extracts for measurement of enzyme II^{bgl} *activity*

Induced W 1895 dl R *bgl*$^+$ in exponential growth was harvested, washed in 50 mM sodium phosphate buffer, pH 7, containing 2 mM $MgSO_4$ 0.5 mM 2-mercaptoethanol and resuspended at 1.4 mg/ml (dry weight) in the same buffer in the presence of 1 μg/ml DNAase. The suspension was broken by Ribi fractionator under a pressure of 1400 bars. The extracts were then submitted to a low speed centrifugation to eliminate survivors and heavy particles. The supernatant was used as a source of membranes and soluble enzymes.

Preparation of soluble enzyme

Cell-free extracts of induced W 1895 dl R bgl^+ were submitted to two times 120 min centrifugation at 165 000 × *g*. The final supernatant completely deprived of membranes was used as a source of the soluble enzymes: enzyme I, HPr, and the two phosphoglucosidases.

In vitro formation of *p*-nitrophenol was followed at 37° C in 50 mM sodium phosphate buffer (pH 7), 2 mM $MgSO_4$, 0.5 mM 2-mercaptoethanol. The reaction mixture contained 5 mM phospho*enol*pyruvate, 1 mM *p*-nitrophenyl-β-D-glucoside, 165 000 × *g* supernatant of *E. coli* W 1895 dl R bgl^+ (5 mg/ml protein) as source of excess enzyme I, HPr, and phosphoglucosidases and variable amounts of crude extracts (up to 0.75 mg protein/ml, approximately up to 0.075 mg membrane protein/ml) as source of enzyme II^{bgl}. The final volume of incubation was 1 ml. The reaction was stopped by addition of 0.2 M NaOH (final concn.). All samples were then submitted to low speed centrifugation to eliminate the turbidity caused by precipitated proteins. The amount of *p*-nitrophenol formed was measured by reading the absorbance at 410 nm of the supernatant.

Chemicals

Phospho*enol*pyruvate was obtained from Boehringer, Mannheim, *N*-ethylmaleimide and *p*-nitrophenyl-β-D-glucoside from Calbiochem and 3 Ci/mol methyl-α-D-[U-^{14}C]glucoside from the Radiochemical Center, Amersham.

Results

Sensitization

Fig. 1 shows the effect on *p*-nitrophenyl-β-D-glucoside uptake of 2 min pretreatment with increasing amounts of *N*-ethylmaleimide either alone or in the presence of arbutin, a substrate of the β-glucoside phosphotransferase system [5].

Relative rates of *p*-nitrophenol formation were measured after arrest of inactivation and elimination of *N*-ethylmaleimide and arbutin. The remaining activities are presented on a logarithmic scale. The difference between inactivation in the presence or absence of arbutin is striking: in the presence of arbutin, 90% inactivation was reached after treatment with 1 mM *N*-ethylmaleimide, while the same concentration of inactivator acting alone produced less than 40% inactivation.

Time course of inactivation

Fig. 2 shows inactivation by *N*-ethylmaleimide at a single concentration (0.15 mM) for variable time. Inactivation was performed in the absence or presence of several substrates of the β-glucoside phosphotransferase system: arbutin, methyl-β-D-glucoside, and *p*-nitrophenyl-β-D-glucoside. Here too, inactivation by *N*-ethylmaleimide alone is inefficient (around 20% inactivation in 5 min) compared to dramatic inactivation in the presence of all substrates used (70—80% inactivation in 5 min).

The same results have been observed after inactivation by fluorodinitrobenzene in the absence or presence of *p*-nitrophenyl-β-D-glucoside (unpublished results).

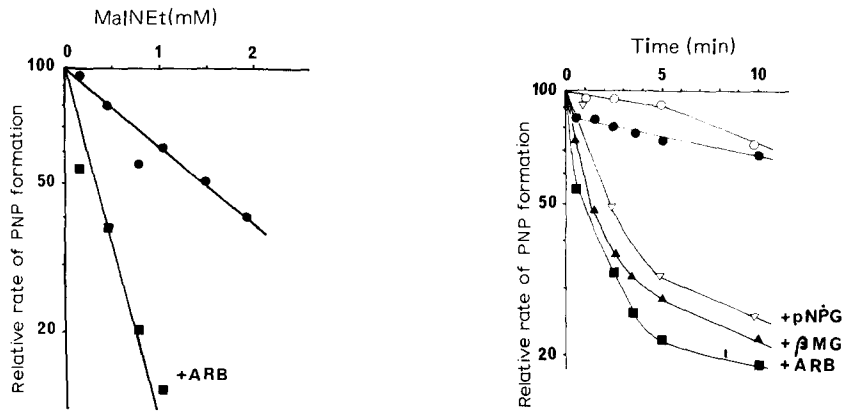

Fig. 1. Inactivation by various concentrations of *N*-ethylmaleimide (MalNet) of *p*-nitrophenyl-β-D-glucoside uptake in the presence or absence of arbutin. *E. coli* strain AJR *bgl*$^+$ was grown in medium 63 gly B_1 supplemented with 100 μg/ml methionine and 2 g/l arbutin. Bacteria were harvested during exponential growth and resuspended at 275 μg/ml (dry weight) in 63 gly B_1 medium. Bacteria were treated at 25°C with various concentrations of *N*-ethylmaleimide in the presence (■) or absence (●) of 20 mM arbutin (ARB). Inactivation was stopped after 2 min by addition of 20 mM 2-mercaptoethanol followed by filtration, wash with cold distilled water, and resuspension in medium 63 gly B_1. In vivo formation of *p*-nitrophenol (PNP) was then measured after 30 min incubation at 37°C in the presence of 2 mM *p*-nitrophenyl-β-D-glucoside. The reaction was stopped by addition of 0.2 M NaOH. Samples were then centrifuged, and the absorbance at 410 nm of the supernatants measured. Rate of *p*-nitrophenol formation in control sample: 37 μM/min per g dry weight.

Fig. 2. Inactivation by *N*-ethylmaleimide for various lengths of time of *p*-nitrophenyl-β-D-glucoside (pNPG) uptake. Experiments performed on *E. coli* AJR *bgl*$^+$ as described in Fig. 1. First experiment: ○, inactivation by 0.15 mM *N*-ethylmaleimide alone; △, inactivation by *N*-ethylmaleimide in the presence of 10 mM *p*-nitrophenyl-β-D-glucoside. Second experiment: ●, inactivation by 0.15 mM *N*-ethylmaleimide alone; ▲, inactivation by *N*-ethylmaleimide in the presence of 10 mM β-methyl-D-glucoside (βMG); ■, inactivation by *N*-ethylmaleimide in the presence of 10 mM arbutin (ARB). Rate of formation of *p*-nitrophenol (PNP) in control sample: 37 μM/min per g dry weight.

Protection by a reversible thiol reagent

Fig. 3 shows the effect of *p*-chloromercuribenzoate on *p*-nitrophenyl-β-D-glucoside uptake. This reagent provided nearly 100% inhibition in the conditions of the experiment. This inhibition was completely reversed by the addition of an excess of 2-mercaptoethanol, even if *N*-ethylmaleimide was added after *p*-chloromercuribenzoate. The same incubation with *N*-ethylmaleimide in the absence of *p*-chloromercuribenzoate caused irreversible inactivation. The protection that *p*-chloromercuribenzoate provided against inactivation by *N*-ethylmaleimide suggests that both reagents act on the same site, probably an -SH group.

These first experiments show that one step involved in *p*-nitrophenol formation from *p*-nitrophenyl-β-D-glucoside is inactivated by thiol reagents and that the presence of β-glucosides sensitizes the target of this inactivation.

Attempts of cross sensitization of two phosphotransferase systems

As it was shown in previous reports that methyl-α-D-glucoside sensitizes its own transport to inactivation by thiol reagents, the question could be raised whether the sensitizing effect of methyl-α-D-glucoside or β-glucosides on inac-

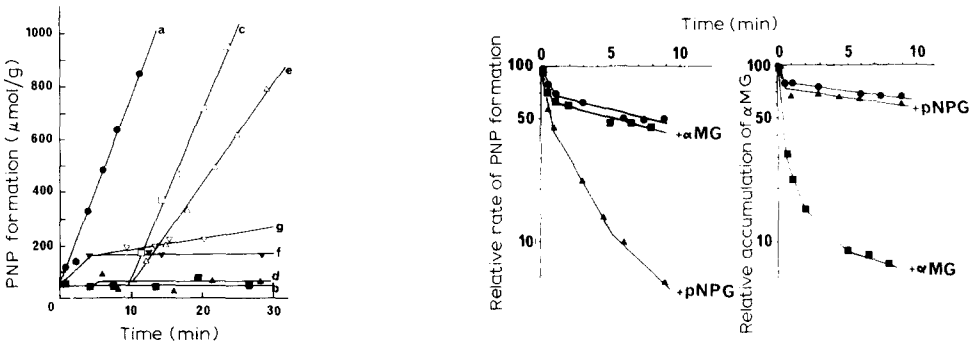

Fig. 3. Protection by *p*-chloromercuribenzoate against inactivation of *p*-nitrophenyl-β-D-glucoside uptake by *N*-ethylmaleimide. *E. coli* strain W 1895 dl R *bgl*$^+$ in 63 gly B_1 medium was supplemented at zero time with 0.5 mM *p*-nitrophenyl-β-D-glucoside. Uptake was followed at 37° C and stopped at different times by addition of 0.2 M NaOH. a, formation of *p*-nitrophenol (PNP) in control sample; b, addition of 0.25 mM *p*-chloromercuribenzoate at zero-time; c, addition of 5 mM 2-mercaptoethanol at 9 min to b; d, addition of 0.1 mM *N*-ethylmaleimide at 5 min to b; e, addition of 2-mercaptoethanol at 10 min to d; f, formation of *p*-nitrophenol after addition of 0.1 mM *N*-ethylmaleimide at zero-time; g, addition of 5 mM 2-mercaptoethanol at 5 min to f.

Fig. 4. Effect of a substrate of enzyme II^{glc} and a substrate of enzyme II^{bgl} on inactivation by *N*-ethylmaleimide of methyl-α-D-glucoside (αMG) and *p*-nitrophenyl-β-D-glucoside (pNPG) uptake. *E. coli* strain AJR *bgl*$^+$ in suspension in medium 63 gly B_1 was submitted during various lengths of time to inactivation by 0.15 mM *N*-ethylmaleimide at 30° C acting alone (●), in presence of 0.5 mM methyl-α-D-glucoside (■), or in presence of 0.5 mM *p*-nitrophenyl-β-D-glucoside (▲). Inactivation was stopped as described in Fig. 1. Steady-state accumulation of methyl-α-D-glucoside was then measured after 15 min incubation at 25° C in presence of 0.05 mM methyl-α-D-[^{14}C]glucoside. *p*-Nitrophenol (PNP) formation was measured after 40 min incubation at 37° C in presence of 1 mM *p*-nitrophenyl-β-D-glucoside. Control sample, total radioactivity accumulated at steady state: 55 μM/g dry weight; rate of *p*-nitrophenol formation: 18 μM/min per g dry weight.

tivation of their transport was specific or a generalized effect of all phosphotransferase system substrates on all phosphotransferase systems.

To answer this question, we chose the following sugars: methyl-α-D-glucoside and *p*-nitrophenyl-β-D-glucoside. Early reports indicated that in vivo methyl-α-D-glucoside uptake in *Salmonella typhimurium* was not inhibited by *p*-nitrophenyl-β-D-glucoside [16]. More recently, it was reported that methyl-α-D-glucoside did not inhibit in vitro phosphorylation of *p*-nitrophenyl-β-D-glucoside by enzyme II^{bgl} [17] in *E. coli*.

We verified in the *E. coli* strains used throughout this study that *p*-nitrophenyl-β-D-glucoside does not inhibit methyl-α-D-glucoside transport, nor did methyl-α-D-glucoside inhibit *p*-nitrophenyl-β-D-glucoside transport (Table I).

Bacteria were submitted for various lengths of time to inactivation by 0.15 mM *N*-ethylmaleimide acting alone, in the presence of *p*-nitrophenyl-β-D-glucoside, or in the presence of methyl-α-D-glucoside. Uptake of these two sugars was subsequently measured after elimination of all reactants present during the preincubation. Fig. 4 shows that methyl-α-D-glucoside and *p*-nitrophenyl-β-D-glucoside specifically sensitized their own transport to inactivation by *N*-ethylmaleimide. Methyl-α-D-glucoside transport was identically inactivated by *N*-ethylmaleimide acting for 5 min alone or in the presence of *p*-nitrophenyl-β-D-glucoside (30% inactivation), while it was inactivated around 90% by *N*-ethylmaleimide acting in the presence of methyl-α-D-glucoside, the

TABLE I

EFFECT OF β-GLUCOSIDES ON METHYL-α-GLUCOSIDE TRANSPORT AND OF α-GLUCOSIDES ON *p*-NITROPHENYL-β-D-GLUCOSIDE TRANSPORT

Steady-state accumulation of 0.05 mM methyl-α-D-[^{14}C]glucoside was measured after 20 min on *E. coli* strain AJ R *bgl*$^+$ induced for the β-glucoside system. Rate of *p*-nitrophenol formation was measured on *E. coli* strain W 1895 dl R *bgl*$^+$ incubated in presence of 0.2 mM *p*-nitrophenyl-β-D-glucoside.

Steady-state accumulation of methyl-α-D-glucoside (μmol/g)			Rate of *p*-nitrophenol formation (μmol/g per min)			
Control	In presence of		Control	In presence of		
	Methyl-α-D-glucoside (1 mM)	*p*-nitrophenyl-β-D-glucoside (1 mM)		Methyl-α-D-glucoside (5 mM)	Glucose (5 mM)	Salicine (5 mM)
40	8	43	7.8	7.5	7.1	0.9

transport substrate, and reciprocally *p*-nitrophenyl-β-D-glucoside enhanced the inactivation of its own transport system which was insensitive to the presence of methyl-α-D-glucoside.

Similar experiments were carried out with methyl-β-D-glucoside, which was reported to inhibit in vivo uptake of methyl-α-D-glucoside (ref. 16 and Table I), as a substrate of enzyme II^{glc} [18]. This sugar which is also a substrate of enzyme II^{bgl} [17] sensitized both transport systems to inactivation by *N*-ethylmaleimide (not shown).

These observations suggest that the target of in vivo inactivation by *N*-ethylmaleimide are the sugar-specific components of phospho*enol*pyruvate hexose phosphotransferase system, that is their membrane-bound part: enzyme II^{glc} and enzyme II^{bgl}.

Localization of the essential thiol on the membrane-bound component

It was previously demonstrated in the case of glucose phosphotransferase system, in cross reconstitution experiments, that the target of in vivo inactivation by *N*-ethylmaleimide was the membrane fraction [2,4]. The experiment reported below was performed to see if the same conclusion holds for the β-glucoside phosphotransferase system. A suspension of *E. coli* W 1895 dl R *bgl*$^+$ was divided into three samples. One was left untreated, the other two were submitted to inactivation by *N*-ethylmaleimide in the presence or absence of arbutin. After wash and resuspension in medium 63 gly Bl containing 2 mM 2-mercaptoethanol, *p*-nitrophenol formation from *p*-nitrophenyl-β-glucoside was measured in each sample. Crude extracts were then prepared from each bacterial sample. Since these contain rate-limiting amount of soluble enzymes, the membrane-bound enzyme II^{bgl} activity of each extract was measured in the presence of an excess of soluble enzymes of untreated induced bacteria.

N-Ethylmaleimide alone produced 18% inhibition of in vivo *p*-nitrophenol formation (Fig. 5A) and 22% inhibition of in vitro enzyme II^{bgl} activity (Fig. 5B). In the presence of arbutin the inhibition increased to 77 and 71%, respectively. We can conclude that the target of in vivo *N*-ethylmaleimide inactivation is enzyme II^{bgl}.

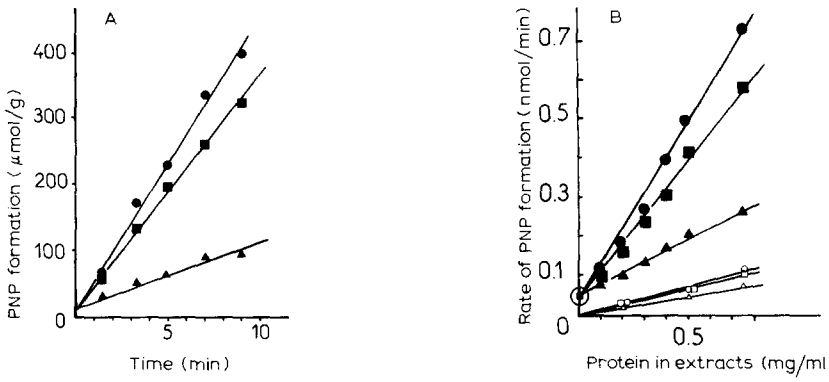

Fig. 5. Substrate-dependent *N*-ethylmaleimide inactivation of enzyme II^{bgl} activity. *E. coli* strain W 1895 dl R *bgi*$^+$ induced for β-glucoside system was resuspended at 1.375 mg/ml dry weight. (a) Control sample; (b) samples were then submitted to 3 min treatment at 25°C by 0.3 mM *N*-ethylmaleimide alone, or (c) in presence of 10 mM arbutin. (A) In vivo *p*-nitrophenol (PNP) formation was measured in each sample by incubation at 37°C in presence of 1 mM *p*-nitrophenyl-β-D-glucoside: ●, activity in a; ■, in b; ▲, in c. (B) In vitro *p*-nitrophenol formation was measured after 90 min incubation at 37°C in presence of increasing amounts of extracts alone (○, activity in a; □, in b; △, in c) or with excess 165 000 × *g* supernatant (●, activity in a; ■, in b; ▲, in c). ⊛, *p*-nitrophenol formation in 165 000 × *g* supernatant alone.

Inactivation in toluene-treated cells; protection by phosphoenolpyruvate

Fig. 6A shows that in vivo *p*-nitrophenol formation is completely inhibited by fluoride acting at pH 6.2. When bacteria are permeabilized to low molecular weight molecules by toluene treatment [14] they do not liberate *p*-nitrophenol upon incubation with *p*-nitrophenyl-β-D-glucoside (Fig. 6B), as shown previously [5], but externally added phospho*enol*pyruvate allows the reaction to proceed. Fluoride does not inhibit this phospho*enol*pyruvate-dependent *p*-nitrophenol formation. It is likely that fluoride inhibits *p*-nitrophenyl-β-D-

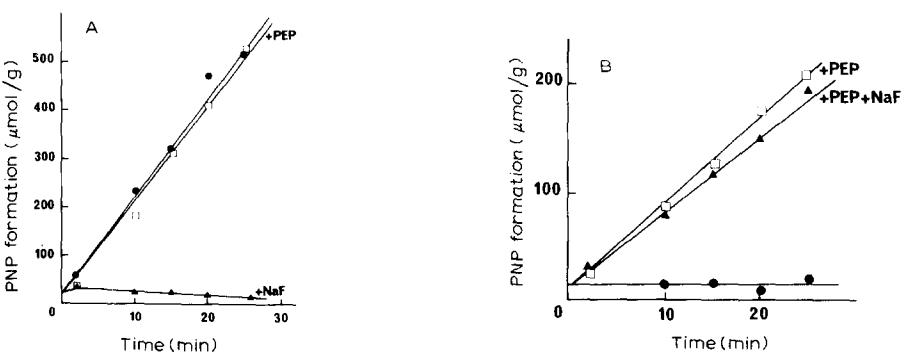

Fig. 6. (A) In vivo inhibition of *p*-nitrophenyl-β-D-glucoside uptake by fluoride. *E. coli* strain W 1895 dl R *bgi*$^+$ in medium 63 gly B_1 at pH 6.2 was supplemented at zero time with 1 mM *p*-nitrophenyl-β-D-glucoside. *p*-Nitrophenol (PNP) formation was followed at 25°C and stopped at different times by addition of 0.2 M NaOH. ●, formation of *p*-nitrophenol in control sample; □, in presence of 1 mM phospho*enol*pyruvate (PEP); ▲, in presence of 50 mM NaF. (B) Phospho*enol*pyruvate-dependent *p*-nitrophenol formation in toluene-treated bacteria. Same bacteria as above, resuspended after growth in medium 63 gly B_1, pH 7.2, were submitted to toluene treatment at 37°C. *p*-Nitrophenol formation was followed at 25°C as in A. ●, formation of *p*-nitrophenol in control sample; □, in presence of 1 mM phospho*enol*pyruvate, ▲, in presence of 50 mM NaF and 1 mM phospho*enol*pyruvate.

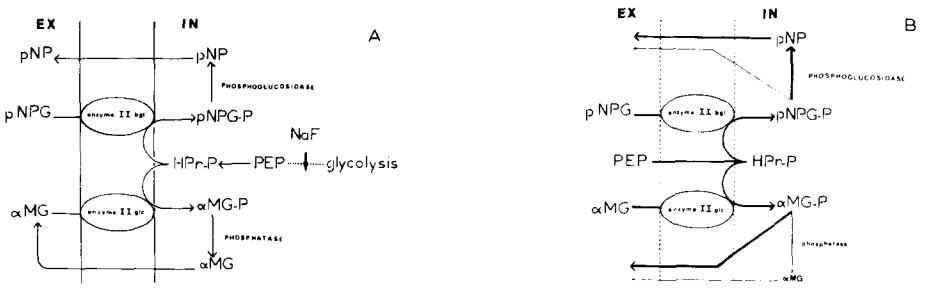

Fig. 7. Diagrammatic representation of phosphotransferase reactions in whole cells (A) and in toluenized cells (B). αMG, methyl-α-D-glucoside; pNPG, *p*-nitrophenyl-β-D-glucoside; pNP, *p*-nitrophenol; PEP, phospho*enol*pyruvate.

glucoside uptake in vivo through its effect on phospho*enol*pyruvate synthesis (Fig. 7A). It does not stop *p*-nitrophenyl-β-D-glucoside phosphorylation by the phosphotransferase system, nor *p*-nitrophenyl-β-D-glucoside phosphate hydrolysis by phosphoglucosidases.

It is noteworthy that these last enzymes still function in toluene-treated bacteria, while the phosphatase which splits methyl-α-D-glucoside phosphate formed by enzyme II^{glc} [4] does not, presumably because of the escape and consequent high dilution of its substrate (Fig. 7B). This difference could result from the low K_m of phosphoglucosidase A and B for *p*-nitrophenyl-β-D-

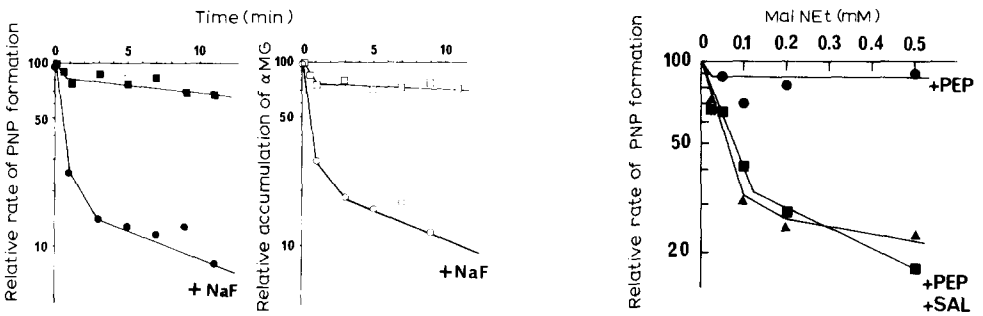

Fig. 8. Fluoride sensitization to *N*-ethylmaleimide inactivation of methyl-α-D-glucoside and of *p*-nitrophenyl-β-D-glucoside uptake. *E. coli* strain AJR *bgl*$^+$ in suspension in medium 63 gly B_1 at pH 6 was submitted during various lengths of time at 30°C to inactivation by 0.1 mM *N*-ethylmaleimide acting alone (■,□) or in the presence of 50 mM NaF (○,●). Inactivation was stopped by addition of 1 mM 2-mercaptoethanol, followed by filtration, wash with distilled water, and resuspension in medium 63 gly B_1 at pH 7. Steady-state accumulation of methyl-α-D-[^{14}C]glucoside (αMG) (open symbols) and *p*-nitrophenol (PNP) formation (full symbols) were then measured as described in Fig. 4. Control sample: total radioactivity accumulated at steady state: 60 μM/g dry weight; rate of *p*-nitrophenol formation: 60 μM/g per g dry weight.

Fig. 9. Inactivation by *N*-ethylmaleimide (MalNEt) of *p*-nitrophenol formation in toluene-treated bacteria. *E. coli* strain W 1895 dl R *bgl*$^+$ grown in medium 63 gly B_1 with 100 μg/ml methionine and 2 g/l arbutin was submitted to toluene treatment at 37°C after resuspension in medium 63 gly B_1. Toluene-treated bacterial samples were submitted to 2.5 min inactivation by increasing amounts of *N*-ethylmaleimide alone (▲), in presence of 2 mM phospho*enol*pyruvate (PEP) (●), or in presence of 2 mM phospho*enol*pyruvate and 1 mM salicine (SAL) (■). *p*-Nitrophenol formation was measured after 10 min incubation at 37°C in presence of 1 mM *p*-nitrophenyl-β-D-glucoside and 1 mM phospho*enol*pyruvate after filtration, washing with medium 63, and resuspension in medium 63 gly B_1. Control sample of untreated bacteria: 67 μM *p*-nitrophenol formed/min per g dry weight.

glucoside phosphate (0.33 and 0.18 mM, respectively) [18], compared to the probably high K_m of phosphatase for methyl-α-D-glucoside phosphate (4–6 mM) [19]. The phospho*enol*pyruvate-dependent phosphorylation of several sugars in toluene-treated bacteria has been utilized to better explore the in vivo kinetics of their phosphorylation by the phosphotransferase system [14,19,20]. We applied this technique successfully in the case of β-glucoside transport.

Fig. 8 shows that fluoride inhibition of phospho*enol*pyruvate synthesis simultaneously sensitized methyl-α-D-glucoside and *p*-nitrophenyl-β-D-glucoside uptake to inactivation by *N*-ethylmaleimide in the absence of any added substrate.

Pretreatment with *N*-ethylmaleimide alone resulted in complete inactivation of phospho*enol*pyruvate-dependent *p*-nitrophenol formation in toluene-treated bacteria (Fig. 9). Preincubation of these permeabilized bacteria with externally added phospho*enol*pyruvate provided a protective effect only in the absence of added β-glucoside.

Discussion

We reported here that *p*-nitrophenol formation in bacteria supplemented with *p*-nitrophenyl-β-D-glucoside is inactivated in vivo by thiol reagents. This inhibition is specifically enhanced by the presence of β-glucosides. The target of this substrate-dependent *N*-ethylmaleimide effect is the membrane-bound enzyme II^{bgl}. We have previously demonstrated that enzyme II^{glc} presents the same property.

Whereas a given substrate of the phosphotransferase system can only promote a conformational change unmasking a reactive thiol of its specific enzyme II, fluoride inhibition of in vivo phospho*enol*pyruvate synthesis induces simultaneously sensitivity to thiol reagents of both enzymes in the absence of added substrate.

The same feature, i.e. inactivation by *N*-ethylmaleimide alone is observed in toluene-treated bacteria depleted of their internal phospho*enol*pyruvate. In the latter case, the addition of external phospho*enol*pyruvate has a protective effect only in the absence of substrate.

We therefore propose, as in the case of enzyme II^{glc}, that enzyme II^{bgl} may present two alternate conformations during β-glucoside uptake.

When the HPr-*P* generating system is functional and the substrate absent enzyme II^{bgl} could be in an energized state resistant to inactivation by thiol reagents. In the presence of substrate or inhibition of the HPr-*P* generating system, enzyme II^{bgl} could be in a deenergized state sensitive to inactivation by thiol reagents.

As the presence of methyl-α-D-glucoside did not sensitize enzyme II^{bgl} to inactivation by thiol reagents, nor did *p*-nitrophenyl-β-D-glucoside promote the inactivation of enzyme II^{glc}, we conclude that each of these enzymes remains in a stable energized state in vivo even during the transport of a substrate of another membrane-bound enzyme II (Fig. 10). This observation indicates that HPr-*P* is probably present in vivo in saturating amounts sufficient to allow one enzyme II to function, and another one to be maintained in its energized form.

That HPr-*P* is not the rate-limiting factor in sugar uptake via the phospho-

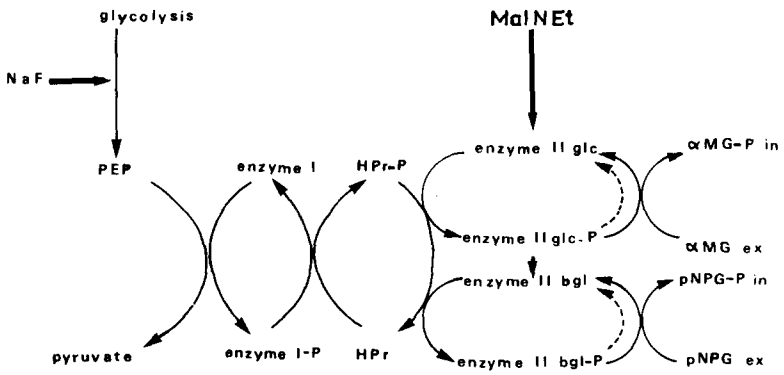

Fig. 10. Phosphate transfer via the phosphotransferase system. PEP, phospho*enol*pyruvate; MalNEt, *N*-ethylmaleimide; αMG, methyl-α-D-glucoside; pNPG, *p*-nitrophenyl-β-D-glucoside.

transferase system can be concluded from the fact that a substrate of one enzyme II does not inhibit in vivo transport of a substrate of another enzyme II (Table I) [16,18]. Actually both reactions can proceed at maximal rate.

The way by which enzyme II^{glc} and enzyme II^{bgl} are both deenergized as soon as phospho*enol*pyruvate synthesis is inhibited remains obscure. This might be due to a reversion of the phosphate group transfer sequence (dotted arrows Fig. 10). But it has been reported that in vitro the enzyme II^{bgl} reaction was irreversible [17].

The energized state of enzyme II^{bgl} might be a phosphorylated form of this membrane-bound enzyme. Such a transient phosphorylated form of both enzyme II^{glc} and enzyme II^{bgl} have been suggested on the basis of bi-bi ping-pong kinetics [17,22]. The question could be raised whether the conformational change of enzyme II^{glc} and enzyme II^{bgl} during phosphorylative uptake of their substrates is a general feature of all enzymes II.

The experiments reported here suggest that the energization-deenergization cycle postulated for these two enzymes II is associated with more extensive conformational changes of these membrane-bound enzymes than a simple covalent change of phosphorylation-dephosphorylation, and that these changes might be a part of the translocation cycles.

Acknowledgements

We thank B. Bachman for *E. coli* strain 1101, S. Schaefler for *E. coli* strain W 1895 dl, J. Jimeno for *E. coli* strain AJ 19, and for fruitful discussion, J. Daniel for help in transducing experiments, and L. Peutat for skillfull technical assistance.

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