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A Conserved Glutamate Residue, Glu-257, Is Important for Substrate Binding and Transport by the *Escherichia coli* Mannitol Permease

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The mannitol permease, or D-mannitol-specific enzyme II of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) of *Escherichia coli*, both transports and phosphorylates its substrate. Previous analyses of the amino acid sequences of PTS permeases specific for various carbohydrates in different species of bacteria revealed several regions of similarity. The most highly conserved region includes a GIXE motif, in which the glutamate residue is completely conserved among the permeases that contain this motif. The corresponding residue in the *E. coli* mannitol permease is Glu-257, which is located in a large putative cytoplasmic loop of the transmembrane domain of the protein. We used site-directed mutagenesis to investigate the role of Glu-257. The properties of proteins with mutations at position 257 suggest that a carboxylate side chain at this position is essential for mannitol binding. E257A and E257Q mutant proteins did not bind mannitol detectably, while the E257D mutant could still bind this substrate. Kinetic studies with the E257D mutant protein also showed that a glutamate residue at position 257 of this permease is specifically required for efficient mannitol transport. While the E257D permease phosphorylated mannitol with kinetic parameters similar to those of the wild-type protein, the V_{max} for mannitol uptake by this mutant protein is less than 5% that of the wild type. These results suggest that Glu-257 of the mannitol permease and the corresponding glutamate residues of other PTS permeases play important roles both in binding the substrate and in transporting it through the membrane.

The phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) carries out the phosphorylation and transport of many carbohydrates in a variety of bacterial species (reviewed in references 16, 23, and 27). The phosphotransfer and transport steps carried out by most PTSs are summarized as follows:

$$\text{PEP} + \text{enzyme I (EI)} \leftrightarrow \text{P-EI} + \text{pyruvate}$$

$$\text{P-EI} + \text{HPr} \leftrightarrow \text{P-HPr} + \text{EI}$$

$$\text{P-HPr} + \text{EIIA (domain or protein)} \leftrightarrow \text{P-EIIA} + \text{HPr}$$

$$\text{P-EIIA} + \text{EIIB (domain or protein)} \leftrightarrow \text{P-EIIB} + \text{EIIA}$$

$$\text{P-EIIB} + \text{carbohydrate}_{(\text{out})} \xrightarrow{\text{EIIC}} \text{EIIB} + \text{carbohydrate-P}_{(\text{in})}$$

EI and HPr are general PTS phospho-carrier proteins located in the cytoplasm. The membrane-bound enzyme II (EII) complexes are carbohydrate specific and have at least three structural and functional domains, named EIIA, EIIB, and EIIC (29). These domains may be in a single polypeptide, as in the mannitol permease, or in two proteins (e.g., the separate $EIICB^{Glc}$ and $EIIA^{Glc}$ components of the glucose permease), or they may each be a separate protein, as in the cellobiose permease (for reviews, see references 9, 10, and 27).

As depicted above, there are two phospho intermediates in the transfer of the phospho group from P-HPr to the carbohydrate. The EIIA domain is the acceptor from P-HPr. The EIIB domain accepts the phospho group from EIIA and is the phospho donor to the carbohydrate. In EII^{Mtl} (EII specific for mannitol), His-554 is the phosphorylated residue in the EIIA domain (24), and that in the EIIB domain is Cys-384 (24, 26). Both of these residues have been shown to be essential for transport and phosphorylation by the mannitol permease (40, 41). Mannitol transport and phosphorylation are distinct steps, and transport without phosphorylation by EII^{Mtl} has been observed (18). However, phosphorylation of the permease by P-HPr greatly increases the rate of mannitol translocation 6, 18, 19). The evidence suggests that the translocation step is mediated by the cycling of the permease through at least three distinct conformations (18).

EII^{Mtl} consists of a single polypeptide (8, 13) which contains all three domains (38, 39, 43). The C-terminal half of the mannitol permease, which contains the EIIA and EIIB domains, is hydrophilic and located in the cytoplasm of the cell (35). EIIC of the mannitol permease is membrane bound (7, 35), and is believed to traverse the membrane at least six times as α-helical segments (37). It is responsible for both mannitol binding (7) and translocation (6, 17).

EII^{Mtl} is most likely dimeric in the membrane (reviewed in references 9 and 27), and intersubunit transfer between His-554 on one subunit and Cys-384 on the other has been demonstrated as one possible pathway of the phospho group through the protein (40, 41). Moreover, the permease dimer has been shown to contain one high-affinity and one low-affinity binding site for mannitol (25). Therefore, each subunit within a dimer may contain a mannitol binding site, and these binding sites may interact in a negatively cooperative fashion.

Several amino acid residues in a large putative cytoplasmic loop of the mannitol permease between transmembrane helices 4 and 5 have been shown to be important for mannitol binding and transport (see references 10 and 27 for reviews).

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
E. coli LGS322	F$^-$ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50* Δ(*mtlA'p*) *mtlD*(Con) Δ(*gutR'MDBAp-recA*)	7
S. typhimurium LJ144	*cpd-401 cysA1150* (F′198)(*pts*$^+$ *on cysA*$^+$)	1
E. coli TG1	*supE hsd*Δ*5thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lac*^{q} *lacZ* Δ*M15*)	32
Plasmids		
pGJ9	Cm^r; *mtlA* on pACYC184	7
pBQ11	Amp^r; *mtlA* on pBR322	41
pBH195A (pAQW6)	H195A on pBR322	41
pBC384H (pAQW7)	C384H on pBR322	41
pBH554A (pAQW4)	H554A on pBR322	41
M13*mtlA*	*mtlA* on M13mp19	This study
pGE257A	E257A mutation on pGJ9	This study
pGE257Q	E257Q mutation on pGJ9	This study
pGE257D	E257D mutation on pGJ9	This study
pGH195N	H195N mutation on pGJ9	42

TABLE 2. Oligonucleotide sequences used for mutagenesis

Mutation[a]	Oligonucleotide[b]	Change of: Codon	Change of: Amino acid
E257A	AGTAGATTGCTGGATAC	GAA→GCA	Glu-257→Ala
E257Q[c]	AGTAGATTTGGTGGATAC GGTATCCACCAAATCTAC	GAA→CAA	Glu-257→Gln
E257D[c]	AGTAGATATCGTGGATAC TATCCACGATATCTACTT	GAA→GAU	Glu-257→Asp
F1[d]	CCCGTCGACTGGACAGTTAA	None	
F2[d]	TTTTACGTACGTGGCTCAGG	None	

[a] The notation E257A, for example, denotes the resultant mutant protein in which a glutamate residue was replaced with an alanine residue at position 257.
[b] The deoxyoligonucleotides are written in the 5′-to-3′ direction. The mutated codons are underlined.
[c] These mutations were constructed by overlap PCR, which requires two mutagenic oligonucleotides, complementary to the opposite strands of the plasmid.
[d] F1 encompassed bases −140 to −120, relative to the start of translation, and was complementary to the region including the *Sal*I restriction site located upstream of the 5′ end of *mtlA*. F2 encompassed bases 1115 to 1135 and was complementary to the region containing a unique *Spl*I restriction site within *mtlA*.

Also in this region is a conserved G-I-X-E (Gly-Ile-X-Glu) sequence that is found in PTS permeases having different substrate specificities in a variety of bacteria (15, 30). In this study, we have used site-directed mutagenesis to investigate the role of Glu-257 of the mannitol permease, the most conserved residue in this motif. Our results show that Glu-257 plays an important role in mannitol binding and transport by this protein.

MATERIALS AND METHODS

Chemicals and enzymes. D-[^{14}C]mannitol (54.5 mCi/mmol), D-[^{3}H]mannitol (22.5 mCi/mmol), and [^{3}H]glycerol (40 mCi/mmol) were purchased from DuPont-New England Nuclear (Boston, Mass.). Restriction enzymes and Vent polymerase were obtained from New England Biolabs (Beverly, Mass.). T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase (Klenow fragment), and DNA sequencing reagents were from U.S. Biochemical Corp. (Cleveland, Ohio). All enzymic and sequencing reactions were carried out as recommended by the supplier. Oligonucleotides for mutagenesis and sequencing were synthesized at the Boston University DNA synthesis facility on a Milligen DNA synthesizer (Bedford, Mass.) or by New England Biolabs. A cytoplasmic fraction from *Salmonella typhimurium*, strain LJ144 (see Table 1), was prepared and used as a source of EI and HPr as previously described (1). Goat anti-rabbit immunoglobulin G-alkaline phosphatase and all other chemicals, which were reagent grade, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed and described in Table 1. Plasmid DNA was isolated by the alkaline lysis method (32) and transformed into competent cells prepared with $CaCl_2$ (22).

Growth of bacteriophage and isolation of single-stranded DNA. Liquid cultures of *Escherichia coli* TG1 cells were grown overnight in M63 medium (31) containing 50 μg of thiamine per ml and 1% glucose. A 50-μl aliquot was mixed with 3 ml of 2× YT medium (1.6% tryptone, 1% yeast extract [both from Difco, Detroit, Mich.], 0.5% NaCl) and one plaque from M13*mtlA* phage (see below). Cultures were grown for 4 h at 37°C with shaking. Single-stranded DNA was prepared as described by Sambrook et al. (32).

Construction of mutants. The E257A mutant was constructed by the method of Sayers and Eckstein (33). The *mtlA* gene (encoding the permease) was subcloned from plasmid pGJ9 (7) into bacteriophage M13mp19 by using the *Sal*I and *Bam*HI restriction sites flanking *mtlA*, to create M13*mtlA*. This plasmid was then transformed into *E. coli* TG1 cells (Table 1), and single-stranded DNA was isolated and used as a template for mutagenesis. The sequence of the mutagenic oligonucleotide complementary to the coding strand is given in Table 2. Clones carrying the appropriate mutation were identified by DNA sequencing with the Sequenase version 2.0 kit (U.S. Biochemical Corp.). The *Sal*I-*Spl*I fragment of the *mtlA* gene (sites located at bp −140 and 1,125 from the start of translation, respectively) was excised from the replicative form of mutant M13*mtlA* and subcloned into pGJ9 from which the corresponding wild-type portion of the gene had been removed.

To construct the E257Q and E257D mutations, overlap PCR mutagenesis with Vent polymerase was performed as described by Deng and Nickoloff (5). PCR was performed in a PTC-100 thermocycler from MJ Research (Watertown, Mass.). Plasmid pGJ9 was used as the template in two different reactions. In the first reaction, the first 900 bp of the *mtlA* gene were amplified by using an oligonucleotide complementary to the sequence flanking a unique *Sal*I site upstream of the 5′ end of the gene (7) and a mutagenic oligonucleotide designed to introduce the desired mutation at codon 257. Next, an overlapping fragment comprising about 400 bp of the gene was amplified by using an oligonucleotide complementary to the sequence flanking a unique *Spl*I restriction site that overlaps codons 374 and 375 and a second mutagenic oligonucleotide complementary to the sequence that includes codon 257 (Table 2). These two PCR products overlapped at the sequence surrounding codon 257 and were used together as the templates in a third amplification reaction with the two oligonucleotides complementary to the *Sal*I and *Spl*I restriction sites. The approximately 1,300-bp PCR product from this reaction was digested with *Sal*I and *Spl*I enzymes, subjected to agarose gel electrophoresis, and purified with a GeneClean kit (Bio-101, La Jolla, Calif.). It was then used to replace the corresponding wild-type portion of the gene in pGJ9 as described above. In the construction of the E257D mutant, an *Eco*RV restriction site was introduced into the mutated codon to facilitate screening for the desired mutation. Potential E257D mutants were screened for the desired mutation by restriction analysis with *Eco*RV, followed by DNA sequencing. E257Q mutants were identified by DNA sequencing to verify the presence of the appropriate mutation.

The plasmids containing the mutated *mtlA* genes (pGE257A, pGE257Q, and pGE257D) (Table 1) were individually transformed into *E. coli* LGS322 cells (Δ*mtlA* [7]) for functional analyses of the mutant proteins. For each of the mutants, the entire fragment cloned into plasmid pGJ9 was subjected to DNA sequencing to verify that other undesired mutations had not been inadvertently created.

In vivo complementation between mutant mannitol permeases. Amp^r plasmids pBH195A, pBC384H, and pBH554A (originally named pAQW6, pAQW7, and pAQW4, respectively 41, 42) (Table 1) were individually transformed into LGS322 cells (Table 1) harboring Cm^r plasmid pGE257A. Transformants were selected for both Amp and Cm resistances. The presence of both plasmids in each doubly transformed strain was verified by agarose gel electrophoresis and restriction analyses.

Growth of cells and preparation of membrane vesicles. Bacteria were routinely grown in liquid culture at 37°C on Luria-Bertani medium (1% tryptone, 0.5% yeast extract [both from Difco], 1% NaCl)] containing 30 μg of chloramphenicol per ml and/or 50 μg of ampicillin per ml, if necessary, for selection. The medium also contained 1% mannitol in all cases in which the cells were used for immunoblots or transport, phosphorylation, or binding assays. Mannitol fermentation was assessed by growing LGS322 cells harboring various plasmids on MacConkey indicator plates containing 1% mannitol and chloramphenicol for 24 h at 37°C.

Membrane vesicles were prepared from *E. coli* LGS322 cells harboring mutant *mtlA* plasmids as described by Grisafi et al. (7). The protein concentration of these vesicles was estimated by the Bradford method (2), using bovine serum albumin as the standard.

Protein electrophoresis and immunoblotting. The expression of wild-type and mutant proteins in membrane vesicles was estimated after electrophoresis on sodium dodecyl sulfate–10% polyacrylamide gels (12) by immunoblotting as described by Stephan and Jacobson (35, 36). Goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Chemical Co.) was used to detect binding of anti-EII^{Mtl}. Prestained, transferable molecular weight standards were obtained from Novex (San Diego, Calif.).

Mannitol binding, phosphorylation, and uptake assays. Low-affinity binding of [^{3}H]mannitol to membrane vesicles was performed as described by Grisafi et al. (7) at 1, 2, 5, and 10 μM mannitol. Parallel assays were performed with [^{3}H]glycerol to correct for nonspecific binding. High-affinity mannitol binding assays were performed at 50 to 500 nM [^{14}C]mannitol according to the procedure of Pas et al. (25), as modified by Weng and Jacobson (42). Dissociation constants were estimated by the method of Scatchard (34).

PEP-dependent mannitol phosphorylation was measured with everted membrane vesicles or permeabilized whole cells as previously described (41–43).

Uptake of [^{14}C]mannitol was measured in whole cells, which were grown to mid-exponential phase (optical density at 550 nm = 0.6 to 0.8) in Luria-Bertani medium containing 1% mannitol, washed twice, and resuspended to the original volume in M63 medium. Uptake was initiated by the addition of various concentrations of [^{14}C]mannitol to this suspension, which was then incubated at room temperature. Aliquots were taken at various times and were filtered on 0.4-μm-pore-size nitrocellulose filters, which were then washed twice with cold M63 medium. The amount of radiolabeled mannitol taken up was determined by counting the air-dried filters in a liquid scintillation counter.

RESULTS

Construction and expression of mutant *mtlA* genes. E257A, E257Q, and E257D mutants of *mtlA* were constructed as described in Materials and Methods and in Table 2, and these plasmids were then transformed into *E. coli* LGS322, which carries a deletion in the *mtlA* gene, for analysis of the properties of the mutant proteins.

To determine whether the three mutant proteins were expressed and inserted into the membrane, membrane vesicles were prepared, extracted in sample buffer containing sodium dodecyl sulfate at 100°C, and subjected to polyacrylamide gel electrophoresis. An immunoblot of a representative gel is shown in Fig. 1A. All three mutant proteins were observed at approximately the same apparent molecular mass (65 kDa) and in amounts comparable to that of the wild-type protein (Fig. 1A, lanes 2 to 5). However, the bands for the E257A and the E257Q proteins exhibited a considerable degree of heterogeneity, while the wild-type mannitol permease exhibited a somewhat smaller amount of heterogeneity, which was not observed at all with the E257D mutant permease. These apparently smaller forms of the wild-type and mutant mannitol permeases may be the result of partial proteolysis within the cell, as similar results were observed in immunoblots performed with whole cells (data not shown).

In addition, the monomer forms of both the E257A and E257Q mutant mannitol permeases had a slightly higher electrophoretic mobility than either the wild-type or the E257D permease (Fig. 1A). This could be attributed to the loss of a negative charge in the E257A and E257Q permeases. Indeed, lower mobilities in polyacrylamide gels have been reported for proteins in which a negatively charged residue was inserted in place of an uncharged residue (4).

Previous work has shown that the mannitol permease forms oligomers in the membrane which are at least partially stable when the membranes are extracted in sodium dodecyl sulfate at 30°C (3, 36). Evidence also suggests that PEP-dependent mannitol phosphorylation is catalyzed optimally by a permease oligomer, probably a dimer (reviewed in reference 9). To examine the ability of each of the mutant proteins to form oligomers, membrane vesicles were incubated at 30°C in sodium dodecyl sulfate sample buffer as described by Stephan and Jacobson (36), and these extracts were also subjected to polyacrylamide gel electrophoresis. Each of the three mutant proteins was able to form oligomers in a proportion comparable to that of the wild-type protein when incubated under these conditions, and little heterogeneity was observed for any of the bands corresponding to the apparent dimers (Fig. 1B, lanes 2 to 5).

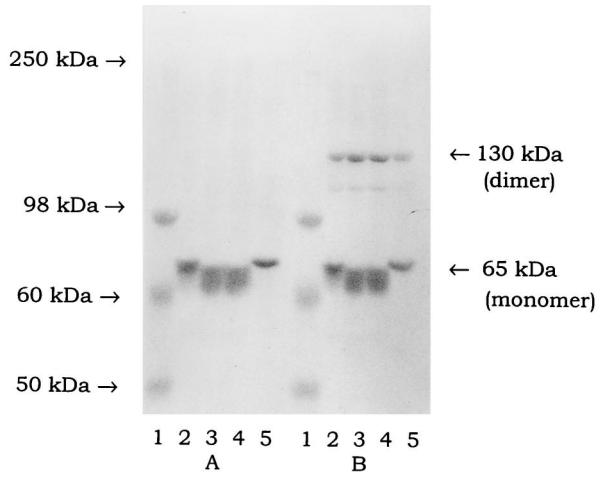

FIG. 1. Immunoblot analysis of membranes containing the wild-type or mutant mannitol permeases. Membrane proteins (25 μg/lane) were extracted in sample buffer containing sodium dodecyl sulfate at either 100 or 30°C, and the samples were separated by polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and blotted with anti-EII^{Mtl} as described in Materials and Methods. (A) Extraction at 100°C. Lane 1, prestained, transferable molecular mass standards (from top to bottom: myosin, 250 kDa; bovine serum albumin, 98 kDa; glutamate dehydrogenase, 60 kDa; alcohol dehydrogenase, 50 kDa); lane 2, membranes containing wild-type mannitol permease (pGJ9); lane 3, membranes containing the mutant E257A permease (pGE257A); lane 4, membranes containing the mutant E257Q permease (pGE257Q); lane 5, membranes containing the mutant E257D permease (pGE257D). (B) Extraction at 30°C. Lane 1, molecular mass standards as in panel A; lane 2, membranes containing wild-type mannitol permease (pGJ9); lane 3, membranes containing the mutant E257A permease (pGE257A); lane 4, membranes containing the mutant E257Q permease (pGE257Q); lane 5, membranes containing the mutant E257D permease (pGE257D).

Phenotypes of cells expressing Glu-257 mutant proteins. *E. coli* LGS322 cells harboring mutant plasmids were grown on MacConkey-mannitol indicator plates at 37°C as a qualitative assay of EII^{Mtl} activity, as described in Materials and Methods. Colonies expressing wild-type EII^{Mtl} were red, while those expressing E257D mutant protein were pink, indicating inefficient transport or metabolism of mannitol. Cells expressing E257A or E257Q mutant proteins produced white colonies, which suggested that these mutant permeases were defective in mannitol transport and/or phosphorylation.

Mannitol binding activity. To determine whether the role of Glu-257 could involve the binding of mannitol, we measured mannitol binding to membrane vesicles containing the wild-type permease or each of the three mutant proteins as described in Materials and Methods. The results are shown as a Scatchard plot in Fig. 2. No binding of mannitol to either the E257A or the E257Q mutant protein was detected at concentrations up to 1 mM (also see reference 10). The E257D mutant, however, did bind mannitol at least at the low-affinity site (Fig. 2). As shown in Table 3, the K_d value obtained for mannitol binding to this site of the E257D mutant was 11 μM, approximately twice that obtained for the wild-type permease (K_d = 5.2 μM). However, high-affinity binding of mannitol ($K_d \cong 40$ nM for the wild-type protein) was not detectable in the E257D mutant (data not shown).

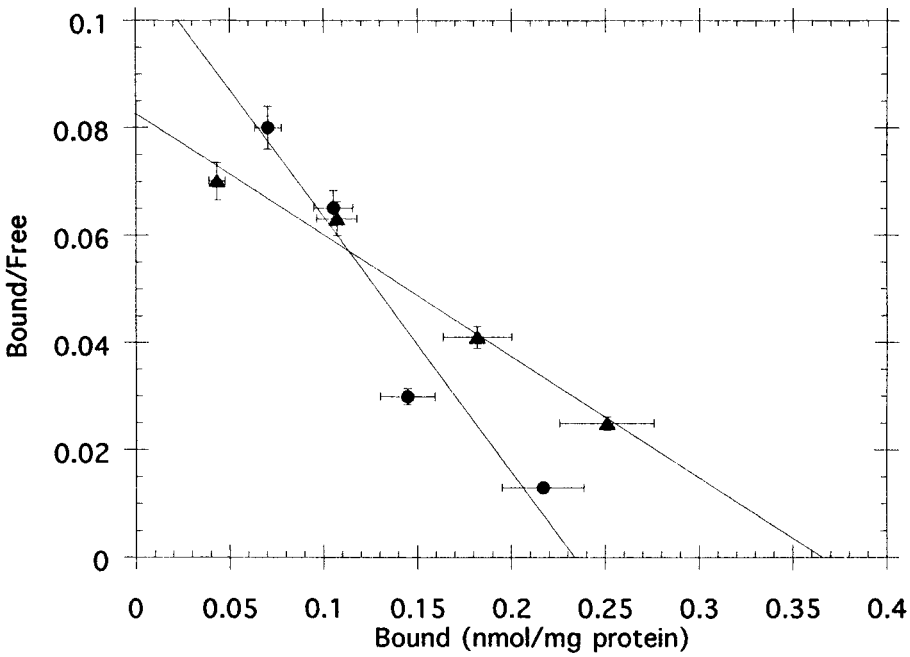

FIG. 2. Scatchard plot of mannitol binding to the mannitol permease and the E257D mutant. Error bars represent the standard deviation from the amount of mannitol bound averaged from five assays at each concentration. Membranes from strain LGS322 (Δ*mtlA*) expressing either wild-type protein (●) or the E257D mutant (▲) were assayed for mannitol binding at 1, 2, 5, and 10 μM [^{3}H]mannitol as described in Materials and Methods. Lines were determined by least-squares analyses.

Surprisingly, the stoichiometry of mannitol binding was somewhat higher in membranes from cells expressing the E257D mutant (0.37 nmol/mg of membrane protein) compared to that in membranes containing the wild-type protein (0.23 nmol/mg of membrane protein) (Fig. 2). One possible explanation could be that this mutation at position 257 increases the stability of the permease protein in the membrane compared to that of the wild-type protein. Indeed, we did routinely observe somewhat higher amounts and less heterogeneity of the E257D protein in the membrane compared to the wild-type protein (Fig. 1). These results also suggest that the high-affinity binding site in the E257D mutant may have been converted into a low-affinity binding site, since the binding stoichiometry at high mannitol concentrations (Fig. 2) is the sum of both high- and low-affinity binding stoichiometries.

TABLE 3. Dissociation constants and kinetic values of wild-type and mutant mannitol permeases

Permease (plasmid)	Mannitol fermentation[a]	Mannitol binding		PEP-dependent phosphorylation[d]		Mannitol uptake[e]	
		K_d[b] (μM)	K_d[c] (nM)	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Wild-type (pGJ9)	Red	5.2	60	17	44	33	550
E257D (pGE257D)	Pink	11	ND	25	30	100	15
H195N[f] (pGH195N)	Red	2.7	ND	31	25	56	67

[a] Mannitol fermentation phenotype was determined as described in Materials and Methods.

[b] Low-affinity mannitol binding measured at 1, 2, 5, and 10 μM [^{3}H]mannitol as described by Grisafi et al. (7). The stoichiometry of mannitol binding at saturation for the wild-type permease was 0.25 nmol/mg of protein, and for E257D it was 0.37 nmol/mg of protein.

[c] High-affinity mannitol binding. ND, not detectable.

[d] Measured at 10, 25, 50, and 100 μM [^{14}C]mannitol as described by White and Jacobson (43). V_{max} units are nanomoles of mannitol 1-P formed per minute per milligram of protein.

[e] Measured at 5, 10, and 25 μM [^{14}C]mannitol for the wild type; 100, 250, and 500 μM for the E257D permease; and 10, 25, and 50 μM for the H195N permease, as described in Materials and Methods. V_{max} units are nanomoles of mannitol taken up per minute per milligram of protein.

[f] Data for mannitol binding and PEP-dependent mannitol phosphorylation by the H195N mutant are from Weng and Jacobson (42).

PEP-dependent mannitol phosphorylation. Everted membrane vesicles derived from cells expressing the different mutant proteins were used to measure PEP-dependent mannitol phosphorylation. The results are shown in Fig. 3, and the derived kinetic values are presented in Table 3. Membranes from mutants E257A and E257Q exhibited only background levels of mannitol phosphorylation (i.e., that exhibited by strain LGS322 containing no plasmids) at concentrations up to 1 mM mannitol. This result was expected, because neither of these mutant permeases could detectably bind mannitol. Replacing Glu-257 with an aspartate residue resulted in a permease which was able to phosphorylate mannitol nearly as well as the wild-type protein. Only minor differences in the K_m and V_{max} values for the two proteins were found (Table 3).

Mannitol uptake. As shown in Fig. 4, LGS322 cells expressing either the E257A or E257Q mutant protein exhibited no detectable mannitol uptake at concentrations up to 1 mM. At 10 μM mannitol, uptake by the E257D mutant protein was detectable, but very low, compared to that of the wild-type protein. Kinetic analyses of mannitol uptake revealed that the E257D mutant protein exhibited a K_m value that was three times higher but a V_{max} value that was less than 5% of the value for the wild-type protein (Table 3). Thus, although replacing Glu-257 with an aspartate residue did not greatly affect mannitol binding or phosphorylation, this mutation drastically impaired mannitol uptake. It was previously found (42) that an H195N mutant mannitol permease, like the E257D mutant permease, was incapable of high-affinity mannitol binding; therefore, the kinetic values for mannitol uptake by the H195N permease were also measured. The V_{max} for mannitol uptake

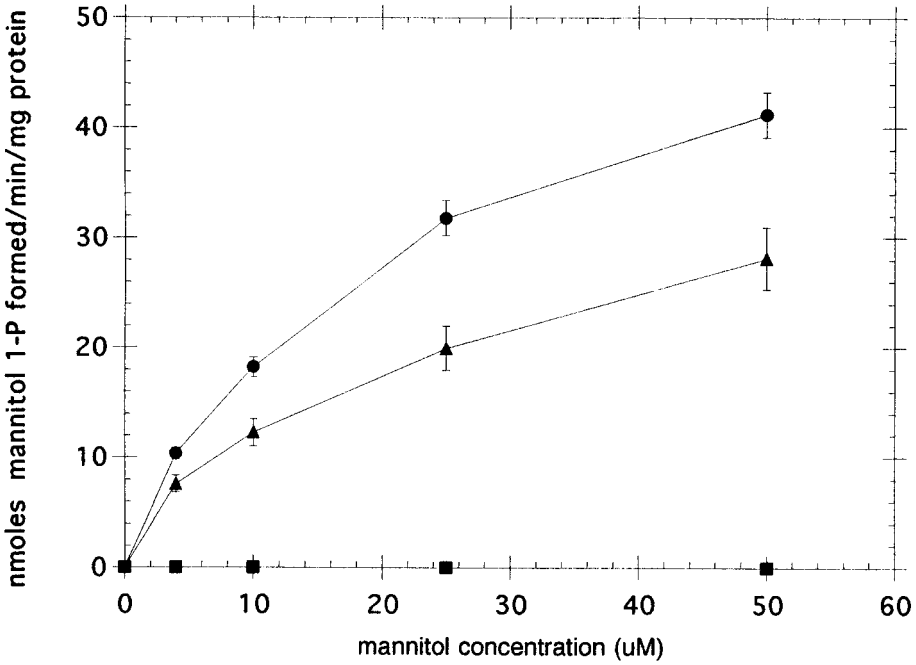

FIG. 3. PEP-dependent mannitol phosphorylation activities of wild-type and mutant permeases. PEP-dependent phosphorylation in membrane vesicles was measured as described in Materials and Methods at 100 μM [^{14}C]mannitol. The standard deviation in each case from the average of four separate assays is represented by error bars. ●, membranes containing the wild-type permease (pGJ9); ▲, membranes containing the E257D mutant permease (pGE257D); ■, membranes containing either the E257A or E257Q mutant permease (pGE257A or pGE257Q, respectively).

by the H195N permease was only about 12% that of the wild-type, while the K_m for mannitol uptake by this mutant permease was 70% higher than that of the wild-type protein (Table 3).

In vivo complementation of mannitol permease mutant proteins. Previous work in our laboratory has shown that various inactive mannitol permease mutants can complement one another when coexpressed in the same cell, presumably through

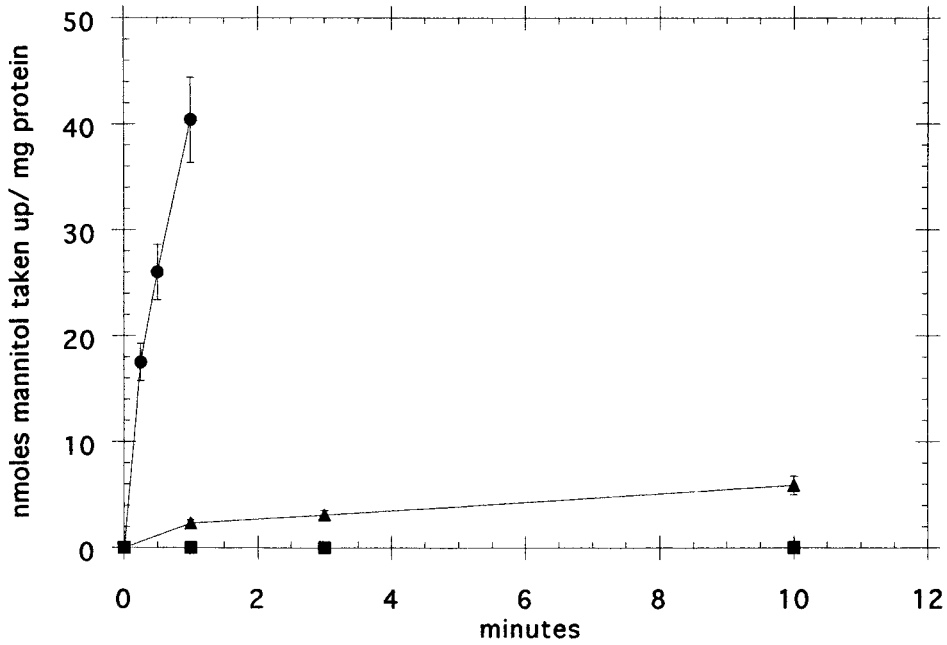

FIG. 4. Mannitol uptake in LGS322 cells. [^{14}C]mannitol uptake was measured at 10 μM in whole cells as described in Materials and Methods. The standard deviation in each case from the average of four separate assays is represented by error bars. ●, cells expressing the wild-type permease (pGJ9); ▲, cells expressing the E257D mutant permease (pGE257D); ■, cells expressing either the E257A or the E257Q mutant permease (pGE257A or pGE257Q, respectively).

TABLE 4. In vivo complementation of mannitol permease mutants

Plasmid(s)	Phenotype[a]	Mannitol phosphorylation[b]	
		K_m (μM)	V_{max}
pBQ11 (wild-type)	Red	25	225
pGE257A/pBC384H	Red	40	75
pGE257A/pBH554A	Red	40	175
pGE257A/pBH195A	Pink	35	50

[a] LGS322 cells harboring the various plasmids were grown on MacConkey-mannitol plates for 24 h at 37°C.

[b] Measured at 10, 25, 50, and 100 μM mannitol in permeabilized LGS322 cells. V_{max} units are nanomoles of mannitol 1-P formed per minute per milligram of protein. It should be noted that we routinely observe four- to fivefold-higher V_{max} values, and slightly higher K_m values, when measuring PEP-dependent mannitol phosphorylation activities in permeabilized whole cells (as in this table) compared to those in membrane vesicles (e.g., Table 3).

the formation of active heterodimers in the membrane (41, 42). Because we were concerned that the inactivity of the E257A and E257Q mutants might be due simply to partial proteolysis (Fig. 1), we asked if one of these mutant proteins, E257A, could functionally complement other inactive permease mutants. The mutants we chose for these complementation experiments contained mutations in the A domain (H554A, phosphorylation site 1), B domain (C384H, phosphorylation site 2), or C domain (H195A, a mutant inactive in mannitol phosphorylation) expressed from pBR322-derived plasmids (41, 42). As described in Materials and Methods, LGS322 cells harboring plasmid pGE257A (pACYC184 derived, Cm^r) were individually transformed with these three plasmids, and these doubly transformed cells were assessed for their abilities to ferment and phosphorylate mannitol.

The results from these complementation studies are presented in Table 4. As can be seen from these results, the E257A mutant was able to complement inactive proteins with mutations in either the A, B, or C domain for both mannitol fermentation and phosphorylation activities. Complementation was poorest, however, in the case of the C-domain H195A mutant. In this case, pink colonies were observed on MacConkey-mannitol plates, and the V_{max} for phosphorylation was about 20% that of the control. Nonetheless, the fact that the E257A mutant could complement, at least to some extent, permeases with mutations in each of the three domains of the protein suggests that the complete inactivity of the E257A protein is not entirely due to its possible instability and/or proteolysis (also see Discussion).

DISCUSSION

In this study, we have investigated the possible roles of Glu-257 in the mannitol permease of *E. coli*. This residue is found in a GIXE (Gly-Ile-X-Glu) motif, which is found in all EII proteins of the phosphotransferase except those of the mannose class (15, 27, 30), and the Glu residue is completely conserved in PTS permeases having this motif (15).

We used site-directed mutagenesis to create E257A, E257Q, and E257D substitutions in the mannitol permease. The E257A and E257Q proteins could not detectably bind mannitol. Replacing Glu-257 with an Asp residue had only a minor affect on low-affinity mannitol binding: the mutant protein had a twofold-higher K_d for mannitol than the wild-type permease. However, the E257D mutant did not bind mannitol at the high-affinity binding site of the permease.

Kinetic analyses of the PEP-dependent mannitol phosphorylation and uptake activities of the three mutant proteins at position 257 were carried out, and the kinetic values were compared to those of the wild type. Consistent with the binding results, neither phosphorylation nor uptake of mannitol could be detected in either the E257A or the E257Q mutant. In contrast, the E257D mutant permease was able to phosphorylate mannitol with kinetic values similar to those of the wild-type protein. Kinetic analysis of mannitol uptake by the E257D mutant revealed a three-fold-higher K_m for the mutant protein, but the V_{max} for uptake by the E257D mutant was only about 3% that of the wild-type protein. This presumably explains why LGS322 cells expressing the E257D mutation show only a low level of mannitol fermentation.

The results discussed above indicate that a carboxylate side chain at position 257, provided by either a Glu or Asp, is necessary for mannitol binding and phosphorylation. A Glu residue, however, is specifically required for efficient transport of the substrate and high-affinity mannitol binding. One direct role of a carboxylate side chain in these processes could be to form a hydrogen bond with the substrate. While this may occur, it seems unlikely that simply a loss of one such interaction could completely explain the inability of the E257A and E257Q mutants to bind mannitol. Therefore, mutations at position 257 may result, directly or indirectly, in a conformational change in the binding or active site of the protein. These are unlikely to be drastic conformational changes, however, because all three of the mutant proteins are inserted into the membrane in amounts similar to that of the wild-type protein and oligomerize normally. However, the apparent greater sensitivity to proteolysis of the E257A and E257Q mutant proteins and the greater resistance to proteolysis of the E257D protein compared to that of the wild type suggest that the mutant proteins do adopt conformations which are somewhat different from those of the wild-type protein.

It cannot be completely ruled out that the inactivity of the E257A and E257Q mutants for the various permease functions is due to the apparent proteolysis observed in Fig. 1. However, if these mutant permease proteins were extensively degraded within the cell, they might not be expected to oligomerize. In addition, the oligomers that were formed migrated at a position indistinguishable from that of the wild-type protein, and little, if any, heterogeneity was evident (Fig. 1).

Moreover, in vivo complementation studies with inactive mannitol permeases carrying mutations at positions 384 and 554 (phosphorylation sites in the A and B domains, respectively) and 195 (a site important for phosphorylation in the C domain) showed that the E257A mutant can rescue the activities of these other mutants, at least to some extent. Therefore, we believe that it is highly unlikely that proteolysis is the sole explanation for the undetectable levels of mannitol binding, phosphorylation, and uptake activities exhibited by the E257A and E257Q permeases.

One explanation for how the residue at position 257 could influence the conformational state of the active site could relate to the conformational model of EII^{Mtl} proposed by Lolkema et al. (20). In this model, for which there is some experimental evidence (11, 20), the mannitol binding site of the permease can be in three different conformations: outward facing, occluded, and inward facing. The outward-facing conformation is necessary to interact with mannitol in the periplasm, the inward-facing conformation would allow mannitol phosphorylation, and the occluded site is an intermediate conformation. For the overall transport process, the permease would cycle through these three conformations. Thus, it is possible that the E257A and E257Q mutant permeases are "locked" in an occluded state, preventing substrate binding. In the E257D mutant, perhaps the inward-facing conformation is

favored, allowing mannitol phosphorylation to occur more or less normally but inhibiting cycling through the different conformations and impairing mannitol transport. In this regard, it is interesting to note that the E257D mutation has a drastic effect both on high-affinity mannitol binding and on the V_{max} of uptake. An H195N mutant permease, which was previously reported by Weng and Jacobson (42) to have lost the high-affinity mannitol binding site, was found in this study to also be significantly impaired in mannitol uptake. This might suggest that these two processes are intimately related. Thus, while Glu-257 obviously plays an important role in binding and transport by the mannitol permease, its exact role awaits further studies.

Finally, several other residues within the same putative cytoplasmic loop in which Glu-257 is found have been shown to be important for the binding and/or transport functions of the mannitol permease. An E218A mutant protein had an increased K_m for mannitol and also was shown to carry out facilitated diffusion (7a). Moreover, a G253E mutant was found to phosphorylate mannitol but had very low uptake activity (21), similar to the E257D mutant protein described here. A mutation in the glucose permease within the conserved GIXE motif (residues 295 to 298) has also been isolated. This I296N mutant has a high K_m for glucose compared to the wild-type protein and carries out facilitated diffusion of this carbohydrate (28).

The results of this study, together with previous work, underscore the importance of the region containing the GIXE sequence in the EIIC domain of the PTS permeases. It has been postulated that this region could fold up into the hydrophilic channel formed by the transmembrane regions of the EIIC domain (14). In any case, the accumulated evidence suggests that it comprises at least part of the substrate binding and translocation site of PTS permeases, which could explain the high degree of conservation of this motif in this region of the protein.

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