The Glucose Transporter of Escherichia coli

MUTANTS WITH IMPAIRED TRANSLLOCATION ACTIVITY THAT RETAIN PHOSPHORYLATION ACTIVITY*

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The glucose transporter of the bacterial phosphotransferase system couples translocation with phosphorylation of the substrate in a 1:1 stoichiometry. It is a complex consisting of a transmembrane subunit (II^Glc) and a hydrophilic subunit (III^Glc). Both subunits are transiently phosphorylated. III^Glc is phosphorylated at a histidyl residue by the cytoplasmic phosphorilating carrier protein phospho-enolpyruvate-sugar phosphotransferase system (PTS); for comprehensive reviews see Meadow et al. (1989), and Erni (1992). The glucose transporter is specific for glucose and α-MG. It consists of two subunits, II^Glc and III^Glc. III^Glc (18 kDa) is a hydrophilic protein that contains the phosphorylation site 3 (His-90; Dorschug et al., 1984). II^Glc consists of a transmembrane domain (approximately 380 N-terminal amino acids) and a hydrophilic domain (approximately 100 C-terminal amino acids) that contains phosphorylating and a hydrophobic domain (approximately 100 C-terminal amino acids) that contains phosphorylating site 4 (Cys-421; Pas and Robillard, 1989; Reibaldi et al., 1991; Nuoffer et al., 1988). III^Glc allosterically enhances the catalytic activity of II^Glc in vitro, but the mechanism of regulation is complex (Erni, 1986). A reversible association between II^Glc and III^Glc is important because III^Glc has regulatory functions that require it to associate with other proteins. By binding to non-PTS transport systems (e.g. for lactose and maltose), as well as adenylyl cyclase, III^Glc regulates and coordinates the activity of other transport systems with the transport activity for glucose and, to a lesser extent, for the other PTS substrates (reviewed in Saier and Chin (1990)). The amino acid sequences of II^Glc and III^Glc are about 40% identical and colinear with the sequence of the N-acetylglucosamine transporter (II^Nac; Peri and Waygood, 1988). II^Nac is a single polypeptide containing independent domains corresponding to II^Glc and III^Glc. II^Nac is also closely related to MalX, a protein that appears to be involved in the regulation of the mal operon and that can complement II^Glc (Reidl and Boos, 1991). The mannitol transporter (II^Mann) has an architecture similar to that of II^Nac but almost no sequence similarity. Biochemically, it is by far the best characterized PTS transporter, and reference to it will be frequent in this report.

Nothing is known about the molecular mechanism of coupling between phosphorylation and vectorial translocation of substrates. There is no transport without phosphorylation by wild-type proteins. However, II^Glc mutants exist that facilitate diffusion of glucose without phosphorylation (Postma, 1981).

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1 The abbreviations used are: PTS, phosphoenolpyruvate-sugar phosphotransferase system; II^Glc, transmembrane subunit of the glucose transporter; III^Glc, cytoplasmic subunit of the glucose transporter; α-MG, methyl-α-D-glucopyranoside; II^Nac, mannitol transporter; III^Nac, N-acetylglucosamine transporter.

Postma and Lengeler (1985). Phosphoryl groups are transferred sequentially from P-enolpyruvate via four phosphorylation sites to the sugar substrates. Sites 1 and 2 are histidines on the cytoplasmic proteins enzyme I and heat-stable phosphorilating carrier protein, respectively. Sites 3 and 4 are on the transporters. Sites 3 are histidines, and sites 4 are cysteines or histidines. The transporters consist of three domains. The hydrophobic domain is predicted to span the membrane between six and eight times. It contains the sugar-binding site. Two hydrophilic domains at the cytoplasmic face of the inner membrane contain phosphorylation sites 3 and 4, respectively. The transporters differ in substrate selectivity, amino acid sequence, the chemical nature of phosphorylation site 4, and in how the three domains are organized as independent domains in a polypeptide chain or polypeptide subunits in a complex (reviewed in Robillard and Lolkema (1988), Erni (1989), and Erni (1992)).

The glucose transporter of Escherichia coli acts by a mechanism that couples translocation with phosphorylation of the substrate. It belongs to the family of structurally and functionally related transporters known as enzymes II of the bacterial phosphotransferase system (PTS); for comprehensive reviews see Meadow et al. (1990), Postma (1987), and

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In contrast to transport without phosphorylation, phosphorylation without transport can be observed under special conditions. In the absence of a kinase, a PTS sugar in the cytoplasm can be phosphorylated by the respective PTS transporter (Thompson and Chassy, 1985; Thompson et al., 1985; Nuoffer et al., 1988). That the sugar does not need to exit first to become phosphorylated during reentry is inferred from the observation that a sugar generated intracellularly from a disaccharide does not equilibrate with the extracellular pool of a competing substrate. Kinetic and binding studies performed with the mannitol transporter (II'Mt) indicate that there is only one binding site/polyepitope and that it is located in the hydrophobic domain (Grasfai et al., 1989; Löffler et al., 1990). More accurate localization of the binding site by affinity labeling has been attempted but not yet been reported for PTS transporters.

Here we describe experiments aimed at identifying regions in the II'Mg subunit of the glucose transporter that are directly involved in binding and translocation of glucose. II'Mg mutants were selected that retained phosphorylation activity but showed poor transport activity. Three protein segments were thus identified that might affect translocation but have little or no effect on binding or phosphorylation of the substrate.

**Experimental Procedures**

Bacterial Strains (E. coli K-12), Plasmids, and Media

A recA derivative of ZSC112L (ptsG ptsM slc; Curtis and Epstein, 1975) was used as the host cell for selection of plasmid encoded II'Mg mutants. This strain does not grow on maltose, although it accumulates maltose and releases into the medium glucose derived from maltose (Nuoffer et al., 1988). This phenotype is not understood. LE-30 (mutDS) was used for in vitro mutagenesis (Fowler et al., 1974). Plasmid pMaG was constructed by ligation into the phagemid vector pMaS (Scha, cat-anbcr; Sandansens et al., 1988) of the EcoRI HindII fragment containing ptsG from pTSG3 (pTSG3 is a derivative of pTSG6 containing two of the two EcoRI sites deleted; Erni and Zanolori, 1986)). Standard minimal salts medium complemented with 0.1% casamino acids, and 1% glycerol. After 8-10 h at 37°C and further incubation at 37°C for 2.5 h. Plasmids were extracted with 20 ml of ice-cold 1% saline and counted in 2 ml of glass fiber filters (GF/F, Whatman) under suction. The filters were washed with 20 ml of ice-cold 1% saline and counted in 2 ml of Rotiszint (Roth). The transport rate was calculated as nmol min⁻¹ mg⁻¹ (dry weight) from the linear part of the uptake curve.

Assay for P-enolpyruvate-Sugar Phosphotransferase Activity

For the initial characterization of the large number of mutants, the photometric assay of Kundig and Roseman (1971) using glucose-6-phosphate dehydrogenase (Boehringer Mannheim) and NADP was used. For the final characterization of the selected mutants and for comparing phosphorylation activity with glucose and α-MG as substrates, the ion exchange method of Kundig and Roseman (1971) was used as specified by Erni et al. (1982). Membranes containing mutant II'Mg were prepared by fractionated centrifugation from cells ruptured in a French pressure cell (Erni et al., 1982). A cytoplasmic extract from a strain overexpressing enzymes I, heat-stable phosphoryl carrier protein, and III'Mg was used as source of soluble phosphoryl carrier proteins and III'Mg (Erni et al., 1982). The substrate concentration ([U-14C]α-MG and [U-3H]α-glucose) was 400 μM in all experiments.

DNA Sequencing and Plasmid DNA Techniques

The mutant ptsG genes were completely sequenced, and the restriction fragments containing the mutation were recloned into wild-type ptsG. All the recombinant plasmids thus obtained conferred the same phenotype as the original mutant plasmids. Double-stranded plasmid DNA was sequenced by the DNA exonuclease chain termination method using the Sequenase™ kit (United States Biochemical Corp.) and conveniently positioned oligonucleotide primers that had been used previously for site-directed mutagenesis. Plasmid DNA was prepared and analyzed by standard techniques (Maniatis et al., 1982).

Other Techniques

To estimate the amount of III'Mg in whole cell extracts and in membrane preparations, 200 and 40 μg, respectively, of total protein were electrophoresed (Aubel et al., 1987) and blotted onto nitrocellulose (Towbin et al., 1979), and III'Mg was quantified by staining with a pool from four monoclonal antibodies and a second peroxidase-conjugated antibody (Müller et al., 1988). To ensure aggregation of III'Mg, the proteins were not boiled in sample buffer prior to electrophoresis. Standard procedures were used for protein determination according to Lowry (Markwell et al., 1978) and dry weight determination (Gerhardt, 1981).

**Results**

Selection of Mutants—E. coli ZSC112L is unable to grow on glucose as the only carbon source, because it lacks a functional glucose transporter (II'Mg). A functional II'-Mg subunit of the mannose transporter and glucokinase. Surprisingly, this strain does not grow on maltose either, although it does accumulate and process it (Nuoffer et al., 1988). We cannot explain this deficiency because ZSC112L should be able to isomerize and metabolize glucose 1-phosphate derived from maltose, even if the glucose moiety derived from maltose cannot be phosphorylated and is therefore lost into the medium (for a review of the maltose metabolism, see Schwartz, 1987). When ZSC112L is transformed with a plasmid encoding the II'Mg subunit (or the mannose transporter), it remains growing on glucose as well as on maltose. It appears that II'Mg can phosphorylate glucose derived from maltose and thus complement the glucokinase defect. This particular phenotype of ZSC112L was exploited to select for III'Mg mutants.
that allowed growth on maltose but left the cells resistant against the nonmetabolizable, toxic glucose analog α-MG (Fig. 1). We presume that these mutants can phosphorylate and hence metabolize intracellular glucose but can no longer efficiently transport extracellular substrates. Knock-out mutations will be selected against because they cannot utilize maltose, and wild-type because they accumulate the toxic analog. In the presence of maltose and α-MG, mutants appeared as large colonies at a frequency of approximately 3 x 10^{-5} transformed cell. When spread on McConkey indicator plates containing 0.4% glucose, they did not ferment or ferment much less than did a wild-type control.

**Characterization of Mutants**—Sixty-two independent clones that formed large colonies were chosen for further analysis. They were characterized as follows. (i) Whole cell extracts were electrophoresed and blotted, and 11'''' was visualized with monoclonal antibodies. 11'''' was not detectable with a-MG analog that inhibits cell growth. (ii) With the membrane preparations with glucose as substrate. The activities varied between 2 and 150% of the wild-type control. Those with less than 10% activity were excluded from further analysis. (iii) Of 21 mutants that expressed normal amounts of IIICα, the in vivo transport activity was determined with α-MG as substrate. 12 mutants had 6% or less and 4 had 25% or more of the control activity. (iv) The 10 mutants with the highest activity ratio of phosphorylation/transport were sequenced. (v) Finally, transport and phosphorylation activities of the 10 selected mutants were measured with both glucose and α-MG as substrates. This appeared appropriate because Stock et al. (1982) found that α-MG and glucose have different affinities for IIICα in the two different assays. (Uptake: Kdp (D-Glc), 20 μM; Kdp (α-MG), 170 μM. Phosphorylation: Kdp (D-Glc), 6 μM; Kdp (α-MG), 10 μM.) We therefore had to exclude the possibility that some of the selected mutants were specificity mutants rather than transport mutants. Uptake of α-MG was measured at both 100 and 400 μM to confirm that the measured rates were concentration-independent (Vmax conditions). All other activity assays were performed in the presence of 400 μM substrate, which is above the Kdp of wild-type IIICα for both substrates.

The results are summarized in Table I and Fig. 2. With one exception, the residual transport activity for α-MG is less than 5% of the wild-type control. In contrast, the transport activity for D-glucose is reduced only to 20-40%. The phosphorylation activity is also reduced but less than uptake activity. The residual phosphorylation activity is between 20 and 70% for α-MG and between 40% and unchanged for D-glucose. The reduction of the transport activity relative to the phosphorylation activity is more pronounced for α-MG (ratio 0.05-0.2) than for D-glucose (ratio 0.2-0.8). All mutations are clustered in three short segments of the polypeptide chain. Met-17 was found mutagenized no less than three times. The second cluster includes 3 residues centered around residue 155. The third cluster is centered around residue 340. All mutations are in hydrophilic stretches within the hydrophobic domain of IIICα. With the exception of the apparently conservative M171 mutation, all other substitutions resulted in a change of charge (K150E, H339Y, and D343G) or at least a change in the potential to form hydrogen bonds (G149S, S157F, and M17T). The D343G mutation leads to a remarkable increase of mobility during gel electrophoresis in the presence of sodium dodecyl sulfate. Asp-343 might be located at the protein surface, and by its negative charge prevent binding. Similar mobility shifts after the loss of a negative charge have been observed with methyl-accepting proteins upon methylation at glutamyl residues (Hazelbauer and Harayama, 1983) and with phospho-IIIα after dephosphorylation (Erni, 1986).

**DISCUSSION**

Mutant forms of the glucose transporter with strongly reduced transport activity but almost intact phosphorylation activity could be isolated. All mutants were also slightly biased against α-MG, which was used for counterselection. A slight change of substrate specificity favoring glucose and disfavoring α-MG is thus superimposed on a pronounced impairment of transport relative to phosphorylation.

The clustering of the mutations within three narrowly defined polypeptide segments is of particular interest. Met-17 was found mutated three times. This residue is flanked on the left by a sequence with a strong helical hydrophobic moment (Erni and Zanolari, 1986). Similar N-terminal structures resembling mitochondrial targeting sequences are also present in other, albeit not all, PTS transporters (Saier et al., 1988; Saier and McCaldon, 1988). However, none of these PTS transporters contains a methionine in the analogous position. A segment of 20-25 residues with sequence similarity to residues 320-344 of the melibiose transporter follows Met-17 (Fig. 3). This sequence of the melibiose transporter overlaps with the segment, including Val-942, Val-345, and Ile-348, which has been proposed to be part of the melibiose-binding site (Botfield and Wilson, 1988). The three Met-17 substitutions are conservative, as are the substitutions observed in the melibiose transporter. It is possible that the segment around Met-17 and the sequence of the melibiose transporter are part of a general carbohydrate-binding site. Residues Gly-149, Lys-150, Ser-157, His-339, and Asp-343 of the glucose transporters are in two hydrophilic segments predicted to be exposed at the periplasmic face of the membrane, according to a model proposed by Erni (1989). The substitutions of these residues are not conservative with respect to charge and size. However, they neither affect the calculated hydropathy profile nor the folding and membrane insertion of the transporter. The region around amino acid 150 of IIICα is highly conserved (12 residues out of 18 are identical) in the related PTS transporter IIICα and in MalX (Peri and Waygood, 1988; Reidl and Boos, 1991). The glycyl residue is present in all three proteins; lysine and serine are in two. Asp-343 is completely conserved, whereas His-339 is not conserved. The latter 2
residues are in a segment of less sequence similarity to II^Glc
and MalX. The two peptide segments might be involved in a
process occurring in all three proteins, such as an isomeriza-
tion step necessary to translocate the bound substrate from
the periplasmic to the cytoplasmic side of the transporter. No
mutants with alterations in the segment including Cys-421
were detected. Although this cysteine is absolutely essential
for function of II^Glc and the β-glucoside transporter (Nuoffer
et al., 1988; Schnetz et al., 1990) and has been shown to be
transiently phosphorylated in II^Mtl (Pas and Robillard, 1988),
it does not appear to be part of the substrate-binding site.
This agrees with the observation that a hybrid protein con-
sisting of the glucose-binding domain of II^Glc and the Cys-
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catalytically essential cysteine may be in a loop that can close
down over the bound substrate.

A phenotypically similar mutant that retained 40% phos-
phorylation but only 3% transport activity was found in the
mannitol transporter (Manayan et al., 1988). It was ther-
mo-labile, had a 20-fold increased affinity for heat-stable phos-
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\[ \text{M177} \]

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\[ \text{C470T} \]

\[ \text{C1015T} \]

\[ \text{T50C} \]

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FIG. 2. Characterization of transport-deficient II^Glc mu-
nants that retained phosphorylation activity. Transport (dark
bars) and phosphorylation activities (open bars) are given in per-
cent of wild-type activities. The locations of the mutants within the
amino acid sequence are indicated in the hydropathy plot (Kyte and
Doolittle, 1982) of the II^Glc subunit. 100% transport activity was
15 ± 0.6 (2) 18.1 ± 4.5 (7) 51 104
1.0 ± 0.0 (2) <0.5 (2) 1 3
2.8 (1) 0.5 ± 0.1 (4) 40 47
2.8 (1) 0.8 ± 0.4 (4) 54 33
3.6 (1) 0.7 ± 0.1 (2) 48 45
6.0 (1) 0.6 ± 0.1 (3) 71 68
5.1 (1) 0.9 ± 0.4 (2) 22 25
2.2 (1) <0.5 (2) 23 20
2.9 (1) 0.8 ± 0.2 (3) 22 34

The data were determined as described under “Experimental Procedures.” Transport activity was measured
several times, as indicated in brackets, with different cell preparations on different days. Phosphorylation activity
was determined in duplicates from one single membrane preparation.

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\[ \text{u. Hummel and B. Erni, unpublished results.} \]

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cessible from both sides of the membrane would be compatible with the results obtained so far. Phosphorylation of the protein might lower the activation energy for isomerization of the binding site; phosphorylation of the sugar might decrease the affinity of the substrate and thus help to empty the binding site.

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