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THE ROLE OF ENZYME I IN THE UNMASKING OF AN ESSENTIAL THIOL OF THE MEMBRANE-BOUND ENZYME II OF THE PHOSPHO-*ENOL*PYRUVATE-GLUCOSE PHOSPHOTRANSFERASE SYSTEM OF *ESCHERICHIA COLI*

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Summary

The membrane-bound component of the phosphotransferase system of *Escherichia coli*, responsible for the phosphorylative uptake of methyl-α-D-glucoside has an essential thiol group which becomes available to inactivation by thiol reagents in the presence of the phosphate-accepting sugar or when phospho*enol*pyruvate synthesis is inhibited. The form resistant to the thiol reagent requires not only the absence of sugar and an intact phospho*enol*-pyruvate generating system, but also an intact system generating phosphorylated Hpr which is impaired by heating of a thermosensitive enzyme I mutant.

Methyl-α-D-glucoside, a non-metabolizable glucose analog, is taken up in *Escherichia coli* through a vectorial phosphorylation mediated by the phospho*enol*pyruvate hexose phosphotransferase system [1—4].

It has been shown in previous reports [5,6] that in vivo inactivation of methyl-α-D-glucoside transport in *E. coli* by thiol reagents such as *N*-ethylmaleimide is strongly enhanced by the substrate, or by the presence of fluoride, an inhibitor of phospho*enol*pyruvate synthesis.

Toluene-treated bacteria are still able to phosphorylate methyl-α-D-glucoside through the phosphotransferase system when they are supplied with

Abbreviations used: Methyl-α-D-glucoside-*P*, methyl-α-D-glucopyranoside 6-phosphate; enzyme I, EC 2.7.3.9, phospho*enol*pyruvate-protein phosphotransferase; enzyme I-*P*, phosphoenzyme I; enzyme II, EC 2.7.1.69, phosphohistidinoprotein-hexose phosphotransferase; enzyme II-*P*, phosphoenzyme II; enzyme IIglc, enzyme II specific for glucose and methyl-α-D-glucoside; enzyme IIbgl, enzyme II specific for β-glucosides; HPr, histidine containing phosphate carrier protein of the phospho*enol*pyruvate dependant phosphotransferase system; HPr-*P*, phospho histidine protein.

phospho*enol*pyruvate [7,8]. This reaction is also inactivated by thiol reagents and the inactivation is enhanced by substrate, but in its absence is completely prevented by phospho*enol*pyruvate [5,6].

The target of in vivo inactivation by thiol reagents was found to be the membrane bound component of the system called enzyme IIglc [5].

These observations suggested that enzyme IIglc during the transport process could exist in two alternative conformational states, energized and deenergized, the energized state being resistant to inactivation by thiol reagents.

In the present article we show that the *N*-ethylmaleimide resistant state depends on the activity of an HPr-*P* generating system. This was made possible by the use of a thermosensitive enzyme I mutant of *E. coli*.

E. coli strain ts 1962 F^- (*pts*-19 *trp*-30 *pro*A23 *lac*-28 *str*A101) was provided by B. Bachmann and resulted from a cross between HfrC ts 19 (*met*$^-$) [9] and F^- J62 (*pro*A23 *his*-51 *trp*-30 *lac*-28 *str*A101) [10]. *E. coli* K12 strain 3000 was from the collection of the Pasteur Institute.

After growth at 30°C in medium 63 [11] with 4 g/l glycerol as carbon source and 100 μg/ml of the required amino acids, a suspension of *E. coli* strain ts 1962 was divided into two parts, one (a) submitted to 1 h thermal inactivation at 45°C and the other (b) used as an unheated control. Each part was then subdivided into three samples. One was left untreated, the other two were treated for 2 min at 30°C with 0.05 mM *N*-ethylmaleimide with or without 1 mM methyl-α-D-glucoside. After washing and resuspension at a density of 275 μg/ml (dry weight) in medium 63 glyBl containing 1 mM 2-mercaptoethanol, the accumulation of methyl-α-D-glucoside was measured in each sample as described in previous reports [5,6].

Each sample was then harvested, washed in 50 mM potassium phosphate buffer, pH 7, containing 2 mM $MgCl_2$ and 1 mM 2-mercaptoethanol and resuspended at 1 mg protein/ml in the same buffer in the presence of DNAase (5 μg/ml). The suspension was broken with a 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 reaction mixture for in vitro measurement of enzyme II activity in these extracts was made in the same buffer as above, in the presence of 0.2 mM [^{14}C]methyl-α-D-glucoside, 15 mM phospho*enol*pyruvate, 15 mM sodium fluoride, supernatant of wild type *E. coli* strain 3000 (1.5 mg protein/ml, centrifuged 3 times for 90 min at 165 000 × *g*) as source of excess enzyme I and HPr, and 0.1 mg/ml of the crude extracts described above as source of enzyme IIglc. Methyl-α-D-glucoside 6-phosphate formation in 0.1 ml samples was measured at regular intervals for 6 h by the barium precipitation technique [12,7]. The reaction was linear with time.

All results are summarized in Table I.

In bacteria not submitted to thermal inactivation, the presence of methyl-α-D-glucoside again enhanced the inactivation of its uptake by *N*-ethylmaleimide.

Thermal inactivation led to complete suppression of methyl-α-D-glucoside transport, so that further effect of *N*-ethylmaleimide on transport was not measurable.

TABLE I

SUSPENSIONS OF HEATED AND UNHEATED ts 1962 MUTANT WERE SUBMITTED TO 2 min INACTIVATION BY *N*-ETHYLMALEIMIDE IN THE PRESENCE OR ABSENCE OF METHYL-α-D-GLUCOSIDE. EXTRACTS WERE THEN PREPARED AND ENZYME II ACTIVITY MEASURED WITH EXCESS 165 000 × *g* SUPERNATANT OF WILD TYPE *E. COLI* 3000 AS SOURCE OF ENZYME I AND HPr AS DESCRIBED IN THE TEXT. BLANK VALUE IN THE ABSENCE OF ADDED EXTRACT WAS 0.2 nmol METHYL-α-D-GLUCOSIDE-*P* IN 5 h.

Treatment	Heated			Unheated		
	Control	*N*-Ethylmaleimide 50 μM, 2 min	*N*-Ethylmaleimide 50 μM, 2 min + methyl-α-D-glucoside 1 mM	Control	*N*-Ethylmaleimide 50 μM, 2 min	*N*-Ethylmaleimide 50 μM, 2 min + methyl-α-D-glucoside 1 mM
In vivo uptake μmol methyl-α-D-glucoside (g dry weight)$^{-1}$ (2 min)$^{-1}$	N.S.	N.S.	N.S.	8.3	2.4	0.88
Per cent of control				100	29	10.5
In vitro methyl-α-D-glucoside 6-phosphate produced*	7.6	1.13	0.86	6.3	5	0.46
Specific activity of enzyme IIglc**	2.5	0.38	0.29	2.1	1.66	0.15
Specific activity, per cent of control	100	15	11	100	79	7

*nmol per sample of 0.1 ml in 5 h.

**nmol (min)$^{-1}$ (mg protein)$^{-1}$ assuming 10% membrane protein in crude extract.

N.S., not significant.

The membrane bound enzyme IIglc activity (Table I, last two lines) was very differently affected by *N*-ethylmaleimide depending on the pretreatment.

When enzyme I was fully active during the treatment, enzyme IIglc was poorly accessible to *N*-ethylmaleimide, which gave only 21% inactivation, whereas the presence of methyl-α-D-glucoside increased the inactivation to 93%. When enzyme I activity was previously suppressed by the thermal inactivation, enzyme IIglc was accessible to *N*-ethylmaleimide to the extent of 80—90% in the presence or absence of methyl-α-D-glucoside.

In Fig. 1, the classic scheme of the phosphotransferase reaction sequence is extended by the cyclic reaction of enzyme IIglc. This includes an energized form obtained at the expense of phosphorylated HPr and a deenergized form obtained after transfer of the phosphate residue to the sugar. Only this deenergized form is sensitive to *N*-ethylmaleimide inactivation; the energized one is resistant. Previous papers have shown that an intact phospho*enol*-pyruvate generating system was required to transform the *N*-ethylmaleimide-sensitive form into the *N*-ethylmaleimide-resistant form; the requirement for an intact enzyme I for the same reaction is shown in the present article.

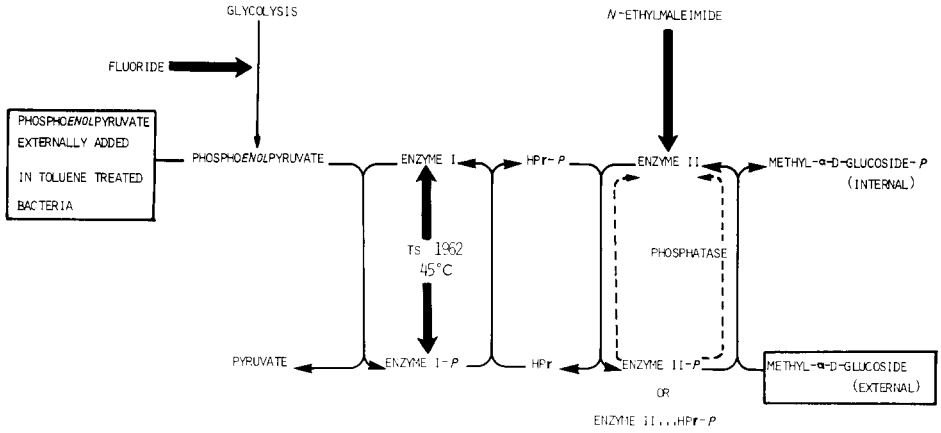

Fig. 1. Schematic representation of the phosphotransferase sequence in vivo and in permeabilized cells showing the sites where experimental action is possible (heavy arrows).

The opposite transformation, going from the *N*-ethylmaleimide-resistant to the *N*-ethylmaleimide-sensitive form of enzyme II can obviously be brought about by the sugar, which has to undergo vectorial phosphorylation (in vivo) or phosphorylation (toluene-treated cells) to be effective. Nevertheless the transition from the *N*-ethylmaleimide-resistant to the *N*-ethylmaleimide-sensitive form also occurs in the absence of added phosphate acceptor sugar after fluoride poisoning or after inactivation of enzyme I. The hypotheses on the mechanism of this transition are dependent on the nature of the *N*-ethylmaleimide-resistant form, here briefly defined as an energized form. If this is a phosphorylated form of enzyme IIglc, its dephosphorylation in the absence of sugar might occur via a more or less specific phosphoprotein phosphatase, or via the reversion of the phosphate transfer sequence: Enzyme II-*P* + HPr → Enzyme II + HPr-*P*.

A phosphorylated transient form has been suggested in the case of another enzyme II of *E. coli*, that specific for β-glucosides, on the basis of ordered bi-bi ping-pong kinetics [14]. The transport of β-glucosides linked to this enzyme IIbgl presents the same changes in accessibility to inactivation by thiol reagents as the glucose phosphotransferase here described (unpublished results). The sensitization of enzyme IIbgl is brought about by β-glucosides such as arbutin, instead of methyl-α-D-glucoside. In favor of a transient phosphorylated form of enzyme IIglc, the labeling of membrane vesicles in the presence of ^{32}P-labelled HPr [15] was also reported.

If the energized form of enzyme II is instead a Michaelis complex with HPr, its deenergization would only require a mechanism able to dephosphorylate HPr-*P*. The reversibility of the reaction catalysed by enzyme I has been described in *Micrococcus aureus* [16].

The experiments reported here also suggest that the energization-deenergization cycle postulated for enzyme IIglc is associated with conformational changes of the enzyme beyond phosphorylation-dephosphorylation. It would be attractive to suppose that this change might be part of the translocation cycle. Our experiments strongly suggest that methyl-α-D-glucoside enters by a single enzymatic system. But they do not allow any speculation on the identity of this system: enzyme IIA/IIB or factor III/enzyme II′B, as recently postulated by Postma and Roseman [14]. It is noteworthy that neither of these combinations has been found for β-glucosides [17].

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