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Isolation and Characterization of a Mutation That Alters the Substrate Specificity of the *Escherichia coli* Glucose Permease

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We isolated 10 mannitol-positive mutants from a mannitol-negative *Escherichia coli* strain. These mutations mapped within *ptsG*, encoding the glucose permease (EII^{Glc}), and resulted in a G-320-to-V substitution that allows EII^{Glc} to transport mannitol. Gly-320 lies within a putative transmembrane helix of EII^{Glc} that may be involved in substrate recognition.

The permeases, or enzymes II (EIIs), of the bacterial phosphoenolpyruvate-dependent phosphotransferase system transport and phosphorylate their carbohydrate substrates, are chemoreceptors, and regulate other assimilation systems (12, 20). Although certain residues in the EIIs have been implicated in various activities of these proteins (5, 13, 18, 19, 22, 31), none has yet been shown to have a role in determining substrate specificity. Here, we report the isolation and characterization of such a mutation in the gene *ptsG*, encoding the *Escherichia coli* glucose permease.

Enzymes were purchased from United States Biochemical Corp. (Cleveland, Ohio), radiolabeled carbohydrates were from Dupont NEN Research Products (Boston, Mass.), and other reagents were from Sigma Chemical Corp. (St. Louis, Mo.). Contaminating glucose was removed from stock (100 mM) mannitol solutions by inoculation with cells from a 3-ml overnight Luria broth (LB) culture of *E. coli* LGS322 (Δ*mtlA* Δ*gut-recA*) (11) followed by incubation for 15 min at 37°C and filter sterilization of the suspension.

Cultures were grown in LB, M63 minimal salts medium (23), or minimal medium A (26) with the appropriate carbon source. For postmutagenic selection, cells were plated on M63 agar containing 0.01% glycerol and 1% mannitol. Fermentation was determined on MacConkey indicator plates containing 1% carbohydrate after incubation at 37°C for 24 h. Antibiotics were added to media at concentrations of 100 μg/ml for ampicillin, 34 μg/ml for chloramphenicol and 5 μg/ml for tetracycline. Membrane-free cell extracts were prepared as described previously (2).

For isolation of chromosomal mutants, cells from a 10-ml LB culture of *E. coli* LGS322 were harvested at exponential phase, washed twice with M63, resuspended in 1 ml of M63, and treated with ethyl methanesulfonate (100 mM). After 5 min at room temperature, 0.1-ml aliquots were spread onto minimal-mannitol plates. These were incubated at 37°C, and large colonies were screened for mannitol (Mtl) fermentation. Two Mtl^+ derivatives, LGS322-1a and LGS322-2a, were isolated. For plasmid-encoded mutants, strain LGS322 containing plasmid pTSG4 (8) ($ptsG^+$) was grown overnight in LB plus tetracycline, and 20 0.1-ml aliquots of this culture were put into 20 separate tubes each containing 5 ml of medium A. A 0.1-ml portion of diethylsulfate was added to each tube, and air was bubbled through the tubes at 37°C for 10 min. Then, a 0.1-ml aliquot was taken from each tube and inoculated into 3 ml of LB. After an overnight incubation (37°C), 0.1-ml aliquots from these tubes were plated onto minimal-mannitol plates, which were incubated at 37°C. Only one Mtl^+ colony from each original mutagenized culture was chosen for subsequent analysis, ensuring that the mutants arose independently. Plasmids isolated from cultures of these colonies were retransformed into strain LGS322. Transformants were then screened for mannitol and glucose (Glc) fermentation. Eight mutant plasmids, pBCP493, pBCP494, pBCP495, pBCP496, pBCP497, pBCP552, pBCP554, and pBCP555, that conferred an Mtl^+ phenotype on strain LGS322 and a Glc^+ phenotype on a Glc^- strain, ZSC112 (8), were isolated.

PCR amplification of chromosomal *ptsG* genes from wild-type and mutant *E. coli* strains was achieved with primers complementary to the first and last 20 nucleotides of *ptsG* plus noncomplementary tails incorporating recognition sequences for the restriction enzymes *Bam*HI and *Sal*I. A 30-cycle PCR (24) was carried out on 100-μl reaction mixtures containing 500 ng of genomic DNA (7) and 1 μM each primer. Products were purified on 1.2% low-melting-point agarose gels, digested with *Bam*HI and *Sal*I, and ligated to a similarly digested expression vector, pCQV2 (21). Plasmids were screened by size and restriction analysis, and three plasmids, pGSB5 (1a mutant), pGSB7 (wild type), and pGSB14 (2a mutant), were selected for further study.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the Laemmli (15) method. Immunoblotting of proteins was performed (28) with anti-EII^{Mtl} (16). Phosphoenolpyruvate-dependent phosphorylation assays on membrane fractions (14) and whole cells (32) were performed as described previously. Protein concentrations were determined by the method of Bradford (3). Plasmid DNA was sequenced by the Sequenase version 2.0 protocol (U.S. Biochemical Corp.) by using the dideoxynucleotide chain termination method (25).

Characterization of mannitol-positive mutants. Two Mtl^+ mutants, LGS322-1a and LGS322-2a, were isolated from *E. coli* LGS322, which lacks the mannitol permease because of a chromosomal deletion of the *mtlA* gene (11). No bands were observed in Western blots (immunoblots) of cell extracts from the mutant strains with antibody raised against EII^{Mtl}, indicating that the mutants were not expressing any protein antigenically related to EII^{Mtl}. Whole-cell mannitol phosphorylation assays showed that this activity in the mutants was dependent on phosphoenolpyruvate, not ATP (data not shown), and thus might be due to a mutation or mutations in a phosphotrans-

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TABLE 1. Kinetic constants for α-MG and mannitol phosphorylation

Strain[a]	α-MG phosphorylation			Mtl phosphorylation	
	K_m (μM)	V_{max} (nmol/min/mg)	K_i (Mtl)[b] (mM)	K_m (mM)	V_{max} (nmol/min/mg)
LGS322	9	1.2	32	5	0.15
LGS322-1a	14	0.8	14	6	0.45

[a] Kinetic constants were determined with membrane preparations from the parental Mtl$^-$ strain LGS322 and the Mtl$^+$ mutant strain LGS322-1a.

[b] K_i values are for mannitol inhibition of α-MG phosphorylation and were calculated assuming competitive inhibition.

ferase system permease. Whole-cell phosphorylation assays on cultures of these mutants grown on LB medium and 1% inducing sugar with a variety of phosphotransferase system substrates showed a lower activity only toward glucose and methyl-α-glucoside (α-MG), suggesting that the mutations may have affected the glucose permease (data not shown).

Mapping of the chromosomal mutations. P1-mediated transduction (1) of a wild-type allele of *ptsG* with a linked Tn*10* insertion from strain CAG12078 (27) into the mutant strains LGS322-1a and LGS322-2a gave rise to predominantly Mtl$^-$ colonies, suggesting that the Mtl$^+$ phenotypes were due to one or more mutations in *ptsG*. Plasmids expressing the amplified and subcloned *ptsG* genes from the two chromosomal mutants and their wild-type parent were transformed into strain ZSC112, and all three conferred a Glc$^+$ phenotype on this strain. Transformation of these plasmids into strain LGS322 gave an Mtl$^+$ phenotype with plasmids carrying *ptsG* genes from either of the mutants (pGSB5 and pGSB14) but not with that carrying the wild-type gene (pGSB7), confirming that the mutation(s) was in *ptsG*.

Kinetic constants of the mutant glucose permeases. Kinetic values were determined for α-MG and mannitol phosphorylation with membranes from LGS322 and one of the Mtl$^+$ mutant strains, LGS322-1a (Table 1). For α-MG phosphorylation, the V_{max} of the mutant is slightly lower than that of the wild-type while the K_m for α-MG is slightly higher, but both are within the range previously reported for the wild-type glucose permease (10, 29). For mannitol phosphorylation, the mutant had a threefold-higher V_{max} but the K_m was similar to that of membranes from the wild-type strain. These results suggest that the ability of this mutant to grow on mannitol is due to the higher V_{max} of mannitol phosphorylation in the mutant than in the wild-type. Moreover, because overexpression of wild-type *ptsG* (strain LGS322/pGSB7) did not result in an Mtl$^+$ phenotype and we could not detect a significant difference between the mannitol phosphorylation rate of this strain and that of strain LGS322 (data not shown), the mannitol-phosphorylating activity detected in strain LGS322 (Table 1) is apparently not due to the wild-type glucose permease. Thus, the V_{max} for mannitol phosphorylation by the mutant glucose permease relative to that for phosphorylation by the wild-type protein is likely to be much greater than the threefold difference shown in Table 1. To determine whether the wild-type glucose permease could bind mannitol at its active site, α-MG phosphorylation assays were carried out with 20 μM [^{14}C]α-MG in the presence of various concentrations (0 to 75 mM) of glucose-depleted, unlabelled mannitol in the reaction mixture. From these experiments we found a K_i for mannitol of 14 mM for the mutant compared with 32 mM for the wild-type protein, assuming competitive inhibition (Table 1).

Sequence analysis. *ptsG* genes from the chromosomal mutants and the wild-type parent were completely sequenced. In both mutants, the only difference from the wild-type was a G→T substitution at the second position of codon 320, giving rise to a Gly-320→Val (G320V) change in the permease. The eight plasmid-derived mutants were partially sequenced in the region of codon 320, and all were found to have the G320V mutation as well.

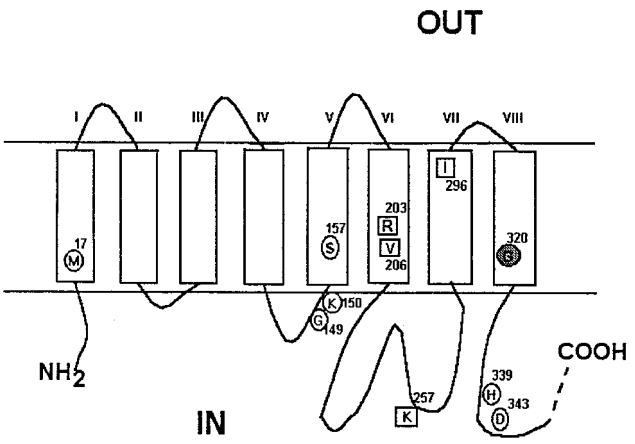

FIG. 1. Locations of mutations in a topological model (6) of the *E. coli* glucose permease. Boxed residues are altered in mutants which carry out facilitated diffusion (22), residues in ovals are altered in mutants which can phosphorylate but translocate very poorly (5), and the circled and shaded glycine is the residue which is changed in the substrate specificity mutants described herein.

Role of Gly-320 in determining substrate specificity. We isolated 10 independent mutants which had a glucose permease that could transport and phosphorylate D-mannitol. In each, a G320V substitution in EII^{Glc} was found. Gly-320 is predicted to lie in a transmembrane portion of the protein (6). Although there is no clear clustering of this mutation and the phosphorylation and transport mutations which have been reported for this protein, in all but one case these mutations lie within the four C-terminal membrane-spanning regions, or within cytoplasmic loops associated with them (Fig. 1).

Gly-320 could reside either in the active site of the protein or in a remote region whose structure affects residues in the active site. Because even single conservative amino acid changes can alter the conformation (9) or completely disrupt the oligomeric form (17) of membrane proteins, an indirect effect is certainly possible. In any case, because the kinetic values for α-MG phosphorylation are not greatly affected in the G320V mutant and mannitol can bind to the wild-type glucose permease nearly as well as to the G320V mutant (Table 1), it is unlikely that the substrate-binding and phosphorylation site has undergone any drastic conformational changes in the mutant protein.

Although strain LGS322, the parent strain of the mutants, can phosphorylate mannitol with a K_m for this substrate similar to that for the mutants but with a lower V_{max} (Table 1), it cannot grow on mannitol. Apparently the threefold increase in V_{max} is sufficient for growth of the mutants. We do not know the source of this activity in strain LGS322, but it is probably not due to the wild-type glucose permease as mentioned earlier. Thus, wild-type EII^{Glc} can bind mannitol, but it cannot phosphorylate it efficiently. Therefore, the major effect of the G320V mutation is to affect the phosphorylation rate of mannitol rather than its recognition by the protein, perhaps by allowing this substrate to assume a more favorable orientation for phosphorylation within the binding site.

wild-type EII^{Glc}	317-I L A G L A F-323
mutant EII^{Glc}	317-I L A **V** L A F-323
wild-type EII^{Mtl}	300-I L A V L A M-306
wild-type EII^{Mtl}	141-I L A I L A F-147

FIG. 2. Alignment of deduced amino acid sequences of *E. coli* glucose (EII^{Glc}) and mannitol (EII^{Mtl}) permeases. The underlined residue in bold is the mutated residue in EII^{Glc} that results in an Mtl^+ phenotype.

Interestingly, two small regions in EII^{Mtl} are very similar in sequence to the stretch of amino acids surrounding the EII^{Glc} mutation. In one, the residue equivalent to Gly-320 is a valine, as in the mutation that confers mannitol specificity on EII^{Glc}, while in the other, the residue equivalent to Gly-320 is an isoleucine (Fig. 2). The latter sequence is predicted to be within the membrane, while the valine-containing sequence is predicted to be in a periplasmic loop between the fifth and sixth transmembrane regions in EII^{Mtl} (30). Whether either of these sequences participates in mannitol recognition by EII^{Mtl} remains to be determined. However, deletion of as little as the last transmembrane region of EII^{Mtl} results in a total loss of mannitol binding (4). Thus, as in EII^{Glc}, the C-terminal end of the transmembrane domain of EII^{Mtl} appears to play a role in substrate recognition.

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