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

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Summary

The addition of *N*-ethylmaleimide (MalNEt), or of fluoro dinitrobenzene to a suspension of *Escherichia coli* during the phosphorylating uptake of methyl-α-D-glucopyranoside (Me-Glc), a glucose analog, stops uptake and phosphorylation and causes the loss of previously accumulated sugar and of its phosphate ester. After removal of the reagents, the phosphotransferase system remains irreversibly inactive.

Pretreatment of the bacteria with the same reagents under the same conditions of concentration, pH, temperature and for the same length of time causes very little inactivation. Mercuric chloride, a reversible inactivator, prevents the phosphotransferase system from reacting simultaneously with MalNEt or with fluorodinitrobenzene. This protection strongly suggests that all three reagents react with the same site, presumably an -SH group.

The change which makes this site available to the reagents depends on the phosphorylative uptake of Me-Glc. Preload of the cells and efflux of Me-Glc do not achieve the same change.

The rate of inactivation is directly proportional to the rate of phosphorylative uptake. When the K_m of phosphorylative uptake is modified by an uncoupling agent, the substrate concentration allowing half maximal rate of inactivation by MalNEt changes accordingly.

The reactive sites of the phosphotransferase system can also be made accessible to the -SH group reagents by fluoride inhibition of phospho*enol*pyruvate

Abbreviations: Me-Glc, methyl-α-D-glucopyranoside; Me-Glc-6-*P*, methyl-α-D-glucopyranoside-6-phosphate; MalNEt, *N*-ethylmaleimide; Enzyme I, E.C. 2.7.3.9. Phospho*enol*pyruvate-protein phosphotransferase; Enzyme II, E.C. 2.7.1.69 phosphohistidinoprotein-hexose phosphotransferase; HPr, histidine containing phosphate carrier protein of the phospho*enol*pyruvate dependant phosphotransferase system.

synthesis. This suggests that the inactivator resistent form is an "energized form" of the enzyme.

The unmasking of the reactive site is not due to a change in transmembrane penetration of the reagents since incubation of toluene treated cells with MalNEt in the presence of phospho*enol*pyruvate fails to inactivate the phosphotransferase activity, while incubation with MalNEt plus Me-Glc causes fast inactivation.

Introduction

The discovery of the phospho*enol*pyruvate hexose phosphotransferase system of bacteria [1] was a decisive step towards the understanding of the molecular mechanism of membrane transport.

Together with the translocation across the membrane, the system performs a useful metabolic reaction with the same expenditure of chemical energy and the impermeability of the cell membrane to sugar phosphates insures a retention and prevents waste of work spent in transport as well as waste of a useful metabolite.

It would be nevertheless an oversimplification to think that the only peculiarity of a vectorial enzyme is to have its active site accessible to its substrate exclusively from outside while its product can only be released on the other side. Obviously, the sugar phosphate cannot leave on the outer face but presumably the sugar cannot approach from the inner face [2]. Moreover the sugar cannot cross the inward pathway unphosphorylated when a phosphate donor or the intermediate phosphate carrier Phospho-HPr is absent [3—5].

During the study of inactivating protein group reagents, looking for possible substrate protection, like that observed in lactose permease [6,7] a strikingly opposite effect was observed [8], namely a protection by the absence of (transport) substrate. This suggested a conformational change during the activity of the membrane bound enzyme.

Observations of similar sensitizing effects of the substrate have been reported in the case of mitochondrial translocators [9], in red blood cell choline [10] and sugar carriers, and in glucuronate active transport in *Escherichia coli* [12].

In the present article, we report experiments which show that the site sensitive to several reagents is common and very likely a thiol group, that this site is unmasked during actual function of the phosphotransferase and not simply due to the presence of the sugar or its phosphate ester at high concentration in the cell and that the reactive conformation is the deenergized conformation of the enzyme. Moreover, it is shown that inaccessibility of the reactive thiol to the thiol reagent in the energized conformation is not due to the permeability barrier of the membrane. It was shown in a previous report [8] that the reactive thiol is on the membrane bound component of the system.

In all the experiments reported below, phosphoenolpyruvate glucose phosphotransferase system was studied in vivo by the use of methyl-α-D-glucopyranoside (Me-Glc); a non metabolisable glucose analog.

The kinetic features of Me-Glc uptake have been repeatedly described [13—15].

Intracellular radioactivity in *E. coli* exposed to [U-^{14}C]Me-Glc is composed by [^{14}C]methyl-α-D-glucopyranoside-6-phosphate (Me-Glc-6-*P* measured in this paper as barium precipitable radioactivity) and [^{14}C]Me-Glc which is calculated as the difference between the total intracellular [^{14}C] and [^{14}C]Me-Glc-6-*P*. As shown previously [13—15], [^{14}C]Me-Glc appears after a short lag while [^{14}C]Me-Glc-6-*P* appears immediately and this is consistent with the sequence:

$$[^{14}C]\text{Me-Glc}_{ex} \xrightarrow{\text{phosphotransferase system}} [^{14}C]\text{Me-Glc-6-P}_{in} \xrightarrow{\text{phosphatase}} [^{14}C]\text{Me-Glc}_{in}$$

Both [^{14}C]Me-Glc-6-*P* and [^{14}C]Me-Glc pools eventually reach a steady-state, which usually corresponds to approximate equality of the two components. The steady-state is due to a passive leak of [^{14}C]Me-Glc. The steady-state level is approximately proportional to the initial rate of phosphorylative uptake, as would be expected if hydrolysis of Me-Glc-6-*P* and the leak of internal Me-Glc are described by pseudo first order kinetics.

This cycle of renewal of intracellular Me-Glc in whole cells is schematized in Fig. 1 (top).

Correlation between in vivo Me-Glc transport and phosphotransferase activity have been well documented, among others with the use of toluene treated bacteria. The complete permeabilization of the membrane by this treatment is still compatible with the phosphotransferase activity, although not with phosphorylative uptake [14,16,17]. Endogenous phospho*enol*pyruvate synthesis is abolished by toluene treatment, but added phospho*enol*pyruvate has free access to enzyme I and HPr which remain in the cell. Similarly Me-Glc-6-*P* escapes freely from the cell and so it does not build up a sufficient concentration to be hydrolyzed by the phosphatase which remains bound to the cell.

The phosphorylation of Me-Glc in toluene treated bacteria is schematized in Fig. 1 (bottom). The identity between specific activity and kinetic features of phosphorylation in toluene treated bacteria and uptake in whole cells, compar-

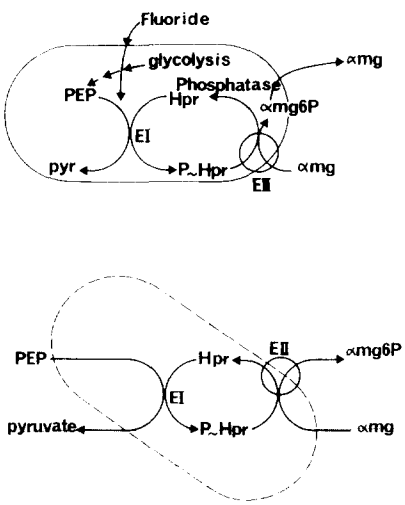

Fig. 1. Diagrammatic representation of phosphotransferase reactions in whole cells (top) and in toluenized cells (bottom).

ed to the important loss of phosphorylative ability of extracts (see for example refs. 1 and 4), were useful in the following study devoted to conformational changes of enzyme II during in vivo phosphorylative uptake of Me-Glc.

Materials and Methods

Bacteria

E. coli K 12 strains 3300 Lac $i^-z^+y^+a^+$, 3000 Lac $i^+z^+y^+a^+$ and AJ19 met^- constitutive for glucuronate permease and deficient in glucuronic acid metabolism [18] were grown at 37°C in medium 63 [14] with 4 g/l glycerol (or in some experiments glucose) as a carbon source, 50 μg/ml thiamine and 100 μg/ml of the required amino acid in aerated Erlenmeyer flasks. Cultures were harvested during exponential growth.

In vivo uptake and phosphorylation

The bacterial culture arrested at a density of 275 μg/ml (dry weight) by addition of chloramphenicol 50 μg/ml was equilibrated at 25°C with aeration. [U-^{14}C]Me-Glc was then added at a final specific activity of 0.5 μCi/μM and a final concentration of 0.05 mM.

(a) *Total intracellular sugar (free and phosphorylated).* Samples usually of 1 ml were withdrawn and filtered on millipore filters HA 0.45 μm pore size, and rinsed twice with medium 63 at 25°C. The filtrate did not contain sugar phosphate in detectable amounts.

(b) *Determination of total Me-Glc-6-P.* Samples of the same volume as above were pipetted into 10 volumes of 90% aqueous ethanol containing 11 mg/ml $BaBr_2$ (13,16,20]. Precipitation was allowed to proceed for 20 min at 0°C, then the alcoholic sample was filtered on a glass fiber filter (Whatman GFB) and rinsed twice with 3 ml 80% ethanol. Both millipore filters and glass fiber filters were dried and counted in a toluene based scintillation mixture in a Mark 1 scintillation spectrometer (Nuclear Chicago).

Toluene treatment was made according to Gachelin [16]. 10 μl of toluene were added to 10 ml samples of a bacterial suspension. The mixture was shaken vigorously for 1 min, and then incubated for 30 min at 37°C with constant agitation.

Phosphorylation in toluene treated bacteria was then measured. The reaction was started after equilibration at 30°C by addition of 1 mM phospho*enol*-pyruvate and 0.05 mM of [^{14}C]Me-Glc. Me-Glc-6-*P* was measured by Ba precipitation as described above.

Inactivation by protein reagents

Unless otherwise stated, inactivation by *N*-ethylmaleimide (MalNEt) was performed in medium 63 at 25°C at a bacterial concentration of 275 μg/ml (dry weight). Inactivation was stopped by the addition of 2-mercaptoethanol in excess. Inactivation by fluorodinitrobenzene was made in similar conditions and was stopped by filtration and wash with cold distilled water. This latter

treatment was used furthermore each time it was necessary to deplete the cells of intracellular solutes used during preincubation [21]. Bacteria were subsequently resuspended in the appropriate medium.

Chemicals

Phospho*enol*pyruvate was obtained from Boehringer, Mannheim, *N*-ethylmaleimide from Calbiochem, 1 fluoro 2-4 dinitrobenzene, from Baker Organic Chemical, and methyl-α-D-glucopyranoside [U-^{14}C] 3 Ci/mol from the Radiochemical Center, Amersham. Other chemicals were reagent grade from conventional commercial sources.

Results

Fig. 2 shows the basic observation which is analyzed in the rest of the report: the phosphorylative uptake of Me-Glc when a suspension was pretreated 2.5 min with 0.2 mM MalNEt, and the treatment terminated by the addition of 50 mM 2-mercaptoethanol before addition of [^{14}C]Me-Glc (Fig. 2A, curve d) is hardly diminished compared to control uptake (curve a). This is in sharp contrast with the strong inactivation observed when treatment with MalNEt was made during the first 2.5 min of uptake (Fig. 2A, curve f) or throughout the experiment starting at time zero (curve e). If during the control experiment MalNEt was added at the steady state for a 2.5-min treatment period (curve c) or throughout the experiment (curve b), the pools decreased and approached

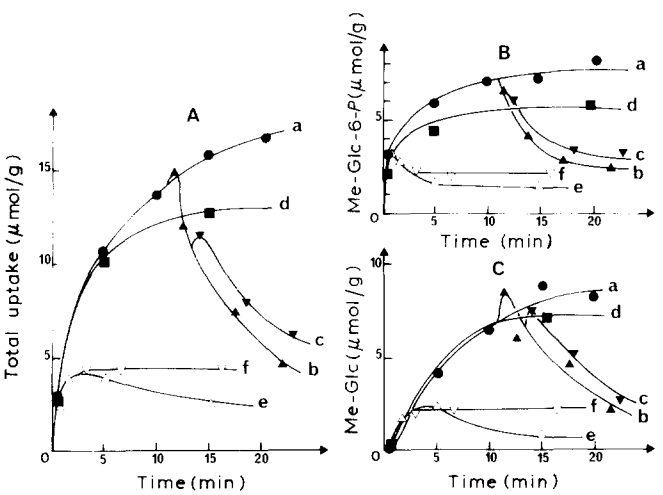

Fig. 2. Effect MalNEt on Me-Glc uptake: *E. coli* strain 3300 275 μg/ml (dry weight) was supplemented at zero time with [^{14}C]Me-Glc 0.05 mM. 1 ml samples were filtered on millipore, dried and counted, (A), total uptake: 1 ml samples were precipitated by 9 ml of an alcoholic solution of $BaBr_2$ 11 mg/ml, and then filtered, washed, dried and counted; (B), Me-Glc-6-*P*. Difference between (A) and (B), free Me-Glc (C). a, (●) control; b, (▲) control supplemented with 0.2 mM MalNEt at 11 min; c, (▼) MalNEt treatment of b was terminated 2.5 min later by addition of 50 mM 2-mercaptoethanol; d, (■) bacteria pretreated with 0.2 mM MalNEt for 2.5 min. At zero time 2-mercaptoethanol was added together with [^{14}C]Me-Glc; e, (△) bacteria supplemented with MalNEt at the time of addition of Me-Glc; f, (▽) MalNEt treatment as in e was terminated at 2.5 min by addition 2-mercaptoethanol.

the same final levels as in the samples treated from the beginning of uptake.

The changes in [^{14}C]MeGlc (Fig. 2C) and in [^{14}C]Me-Glc-6-*P* (Fig. 2B) were roughly parallel to each other and to the total radioactive pool as if the dephosphorylation and leak were not significantly affected by the treatments. The important features of the experiment are the inefficiency of pretreatment with MalNEt compared to the dramatic inactivation when MalNEt treatment is made during uptake, and secondly, the arrest but not the reversal of inactivation by 2-mercaptoethanol.

This irreversibility of inactivation can be used to compare the effect of pretreatments under a variety of conditions, the assay being made in a separated test. Two experiments of this kind are summarized in Fig. 3. These represent the effect of pretreatments for variable times with MalNEt 0.15 mM or with fluorodinitrobenzene 0.5 mM.

The remaining activities are represented on a logarithmic scale. Although the inactivation deviates from first order kinetics, the difference in the rate of inactivation with or without substrate present is striking. In the presence of substrate, 80% inactivation was reached in less than 2 min with either reagent

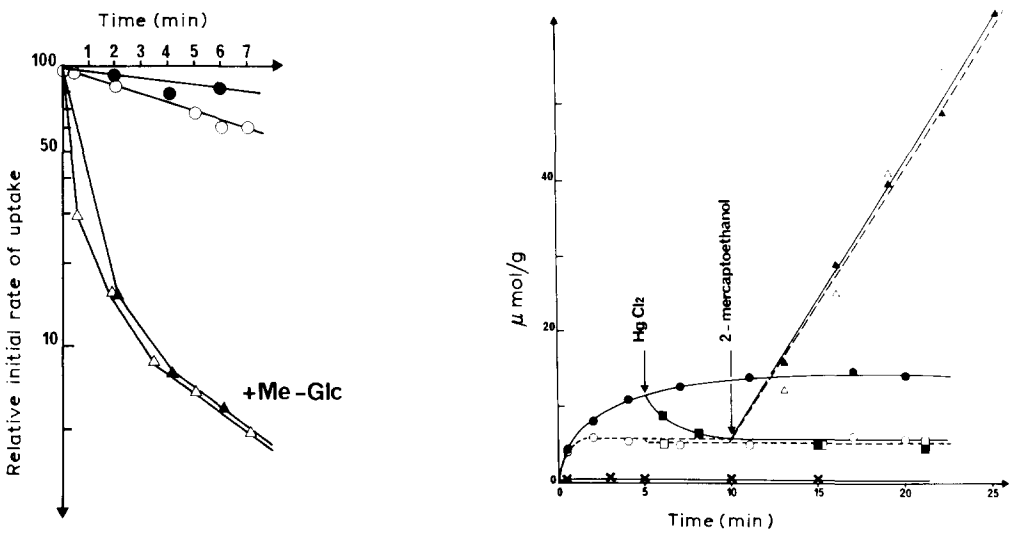

Fig. 3. Inactivation by MalNEt or fluorodinitrobenzene for various lengths of time. Inactivation by MalNEt: glucose grown *E. coli* 33C0 resuspended in medium 63 were treated by MalNEt 0.15 mM for various lengths of time in the presence, (▲) or absence, (●) of Me-Glc 1 mM. Treatment was terminated by addition of 2-mercaptoethanol 10 mM, followed by filtration, wash with distilled water, and resuspension in medium 63. Inactivation by fluorodinitrobenzene. Glycerol grown *E. coli* AJ19 resuspended in medium 63 glyB_1 at 15°C were treated by fluorodinitrobenzene 0.5 mM for various lengths of time in the presence, (△) or absence, (○) of Me-Glc 1 mM. Treatment was terminated by filtration, wash with cold distilled water, and resuspension in medium 63. Relative initial rate of uptake of [^{14}C]Me-Glc 0.05 mM was then measured in both experiments. Initial rate of uptake in untreated bacteria was 18 μmol · min^{-1} · g^{-1} and 16 μmol · min^{-1} · g^{-1} respectively.

Fig. 4. Effect of $HgCl_2$ on Me-Glc uptake and phosphorylation. Experiment was performed on *E. coli* strain 3000. [^{14}C]Me-Glc 0.05 mM was added at zero time. ○, ●, control; □, ■, addition of $HgCl_2$ 1 mM at 5 min to control; △, ▲, addition of 2-mercaptoethanol 5 mM at 10 min to the Hg inhibited suspension. Total uptake, full symbols and lines; Me-Glc-6-*P*, open symbols and dotted lines. X total uptake in bacteria pretreated 5 min with $HgCl_2$ 1 mM before adding [^{14}C]Me-Glc.

while without substrate the inactivation was less than 20% after 5 min.

The remaining phosphotransferase activity can be determined indifferently by measuring initial rate of uptake, steady-state accumulation, or rate of phosphorylation in toluene-treated bacteria, as shown in Table I.

Several types of strains, wild type in the phosphotransferase genes, showed rates of inactivation increased by a factor of 5—10 when substrate was present together with inactivating agent.

Fig. 4 shows the inactivation of phosphotransferase system by $HgCl_2$, and its reversibility.

$HgCl_2$ inactivation was somewhat different from that produced by MalNEt and fluorodinitrobenzene, in that it caused rapid efflux of free Me-Glc with no decrease of total Me-Glc-6-*P*. When added before Me-Glc, $HgCl_2$ caused complete inhibition of phosphorylative uptake. Thus $HgCl_2$, contrary to MalNEt and fluorodinitrobenzene, does inhibit the hexose phosphatase responsible for the intracellular dephosphorylation of the sugar. The most striking difference with the previous reagents is the reversibility of $HgCl_2$ inhibition by excess 2-mercaptoethanol. After addition of this thiol compound, phosphorylative uptake of Me-Glc resumed. On the other hand, the hydrolysis of Me-Glc-6-*P* did not approach a new steady state. Instead, the level of intracellular Me-Glc-6-*P* increased linearly at a rate somewhat lower than before inhibition, but accumulated well beyond the previous steady state: 10 min after reversion of $HgCl_2$ inhibition, there was a 3-fold increase in the total radioactive pool. We observed that total uptake equalled Me-Glc-6-*P* pool. It is, to our knowledge, the only example of transformation of the "permease like" in vivo uptake of Me-Glc, characterized by a steady-state accumulation, into a linear vectorial phosphorylation.

Fig. 5 shows the protection that $HgCl_2$ provides against inactivation by MalNEt: phosphorylation was reactivated by 2-mercaptoethanol even if MalNEt was added after $HgCl_2$, while the same incubation with MalNEt in the absence of $HgCl_2$ caused more than 90% inactivation.

TABLE I

EFFECT OF FLUORODINITROBENZENE ON VARIOUS PARAMETERS OF UPTAKE IN PRESENCE AND ABSENCE OF Me-Glc

Experiment was performed on *E. coli* strain AJ19 in medium 63glyB$_1$ as described in legend of Fig. 3. Initial rate of uptake and steady-state accumulation were measured respectively, after 20 s and 18 min incubation at 30°C in the presence of [^{14}C]Me-Glc 0.05 mM. Toluene treatment was made after chemical inactivation as described in the Materials and Methods. Me-Glc-6-*P* was formed in toluene treated bacteria measured by baryum precipitation after 6 min incubation at 30°C in presence of 1 mM phospho-*enol*pyruvate and 0.05 mM [^{14}C]Me-Glc.

	Value in untreated bacteria	Per cent after 0.5 min treatment by 0.5 mM fluorodinitrobenzene	
		Alone	In presence of 1 mM Me-Glc
Initial rate of uptake	16 μmol · min^{-1} · g^{-1}	89	36
Steady-state accumulation	86 μmol · g^{-1}	81	34
Phosphorylation in toluene treated bacteria	9 μmol · min^{-1} · g^{-1}	100	37

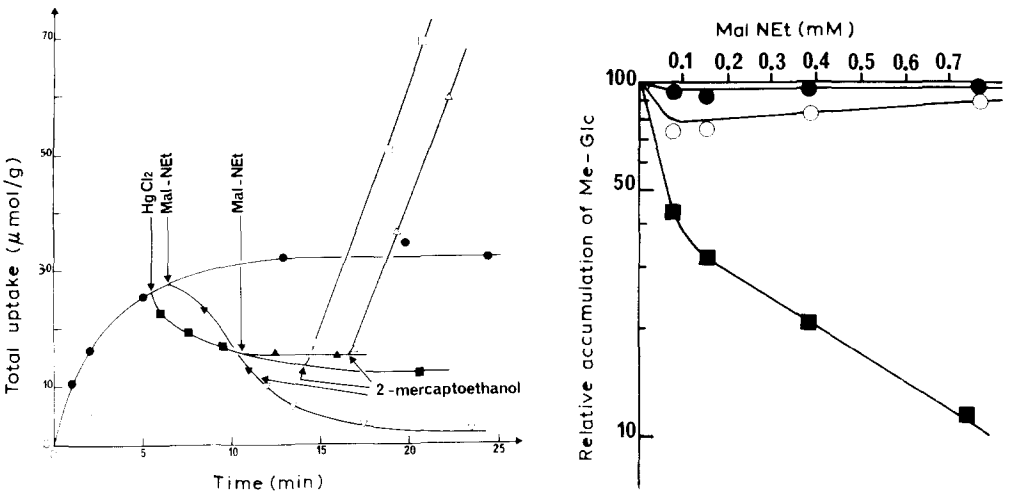

Fig. 5. Protection by $HgCl_2$ against inactivation of Me-Glc uptake by MalNEt. Experiment was performed on *E. coli* strain AJ19. [^{14}C]Me-Glc was added at zero time. (●), control; (■), addition of $HgCl_2$ 1 mM at 5.5 min to control; (⊔), addition of 2-mercaptoethanol 10 mM at 14.5 min to Hg inhibited suspension, (▲), addition of MalNEt 0.3 mM at 10.5 min to the Hg inhibited suspension; (△), addition of 2-mercaptoethanol at 16.5 min to the former to remove unreacted MalNEt and $HgCl_2$; (▼) addition of MalNEt 0.3 mM at 6.5 min to the control followed by addition of 2-mercaptoethanol 10 mM at 11.5 min (▽)

Fig. 6. Comparison between effect of intracellular and extracellular Me-Glc on MalNEt inactivation. *E. coli* strain AJ19 was suspended in medium 63 $glyB_1$ at a density of 1.4 mg/ml (dry weight) in absence or presence of Me-Glc 0.02 mM. 15 min later, bacteria were submitted to a 50-fold dilution and inactivated by various concentrations of MalNEt for 1 min. Inactivation was stopped by addition of 2-mercaptoethanol 20 mM, followed by filtration wash and resuspension in 63 $glyB_1$ with 2-mercaptoethanol 20 mM. Steady-state accumulation of Me-Glc was measured after 30 min incubation in the presence of 0.05 mM [^{14}C]Me-Glc. (●) inactivation by MalNEt alone; (○), inactivation by MalNEt during efflux of preloaded Me-Glc; (■), inactivation by MalNEt in presence of Me-Glc. Steady-state accumulation in untreated bacteria was 100 μmol/g.

The same result was obtained using fluorodinitrobenzene instead of MalNEt. This parallelism, together with that shown on Fig. 3 strongly suggests that MalNEt and fluorodinitrobenzene act on phosphotransferase at the same site.

Since the presence of substrate appeared necessary to make the phosphotransferase system sensitive to inactivation by MalNEt, the question could be asked, whether extracellular Me-Glc, intracellular Me-Glc-6-*P* or intracellular Me-Glc was responsible for this effect. To answer this question, cells were preloaded with Me-Glc (and Me-Glc-6-*P*) then diluted into inactivation medium so that total Me-Glc concentration dropped to 4.10^{-7} M, insufficient to promote inactivation by MalNEt (cf. Fig. 7). Samples were submitted to 1 min incubation with MalNEt at variable concentrations. All samples were exposed to MalNEt during the same short early period of exit of Me-Glc. Control inactivations were run in the presence of 10 mM Me-Glc in the medium or in its complete absence. Fig. 6 shows that internal Me-Glc and Me-Glc-6-*P* did not alter the resistance of the system to MalNEt as did external Me-Glc.

When taken together with results reported below on toluene-treated cells, the experiment of Fig. 6 means that intracellular Me-Glc cannot significantly play the role of phosphate acceptor in the phosphotransferase system.

Fig. 7 establishes an even stronger correlation between activity of phosphotransferase and sensitivity to inactivation by MalNEt. Here the Me-Glc concentration dependence of the rate of phosphorylative uptake and the rate of inactivation by MalNEt were compared in two conditions, the absence or the presence of sodium azide. It was demonstrated by Englesberg and his group [22, 23], that "glucose permease" activity was stimulated by energy starvation or by energy inhibitors such as uncouplers. The stimulation was due to a decrease in K_m of the system for the sugar [24,25]. This is confirmed by the first part of the experiment reported on Fig. 7, while the second part of the experiment shows variations of the rate of inactivation under the same conditions. When plotted on a double reciprocal scale, the results of Fig. 7 show that both K_m of Me-Glc uptake and the concentration of Me-Glc involving half maximal enhancement of MalNEt inactivation were 0.2 mM and 0.066 mM in the absence and presence of 40 mM sodium azide respectively. Besides the strong correlation between activity and unmasking of a site sensitive to MalNEt, this experiment suggests that the stimulating effect of the "energy uncoupled state of the membrane", brought about by a H^+ conducting agent like sodium azide on phosphotransferase activity, occurs via the membrane bound sugar specific component and not as sometimes hypothesized [26] indirectly by modifying

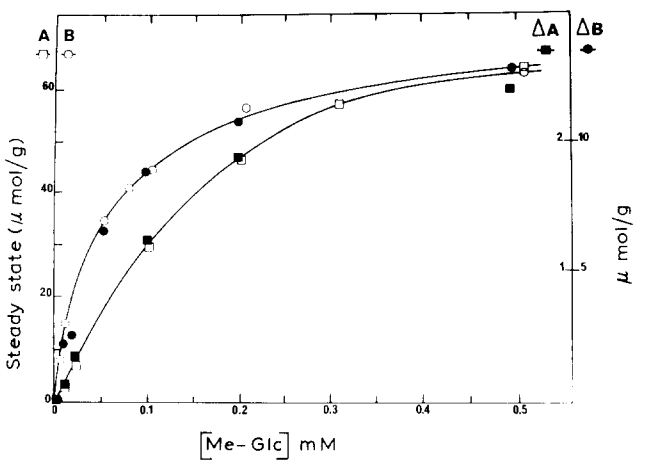

Fig. 7. Substrate concentration dependance of steady-state accumulation and of sensitivity to MalNEt inactivation in the presence or absence of sodium azide. *E. coli* strain 3000 was equilibrated at 25°C in the presence or absence of sodium azide 40 mM. Aliquots were supplemented with various concentrations of [^{14}C]Me-Glc. Steady-state accumulation was determined after 15 min. A (□), steady-state accumulation in the absence of sodium azide; B (○), steady-state accumulation in the presence of sodium azide. The same bacteria were submitted to 1 min inactivation by MalNEt 0.15 mM in the presence of various concentrations of Me-Glc. Inactivation was stopped by addition of 2-mercaptoethanol 1 mM, followed by filtration, wash with water, and resuspension in medium 63 glyB_1. Steady-state accumulation was then measured after 15 min incubation in the presence of [^{14}C]Me-Glc, 0.05 mM and in the presence of sodium azide 40 mM (for bacteria inactivated in the presence of sodium azide) and in the absence of this agent (for bacteria inactivated in its absence; hence the different scale of ordinates for ΔA and ΔB). For each concentration of Me-Glc present during inactivation the increment of inactivation due to its presence was calculated. ΔA (■), substrate dependant increment of inactivation produced by MalNEt; ΔB (●), same as ΔA in the presence of sodium azide. Steady-state accumulation after inactivation by MalNEt alone were respectively 20 μmol/g and 5 μmol/g in the presence and absence of sodium azide.

the level of the intracellular phospho*enol*pyruvate pool, which should not alter the apparent affinity for the sugar.

One more correlation had to be explored between activity and sensitivity to MalNEt, namely the modulation of activity with an inhibitor of phospho*enol*-pyruvate synthesis. According to our previously reported observations [27]

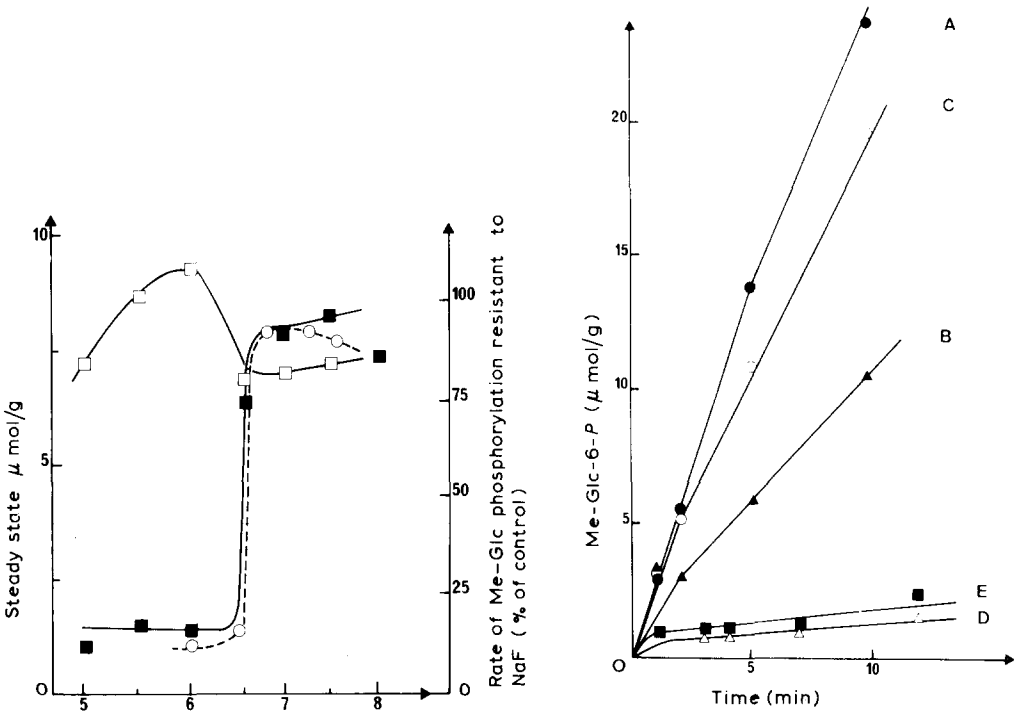

Fig. 8. pH dependance of fluoride inhibition of phosphorylation of Me-Glc and of fluoride sensitization to MalNEt. (1) Bacteria harvested during exponential phase of growth and resuspended in 50 mM K phosphate buffer complemented with glycerol 4 g/l at the appropriate pH were incubated 10 min in the presence or absence of sodium fluoride 50 mM. [^{14}C]Me-Glc 0.05 mM was then added. 2 min later, samples were precipitated by addition of $BaBr_2$ 11 mg/ml. (○), rate of Me-Glc phosphorylation resistant to fluoride inhibition, per cent of control rate (control rate, 3 μmol/min per g). (2) Bacteria harvested during exponential phase of growth were resuspended in K phosphate buffer at the appropriate pH complemented with glycerol and preincubated 10 min in the absence or presence of sodium fluoride 50 mM. Samples were then submitted to 2 min inactivation by MalNEt 0.4 mM terminated by addition of 2-mercaptoethanol 4 mM, and followed by filtration, wash with medium of same pH, and resuspension in 63 $glyB_1$ (pH 7.2). Steady-state accumulation of Me-Glc was measured in each sample after 15 min incubation in the presence of [^{14}C]Me-Glc 0.05 mM. (□), steady-state after inactivation by MalNEt alone; (■), steady-state after inactivation by MalNEt in the presence of sodium fluoride.

Fig. 9. Inactivation by MalNEt of Me-Glc phosphorylation in toluene treated bacteria. *E. coli* strain 3300 harvested in the exponential phase of growth and resuspended in medium 63 supplemented with 4 g/l glycerol and 50 μg/ml chloramphenicol was treated by 1 μl/ml toluene for 30 min at 37°C, and then equilibrated at 30°C. A, (●) control, bacteria supplemented at zero time with phospho*enol*pyruvate 1 mM and [^{14}C]Me-Glc 0.05 mM; B, (▲) bacteria pretreated for 2 min with 0.2 mM MalNEt were supplemented at zero time with 2-mercaptoethanol 1 mM, phospho*enol*pyruvate 1 mM, and [^{14}C]Me-Glc 0.05; C, (○) bacteria pretreated for 2 min with 0.2 mM MalNEt in the presence of 1 mM phospho*enol*pyruvate were supplemented at zero time with 2-mercaptoethanol 1 mM and [^{14}C]Me-Glc 0.05 mM; D, (△) at zero time, addition of 0.2 mM MalNEt and 0.05 mM [^{14}C]Me-Glc, 2 min later addition of 2-mercaptoethanol 1 mM and phospho*enol*pyruvate 1 mM; E, (■) at zero time, addition of 0.02 mM MalNEt 1 mM phospho*enol*-pyruvate, and 0.05 mM [^{14}C]Me-Glc, 2 min later addition of 2-mercaptoethanol 1 mM.

fluoride is such an inhibitor at a pH below 6.5 in the medium (or below pH 7.2 in the cytoplasm). Fig. 8 shows this pH dependence of fluoride inhibition of phosphorylative uptake [8] and also the pH dependence of the rate of inactivation by MalNEt in the presence and the absence of fluoride. The inhibition of phosphotransferase activity by fluoride here parallels its effect promoting inactivation by MalNEt, in the absence of Me-Glc. Fluoride inhibition of phospho*enol*pyruvate synthesis therefore causes the same conformational change as some step of the phosphate transfer process. Both conditions favor a dephosphorylated or energy poor state of the system.

The following experiment further supports this interpretation. When toluene-treated cells were incubated with MalNEt under various conditions the results represented in Fig. 9 were obtained.

The presence of Me-Glc with or without phospho*enol*pyruvate permitted a more than 90% inactivation by 2-min exposure to 0.5 mM MalNEt while MalNEt alone gave about 50% inactivation. The presence of phospho*enol*pyruvate without Me-Glc provided a protective effect so that inactivation was less than 20%.

It is likely that the presence of phospho*enol*pyruvate affected enzyme II through the presence of Phospho-HPr. Fluoride did not provide any inhibition of phospho*enol*pyruvate dependant phosphorylation of Me-Glc in toluene treated bacteria. Neither did it sensitize to MalNEt inactivation in this system in the presence of added phospho*enol*pyruvate (experiments not shown). These observations confirm that its sensitizing effect in vivo occurred through its effect on phospho*enol*pyruvate synthesis. Substrate alone sensitized to inactivation in the absence of any significant activity.

Sensitization to MalNEt in toluene treated cells depends on a minimal amount of phosphate group transfer as opposed to a phosphate group transfer rate in substrate amounts in vivo. Here the conformational change is carried out in less than a single cycle. Me-Glc alone sensitizes completely and phospho*enol*pyruvate alone protects completely.

Discussion

Like *N*-ethylmaleimide, fluorodinitrobenzene, usually an amino group reagent, can react with thiols [28]. Their similarity in the inactivation of phosphotransferase, as well as the similar protection provided by $HgCl_2$ against both, strongly suggest that they react with the same essential thiol of the phosphotransferase system.

The change in reactivity of this essential thiol group can be attributed to a conformational change of the membrane bound phosphotransferase Enzyme II during its functional cycle.

A conformational change is expected in an enzyme which catalyses a vectorial reaction since a hydrophilic hexosyl radical is translocated across a hydrophobic membrane and this translocation is strongly linked to a phosphate transfer reaction. Out of the 4 reactants, the phosphate donor and the phosphate acceptor substrate and the corresponding two products, one only, the phosphate acceptor sugar has access to the enzyme site from outside the membrane. The three others react from inside, a set up which does not suggest that a bulk

movement of the enzyme is linked to sugar translocation. The nature and the possible steps of the conformational change are presently unknown, but the unmasking of an essential thiol and its trapping by a covalent reaction provide a valuable index for a conformational change.

The conformation in which the thiol group is unreactive predominates when phosphate donor (Phospho HPr) is high and phosphate acceptor sugar absent. When phosphate acceptor is added the transition to the state where the thiol group is reactive occurs with a probability which is proportional to the phosphotransferase activity. Therefore, this state is one among those which are characteristic of the functional cycle of the enzyme.

The Michaelis complex formation of the enzyme with outer acceptor sugar but not with cytoplasmic sugar or with cytoplasmic sugar phosphate product can lead to this conformation. This strongly suggests that intracellular sugar cannot be phosphorylated.

The unmasking of the thiol during fluoride inhibition tends to indicate that the Michaelis complex formation is not necessary for the transition to occur. It can be brought about by lowering the phosphate donor.

That the same conformational cycle can be observed in toluene treated bacteria shows first that the unreactive conformation is not due to the exclusion of the thiol reagent by the membrane barrier but to molecular changes of the enzyme proper, and second, that neither the reactive nor the unreactive configuration is due to a transmembrane potential difference or to the chemical or electrochemical gradient of a solute, since all these are abolished by the toluene treatment.

The conformation with the unreactive thiol group could be roughly described as the energized state of the phosphotransferase enzyme and the conformation with the thiol exposed as the deenergized state. In a sequence of phosphate group transfer reactions from phospho*enol*pyruvate to Enzyme I from Phospho-Enzyme I to HPr, from Phospho-HPr ultimately to the sugar, it would be tempting to consider the phosphorylated form of the membrane bound enzyme II as its energized form. The ordered ping-pong kinetics of the Enzyme II specific for β-glucosides makes the existence of the phosphorylated Enzyme II plausible [29]. No similar argument is available in favor of the phosphorylated form of *E. coli* Enzyme II specific for glucose. Another alternative could be a Michaelis complex between Enzyme II and Phospho-HPr as the energized state.

In both of these hypotheses it is easy to see how the acceptor sugar causes the transition toward the deenergized state. In contrast, when the transition is caused by fluoride inhibition, the mechanism remains unclear. Phosphorylated Enzyme II or phospho-HPr should lose a phosphate residue without the natural phosphate acceptor being present. Is this dephosphorylation due to the reversibility of the phosphorylation step(s) or to some trivial phosphatase activity? In this respect it is important to state that the energized form postulated seems to be relatively stable in the toluene treated bacteria since after more than 30 min incubation without phospho*enol*pyruvate, more than half of the Enzyme II remains in the form unreactive with MalNEt.

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