JOURNAL OF BACTERIOLOGY, Aug. 1997, p. 5171–5177 Vol. 179, No. 16
0021-9193/97/$04.00+0

Subunit and Amino Acid Interactions in the *Escherichia coli* Mannitol Permease: a Functional Complementation Study of Coexpressed Mutant Permease Proteins

CYNTHIA A. SARACENI-RICHARDS† AND GARY R. JACOBSON*
Department of Biology, Boston University, Boston, Massachusetts 02215

Received 7 February 1997/Accepted 9 June 1997

Mannitol-specific enzyme II, or mannitol permease, of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system of *Escherichia coli* carries out the transport and phosphorylation of D-mannitol and is most active as a dimer in the membrane. We recently reported the importance of a glutamate residue at position 257 in the binding and transport of mannitol by this protein (C. Saraceni-Richards and G. R. Jacobson, J. Bacteriol. 179:1135–1142, 1997). Replacing Glu-257 with alanine (E257A) or glutamine (E257Q) eliminated detectable mannitol binding and transport by the permease. In contrast, an E257D mutant protein was able to bind and phosphorylate mannitol in a manner similar to that of the wild-type protein but was severely defective in mannitol uptake. In this study, we have coexpressed proteins containing mutations at position 257 with other inactive permeases containing mutations in each of the three domains of this protein. Activities of any active heterodimers resulting from this coexpression were measured. The results show that various inactive mutant permease proteins can complement proteins containing mutations at position 257. In addition, we show that both Glu at position 257 and His at position 195, both of which are in the membrane-bound C domain of the protein, must be on the same subunit of a permease dimer in order for efficient mannitol phosphorylation and uptake to occur. The results also suggest that mannitol bound to the opposite subunit within a permease heterodimer can be phosphorylated by the subunit containing the E257A mutation (which cannot bind mannitol) and support a model in which there are separate binding sites on each subunit within a permease dimer. Finally, we provide evidence from these studies that high-affinity mannitol binding is necessary for efficient transport by mannitol permease.

The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) phosphorylates and transports many carbohydrates in various species of bacteria (reviewed in references 16 and 20). The phosphotransfer steps involved in the transport of carbohydrates by all known PTSs are as follows:

$$\text{PEP} + \text{EI} \leftrightarrow \text{P-EI} + \text{pyruvate}$$
$$\text{P-EI} + \text{HPr} \leftrightarrow \text{P-HPr} + \text{EI}$$
$$\text{P-HPr} + \text{EIIA (domain or protein)} \leftrightarrow \text{P-EIIA} + \text{HPr}$$
$$\text{P-EIIA} + \text{EIIB (domain or protein)} \leftrightarrow \text{P-EIIB} + \text{EIIA}$$
$$\text{P-EIIB} + \text{carbohydrate}_{(\text{out})} \xrightarrow{\text{EIIC}} \text{EIIB} + \text{carbohydrate-P}_{(\text{in})}$$

where EI and EII are enzymes I and II, respectively. Transfer of the phospho group from PEP to the substrate-specific EII complexes is mediated by the general cytoplasmic PTS proteins, EI and HPr. All known EII's consist of at least three structural and functional domains, designated A, B, and C (21). These three domains may comprise a single protein as in *Escherichia coli* mannitol permease (7, 31) or may exist as two (e.g., glucose permease) or three distinct proteins (e.g., cellobiose permease) (reviewed in references 9 and 20).

Phosphotransfer from P-HPr to the substrate occurs in two steps via the EII: EIIA is the phosphoacceptor from P-HPr, and EIIB (which is phosphorylated by P-EIIA) is the phosphodonor to the carbohydrate. The phosphorylated residues of mannitol permease are His-554 in the EIIA domain (17) and Cys-384 in the EIIB domain (17, 19). Site-directed mutagenesis studies have shown that both of these residues are essential for mannitol phosphorylation and transport (28, 29).

Together, the A and B domains comprise the C-terminal half of mannitol permease, which is hydrophilic and exposed to the cytoplasm (26). The EIIC domain, which is responsible for both binding (5) and translocation (4, 12) of mannitol, is membrane bound and is probably comprised of at least six membrane-spanning α-helices (27).

Mannitol permease forms oligomers in the membrane, most likely dimers (reviewed in references 8 and 20), through which intersubunit phosphotransfer between His-554 on one subunit and Cys-384 on the other subunit can occur (28, 29). The formation of a permease dimer probably is necessary for optimal PEP-dependent phosphorylation (11) and transport (2) of mannitol by mannitol permease. It has been demonstrated that the permease dimer contains one high-affinity and one low-affinity mannitol binding site (18), suggesting that each subunit within a permease dimer forms separate binding and translocation sites, and these sites may interact in a negatively cooperative fashion. Additional mutational analyses also previously demonstrated the importance of His-195 in mannitol permease functions (30). Replacing His-195 with Ala or Arg (H195A or H195R, respectively) resulted in permeases which were defective in high-affinity mannitol binding and PEP-dependent mannitol phosphorylation. A mutant permease with an Asn residue at position 195 demonstrated nearly wild-type PEP-dependent mannitol phosphorylation activity but was defective in high-affinity mannitol binding. In addition, H195A and C384H mutant proteins were not able to complement one another in PEP-dependent mannitol phosphorylation when

* Corresponding author. Mailing address: Department of Biology, Boston University, 5 Cummington St., Boston, MA 02215. Phone: (617) 353-4708. Fax: (617) 353-6340. E-mail: jacobson@bio.bu.edu.
† Present address: Tufts University School of Medicine, Boston, MA 02111.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
E. coli strain		
K-12	F$^-$ *thi-1 hisG1 argG6*	5
LGS322	*metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50* Δ(*mtlA′p*) *mtlD*^{c} Δ(*gutR′MDBAp-recA*)	
Plasmids		
pGJ9	Cm^r, *mtlA* on pACYC184	5
pCSR9	Amp^r, *mtlA* on pBR322	This study
pGE257A	E257A on pGJ9	24
pGE257D	E257D on pGJ9	24
pBH195A (pAQW6)	H195A on pBR322	29
pBC384H (pAQW7)	C384H on pBR322	29
pBH554A (pAQW4)	H554A on pBR322	29

TABLE 2. Complementation of mannitol permease function[a]

Plasmid	Phenotype[b]	Mannitol phosphorylation[c]		Mannitol uptake[d]	
		K_m (μM)	V_{max}	K_m (μM)	V_{max}
pCSR9	Red	25	225	50	525
pGE257A/pCSR9	Red	30	200	50	500
pGE257A/pBC384H	Red	40	175	60	300
pGE257A/pBH554A	Red	40	175	60	350
pGE257A/pBH195A	Pink	35	50	75	100
pGE257D/pBH195A	Red	ND[e]	ND	ND	ND

[a] The phenotype and mannitol phosphorylation data for pGE257A/pBC384H, pGE257A/pBH554A, and pGE257A/pBH195A have been published previously (24) and are presented here for comparative purposes.

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

[c] Measured at 10, 25, 50, and 100 μM mannitol in permeabilized LGS322 cells (24). V_{max} is expressed as nanomoles of mannitol-1-P formed per minute per milligram of protein.

[d] Measured at 5, 10, and 25 μM mannitol in LGS322 cells. V_{max} is expressed as nanomoles of mannitol taken up per minute per milligram of protein.

[e] ND, not determined.

they were coexpressed in the same cell, suggesting that a His at position 195 and a Cys at position 384 must be present on the same subunit within a permease dimer for this function of the protein (30).

Accumulated evidence suggests that mannitol permease exists in different conformations and that phosphorylation of the permease by P-HPr converts it from a form which carries out mannitol transport very slowly to one that is capable of rapid mannitol transport (4, 14). Phosphorylation and transport are thus two separate but related steps, and mannitol phosphorylation without transport has been documented (14).

There is considerable evidence that a relatively hydrophilic region of mannitol permease, encompassing amino acid residues 185 to 270 in the EIIC domain, is important for coupling mannitol phosphorylation and transport (9, 20). In order to understand the mechanisms behind these functions, it will be necessary to understand the interactions between amino acid residues within this region of the protein, as well as between EIIC and the other functional domains. We recently reported that a glutamate residue which is highly conserved in most PTS permeases (Glu-257 in mannitol permease) appears to have an important role in the binding and transport of mannitol by this protein (24). When Glu-257 was changed to an Ala or a Gln residue, binding, phosphorylation, and uptake of mannitol by the permease were abolished. In contrast, a mutant permease in which this Glu residue was replaced by Asp was able to phosphorylate mannitol with kinetic values comparable to those of the wild-type protein, but this mutant was severely defective in mannitol transport. In this report, we use in vivo complementation studies to further investigate the role of Glu-257 in *E. coli* mannitol permease. The results of our studies suggest that this residue may interact with His-195 within the same putative cytoplasmic loop of the protein, that phosphotransfer can occur between Cys-384 on the EIIB domain and mannitol bound to the EIIC domain on the opposite subunit of a permease dimer, and that high-affinity mannitol binding by the permease may be important for its transport function.

MATERIALS AND METHODS

Chemicals and enzymes. D-[^{14}C]mannitol (54.5 mCi/mmol) was purchased from DuPont-New England Nuclear (Boston, Mass.). Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.). Enzymic reactions were carried out as recommended by the supplier. Other chemicals were reagent grade and 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. The *mtlA* gene (encoding mannitol permease) was isolated from plasmid pGJ9 on a *Sal*I/*Bam*HI fragment (5) and subcloned into the tetracycline resistance gene of plasmid pBR322 via *Sal*I/*Bam*HI restriction sites in order to construct the Amp^r plasmid pCSR9. Plasmid DNA was isolated by the alkaline lysis method (23) and transformed into competent cells prepared with $CaCl_2$ (15).

Growth of cells. Bacteria were routinely grown in liquid culture at 37°C on LB medium (1% tryptone, 0.5% yeast extract [both from Difco, Detroit, Mich.], 1% NaCl) containing 30 μg of chloramphenicol per ml and/or 50 μg of ampicillin per ml if necessary for selection. The protein concentration of each cell suspension was estimated by the Bradford method (3) with bovine serum albumin as the standard.

In vivo complementation between mutant mannitol permeases. Amp^r plasmids pCSR9 (this study), pBH195A, pBC384H, and pBH554A (originally named pAQW6, pAQW7, and pAQW4, respectively [29 and 30]) (Table 1) were individually transformed into LGS322 cells (Table 1) harboring a compatible pACYC184-derived plasmid (Cm^r) encoding either an E257A or an E257D mutant mannitol permease (24) (Table 1). Transformants were selected for both ampicillin and chloramphenicol resistances. The presence of both plasmids in each doubly transformed strain was verified by agarose gel electrophoresis and restriction analyses. These transformants were initially tested for mannitol utilization on MacConkey mannitol plates containing ampicillin and chloramphenicol.

Mannitol phosphorylation, uptake, and binding assays. PEP-dependent mannitol phosphorylation was measured in whole permeabilized LGS322 cells harboring various plasmids as previously described (31).

Uptake of [^{14}C]mannitol was measured in whole cells which had been grown to mid-exponential phase (optical density at 550 nm of 0.6 to 0.8) in LB medium containing 1% mannitol, washed twice, and resuspended to the original volume in M63 medium (24). Uptake was initiated by the addition of [^{14}C]mannitol to this suspension, which was then incubated at room temperature. Aliquots were taken at various times and filtered by using 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 radioactivity in the air-dried filters in a liquid scintillation counter.

High-affinity mannitol binding assays were performed at 20 to 150 nM [^{14}C]mannitol as described in Weng and Jacobson (30). Dissociation constants were estimated by the method of Scatchard (25).

RESULTS

Complementation of fermentation and phosphorylation. We previously found that an E257A mutant, which is defective in mannitol binding, uptake, and phosphorylation, could complement H554A, C384H, and, to a lesser extent, H195A, mutant permeases inactive in mannitol fermentation and phosphorylation (24) (Table 2). Interestingly, coexpression of the E257A mutant with the wild-type permease (pCSR9) had little effect on mannitol phosphorylation by the wild-type enzyme (Table 2). Therefore, if heterodimers are formed between E257A and wild-type monomers, the phosphorylation activity of the wild-type subunits in these heterodimers must be similar to that in wild-type homodimers. Expression of C384H, H554A, or H195A mutant mannitol permease with an E257D mutant

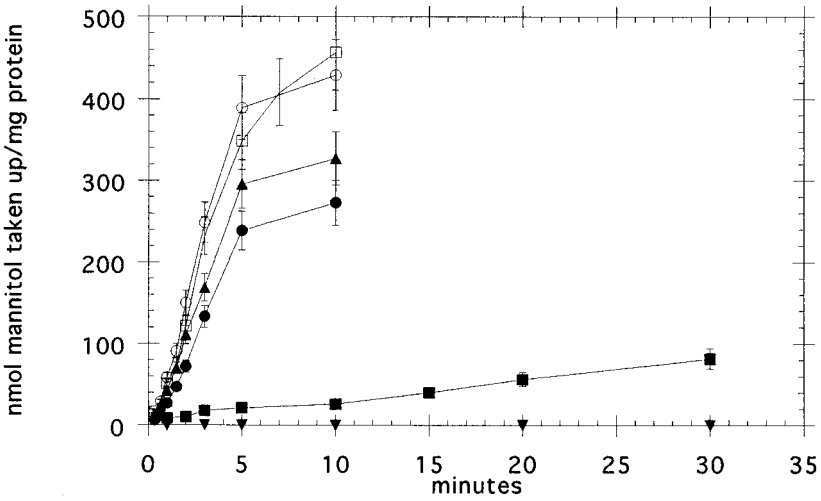

FIG. 1. In vivo complementation of mannitol uptake in LGS322 cells expressing both E257A mutant permease and either wild-type or another mutant permease. Mannitol uptake was measured at 10 μM as described in Materials and Methods. Error bars represent the standard deviations from the average amount of mannitol taken up in four different experiments. □, cells expressing wild-type permease (pCSR9); ○, cells coexpressing E257A and wild-type permeases (pGE257A/pCSR9); ●, cells coexpressing E257A and C384H permeases (pGE257A/pBC384H); ▲, cells coexpressing E257A and H554A permeases (pGE257A/pBH554A); ■, cells coexpressing E257A and H195A permeases (pGE257A/pBH195A); ▼, cells expressing either H195A (pBH195A), C384H (pBC384H), or H554A (pBH554A) permease alone.

(which by itself is pink on these indicator plates and transports mannitol very poorly [24]) resulted in the growth of dark red colonies on fermentation plates, indicating efficient complementation of the transport function of this mutant (also see below).

Complementation of mannitol uptake. Mannitol uptake was measured in LGS322 cells coexpressing both the inactive E257A mutant permease and other mutant permease proteins. The results, presented in Fig. 1, demonstrate that mannitol uptake by permeases carrying a mutation at position 384 or 554 can be rescued by the E257A permease (and vice versa). Compared to the results obtained when E257A and wild-type permeases were expressed together, the K_m value for mannitol uptake was slightly higher (as in phosphorylation) in cells coexpressing the E257A permease with either of the phosphorylation site mutants (Table 2). Moreover, the V_{max} for mannitol uptake in these cells was 75% of that in cells expressing E257A in combination with the wild-type permease (Table 2). These results are consistent with the observation that the mannitol fermentation and phosphorylation phenotypes of cells expressing the E257A mutant permease in combination with either of the phosphorylation site mutants were similar to those of cells expressing E257A with the wild-type permease. In addition, the kinetic values for mannitol uptake were similar for cells expressing both the wild-type permease and the E257A mutant and cells expressing only the wild-type protein (Fig. 1) (Table 2).

Similar to the results obtained in phosphorylation assays, the E257A mutant complemented the H195A mutant only very poorly in mannitol uptake (Fig. 1). When the H195A and E257A permeases were expressed together in the same cells, the K_m value for mannitol uptake was slightly higher than that in cells expressing E257A and the wild-type permease together, while V_{max} was only about 20% of that observed in cells expressing the E257A–wild-type combination (Table 2). In agreement with the observed fermentation phenotypes and the kinetic values for PEP-dependent mannitol phosphorylation, these results suggest that an unmutated Glu-257 and an unmutated His-195 must be on the same subunit within a permease dimer in order for efficient mannitol transport to occur.

Complementation of E257D mutant mannitol permease. Because the E257D mutant permease was found to have nearly normal mannitol phosphorylation activity but very low mannitol uptake activity (24), it was of interest to examine the ability of this mutant protein to be functionally complemented for uptake by the other mutant proteins. The results, presented in Fig. 2, show that when the E257D permease was expressed in the same cell with the H195A, C384H, or H554A mutant protein, the level of mannitol uptake was similar in each case and was only slightly lower than that in cells containing only the wild-type permease or coexpressing both the E257D and the wild-type permeases.

Both the E257D (24) and H195A (30) mutant permeases are incapable of high-affinity mannitol binding (the K_d value for high-affinity mannitol binding by the wild-type permease has been estimated at 40 to 50 nM [reference 30 and this study]). However, a high level of mannitol transport was observed in LGS322 cells in which these two mutant permeases were coexpressed (Fig. 2). Because a correlation between high-affinity mannitol binding and mannitol transport has been observed (24), the ability of these proteins to complement one another for high-affinity mannitol binding was examined (Fig. 3). The K_d value for high-affinity mannitol binding to membrane vesicles prepared from LGS322 cells expressing both the E257D and the H195A mutant permeases was about 260 nM, or approximately fivefold higher than that of the wild-type mannitol permease. Thus, coexpression of the H195A and the E257D mutant proteins, each of which alone is capable of only low-affinity mannitol binding (K_d = 3 and 11 μM, respectively [24, 30]), partially restores both high affinity binding (Fig. 3) and efficient mannitol uptake (Fig. 2).

DISCUSSION

In this report, we have further investigated the potential roles of Glu-257 in the mannitol permease of *E. coli* and its

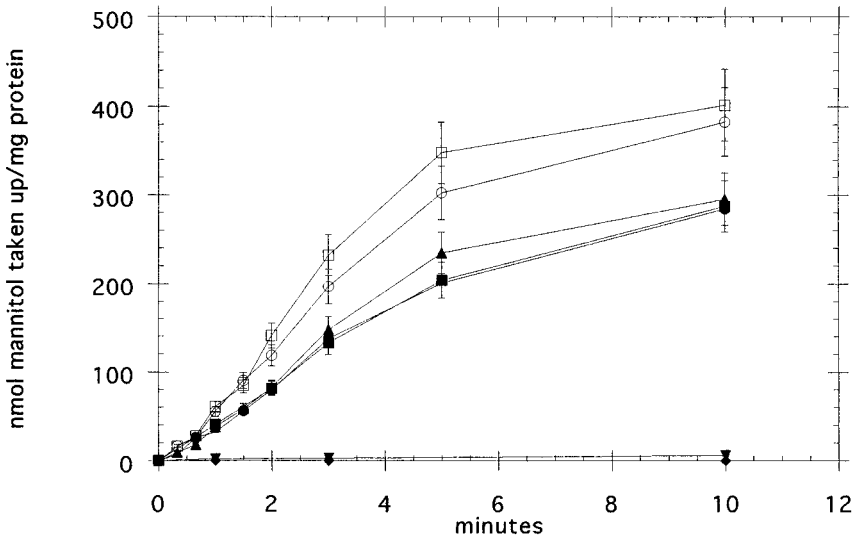

FIG. 2. In vivo complementation of mannitol uptake in LGS322 cells expressing both E257D mutant permease and either wild-type or another mutant permease. Mannitol uptake was measured at 10 μM as described in Materials and Methods. Error bars represent the standard deviations from the average amount of mannitol taken up in four different experiments. □, cells expressing the wild-type permease (pCSR9); ○, cells coexpressing E257D and wild-type permeases (pGE257D/pCSR9); ■, cells coexpressing E257D and H195A permeases (pGE257D/pBH195A); ●, cells coexpressing E257D and C384H permeases (pGE257D/pBC384H); ▲, cells coexpressing E257D and H554A permeases (pGE257D/pBH554A); ▼, cells expressing E257D permease; ◆, cells expressing H195A permease.

interactions with other important residues in this protein. This residue is located in putative cytoplasmic loop 5 between transmembrane regions 4 and 5 of this protein (27). Glu-257 is contained within a GIXE motif, a sequence which is conserved among many PTS permeases (9, 10, 22). Previous site-directed mutagenesis studies suggested the importance of a carboxylate side-chain at position 257 of this protein, because E257A and E257Q mutants could not detectably bind, phosphorylate, or transport mannitol (24). A mutant permease with an Asp residue at position 257 was capable of low-affinity mannitol binding and PEP-dependent phosphorylation of mannitol in a manner similar to that of the wild type, but was incapable of high-affinity mannitol binding, and exhibited a severe defect in mannitol uptake (24).

Inactive proteins with mutations at positions 384 and 554 have been shown to complement one another in both PEP-dependent mannitol phosphorylation and transport when they are expressed in the same cell (27). These results suggested the formation of active permease heterodimers of the coexpressed mutant monomers in which intersubunit transfer of the phos-

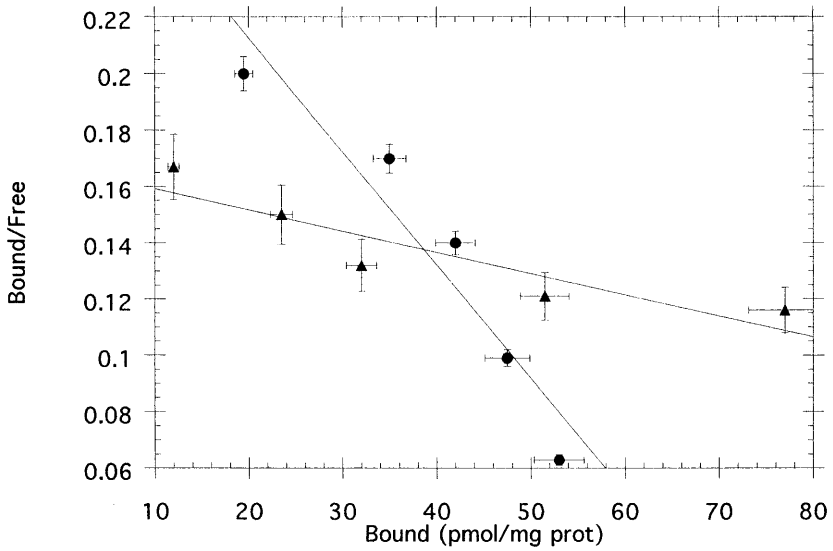

FIG. 3. Scatchard analysis of high-affinity mannitol binding to membrane preparations from cells expressing wild-type mannitol permease (●) or coexpressing E257D and H195A mutant permeases (▲). High-affinity mannitol binding was measured at 20 to 150 nM as described by Weng and Jacobson (30). Error bars represent the standard deviations from the average amount of mannitol bound in four different experiments. Lines were determined by least-squares analyses. The K_d value of high-affinity mannitol binding was determined to be 50 nM with wild-type permease and 260 nM when the E257D and H195A mutant permeases were expressed together. High-affinity mannitol binding was not detected in membranes from cells expressing either the E257D (23) or the H195A mutant permease alone (29).

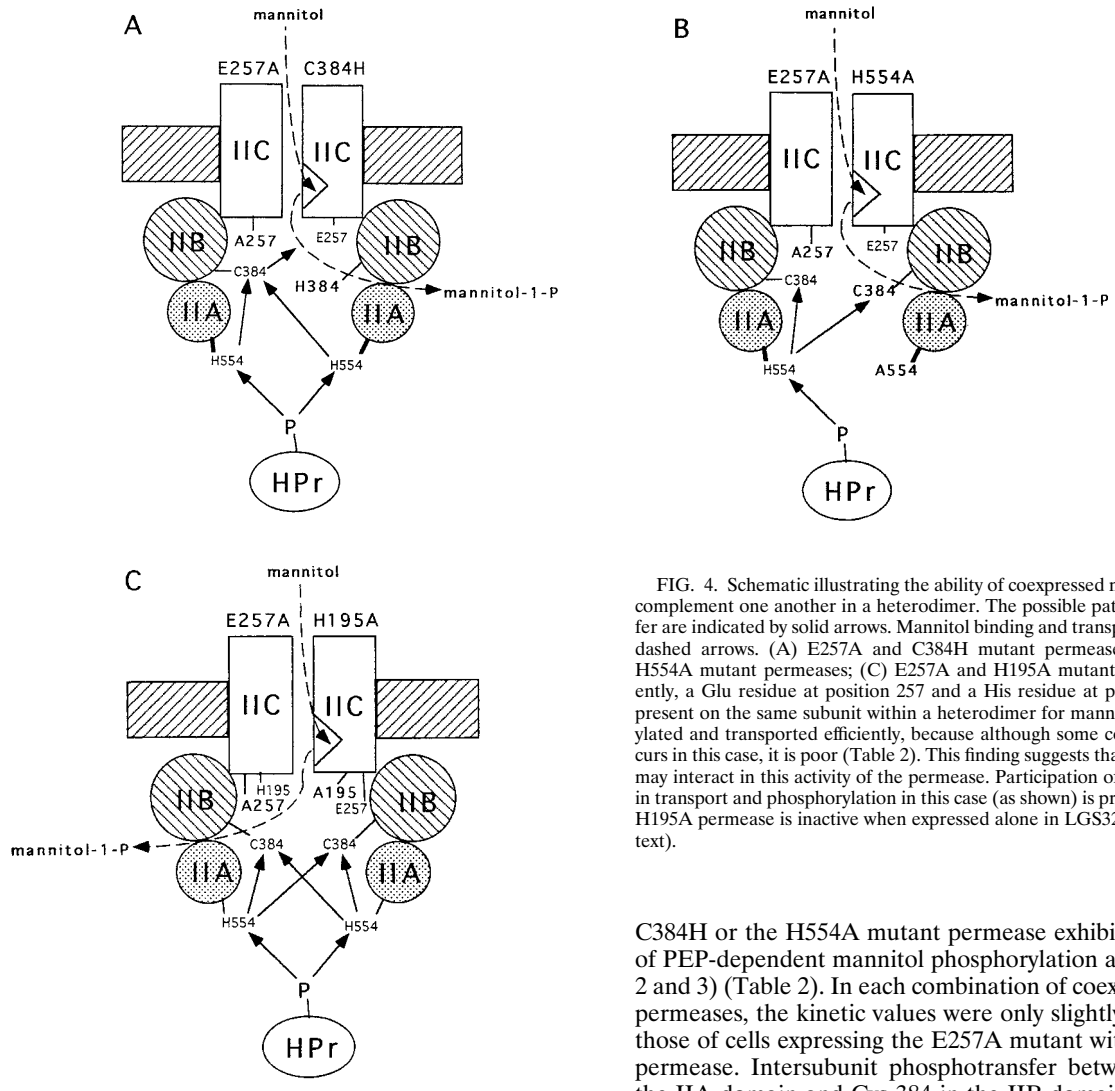

FIG. 4. Schematic illustrating the ability of coexpressed mutant permeases to complement one another in a heterodimer. The possible paths of phosphotransfer are indicated by solid arrows. Mannitol binding and transport are indicated by dashed arrows. (A) E257A and C384H mutant permeases; (B) E257A and H554A mutant permeases; (C) E257A and H195A mutant permeases. Apparently, a Glu residue at position 257 and a His residue at position 195 must be present on the same subunit within a heterodimer for mannitol to be phosphorylated and transported efficiently, because although some complementation occurs in this case, it is poor (Table 2). This finding suggests that these two residues may interact in this activity of the permease. Participation of the E257A subunit in transport and phosphorylation in this case (as shown) is presumed because the H195A permease is inactive when expressed alone in LGS322 cells (also see the text).

pho group from His-554 on the monomer with a mutation at position 384 to Cys-384 on the monomer with a mutation at position 554 had occurred. It was also demonstrated that while an H195A mutant could be functionally complemented in vivo when expressed in the same cell with an H554A mutant, it could not be complemented by a C384H mutant protein for mannitol fermentation or PEP-dependent mannitol phosphorylation (30). The most reasonable explanation for these results is that both a His at position 195 and a Cys at position 384 must be present on the same subunit within a heterodimer to achieve a high rate of PEP-dependent mannitol phosphorylation, implying an interaction between these two amino acid residues in the function of the permease.

Because Glu-257 lies in the same putative cytoplasmic loop as His-195, we were interested in determining the possible interactions between these two residues, as well as between Glu-257 and the two phosphorylation sites of the permease, Cys-384 and His-554. Thus, we studied the ability of an E257A mutant in vivo to complement H195A, C384H, and H554A mutants in fermentation, PEP-dependent phosphorylation, and uptake of mannitol.

Cells expressing the E257A mutant permease with either the C384H or the H554A mutant permease exhibited a high level of PEP-dependent mannitol phosphorylation and uptake (Fig. 2 and 3) (Table 2). In each combination of coexpressed mutant permeases, the kinetic values were only slightly different from those of cells expressing the E257A mutant with the wild-type permease. Intersubunit phosphotransfer between His-554 in the IIA domain and Cys-384 in the IIB domain has been well documented. The results of our studies of complementation between the E257A and C384H mutants indicate that phosphotransfer also can occur from Cys-384 in the IIB domain on the E257A subunit to mannitol bound to the IIC domain on the C384H subunit, because this mutant (30), but not the E257A mutant (24), can bind mannitol (Fig. 4A). For complementation of the E257A and H554A mutants, phosphotransfer could occur from Cys-384 of either subunit to mannitol bound to the H554A subunit (Fig. 4B). In recent studies from another laboratory (1), similar results were obtained with a G196D mutant, which also cannot bind mannitol. The results of our study with the E257 mutant are consistent with the results of these studies.

It was previously determined that an H195A mutant permease had lost the high-affinity mannitol binding site and was not capable of PEP-dependent mannitol phosphorylation (29). The results of our fermentation studies showed that the E257A protein could functionally complement the H195A protein only weakly. The levels of PEP-dependent mannitol phosphorylation and mannitol uptake that we observed in cells coexpressing the E257A and H195A proteins were also relatively low. The V_{max} values for both mannitol phosphorylation and mannitol uptake were fivefold lower than those of cells coexpressing the E257A mutant and the wild-type permeases.

While it might be argued that these lower levels of activities are due to inefficient heterodimer formation with these mutant monomers, both E257A and H195A mutants appear to efficiently dimerize with other mutant proteins (Table 2) (24), and therefore this explanation is unlikely. We previously reported that the E257A mutant does not bind mannitol or phosphorylate mannitol (24). For any complementation to occur between E257A and H195A mutant permeases, mannitol would most likely have to be bound to the H195A subunit of the heterodimer, while mannitol phosphorylation would be carried out by the E257A subunit (Fig. 4C).

Together, these results imply that the presence of a unmutated Glu residue at position 257 on one subunit and an unmutated His-554 (Fig. 4A) or Cys-384 (Fig. 4B) on the other subunit within a heterodimer is sufficient for mannitol phosphorylation and transport. However, His at position 195 and Glu at position 257 must be located on the same subunit in order for high-level mannitol phosphorylation and transport to occur (Fig. 4C), implying a possible interaction between these residues during these processes.

We previously reported that the E257D mutant mannitol permease was incapable of high-affinity mannitol binding and was defective in mannitol uptake (24). This phenotype was also observed for an H195N mutant permease, suggesting that high-affinity mannitol binding might be linked to efficient transport in this protein (24, 30). In this study, a high level of mannitol uptake was observed when the E257D and H195A mutant permeases were coexpressed (Fig. 2). This result was surprising because the H195A mutant also lacks high-affinity mannitol binding (30). However, membranes from cells coexpressing the E257D and H195A mutant proteins bound mannitol with a K_d value of 260 nM, compared to K_d values of 3 and 11 μM, respectively, for the H195A and E257D mutants (24, 30). While this value is fivefold higher than the high-affinity K_d value for the wild-type permease (Fig. 3), these results nonetheless support our previous proposal for a role of high-affinity mannitol binding in efficient transport by the mannitol permease. Presumably, formation of an E257D-H195A heterodimer somehow at least partially restores high-affinity mannitol binding. However, the mechanism by which this occurs remains to be determined.

Finally, with the exception of the E257A and H195A pair, the K_m and V_{max} values for PEP-dependent mannitol phosphorylation and for mannitol uptake do not vary greatly among the various pairs of coexpressed mutant and wild-type permeases or the wild-type permease expressed alone. This finding suggests that the inability of the E257A mutant to interact with mannitol did not significantly affect the ability of the other mutant or wild-type subunits within the heterodimers to interact with and transport mannitol. These results support previously proposed models (1, 9, 13) in which each subunit within a permease dimer contains separate mannitol binding and transport sites, rather than each subunit contributing to a single mannitol binding site formed at the interface between the two permease monomers.

The results of this and previous studies suggest that Glu-257 and His-195 may interact not only with each other within putative cytoplasmic loop 5 of the IIC domain but also with Cys-384 in the IIB domain. For example, phosphoexchange between mannitol-1-P and mannitol has been shown to occur via Cys-384 in the permease (29), and His-195 mutants were defective in this activity (30). In addition, inactive C384H and H195A mutants do not functionally complement one another in PEP-dependent phosphorylation (30), and in this study we have shown that inactive E257A and H195A mutants can complement one another only partially in mannitol phosphorylation and uptake. Finally, a carboxylate side-chain at position 257 has been shown to be important for mannitol binding, and specifically, a Glu residue at this position is necessary for high-level mannitol uptake (24). It is possible, then, that in the overall function of the permease, Glu-257 could be involved in the mannitol binding and transport steps and that His-195 may somehow interact with either Glu-257 or Cys-384 (or both residues) to facilitate transport and the transfer of the phospho group from the IIB domain to mannitol.

ACKNOWLEDGMENT

This research was supported by U.S. Public Health Service grant GM28226 from the National Institute of General Medical Sciences to G.R.J.

REFERENCES

1. **Boer, H., R. H. ten Hoeve-Duurkens, and G. T. Robillard.** 1996. Relation between the oligomerization state and the transport and phosphorylation function of the *Escherichia coli* mannitol transport protein: interaction between mannitol-specific enzyme II monomers studied by complementation of inactive site-directed mutants. Biochemistry **35:**12901–12908.
2. **Boer, H., R. H. ten Hoeve-Duurkens, G. K. Schuurman-Wolters, A. Dijkstra, and G. T. Robillard.** 1994. Expression, purification, and kinetic characterization of the mannitol transport domain of the phosphoenolpyruvate-dependent mannitol phosphotransferase system of *Escherichia coli*. J. Biol. Chem. **269:**17863–17871.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Elferink, M. G. L., A. J. M. Driessen, and G. T. Robillard.** 1990. Functional reconstitution of the purified phosphoenolpyruvate-dependent mannitol-specific transport system of *Escherichia coli* in phospholipid vesicles: coupling between transport and phosphorylation. J. Bacteriol. **172:**7119–7125.
5. **Grisafi, P. L., A. Scholle, J. Sugiyama, C. Briggs, G. R. Jacobson, and J. W. Lengeler.** 1989. Deletion mutants of the *Escherichia coli* K-12 mannitol permease: dissection of transport, phosphorylation, phospho-exchange, and mannitol binding activities. J. Bacteriol. **171:**2719–2727.
6. **Jacobson, G. R.** 1992. Interrelationships between protein phosphorylation and oligomerization in transport and chemotaxis via the *Escherichia coli* mannitol phosphotransferase system. Res. Microbiol. **143:**113–116.
7. **Jacobson, G. R., C. A. Lee, and M. H. Saier, Jr.** 1979. Purification of the mannitol-specific enzyme II of the *Escherichia coli* phosphoenolpyruvate: sugar phosphotransferase system. J. Biol. Chem. **254:**249–252.
8. **Jacobson, G. R., and C. Saraceni-Richards.** 1993. The *Escherichia coli* mannitol permease as a model for transport via the bacterial phosphotransferase system. J. Bioenerg. Biomembr. **25:**621–626.
9. **Lengeler, J. W., K. Jahreis, and U. F. Wehmeier.** 1994. Enzymes II of the phospho*enol*pyruvate-dependent phosphotransferase systems: their structure and function in carbohydrate transport. Biochim. Biophys. Acta **1188:** 1–28.
10. **Lengeler, J. W., F. Titgemeyer, A. P. Vogler, and B. M. Wöhrl.** 1990. Structure and homologies of carbohydrate:phosphotransferase system (PTS) proteins. Philos. Trans. R. Soc. Lond. B **326:**489–504.
11. **Lolkema, J. S., and G. T. Robillard.** 1990. Subunit structure and activity of the mannitol-specific enzyme II of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system solubilized in detergent. Biochemistry **29:**10120–10125.
12. **Lolkema, J. S., D. Swaving-Dijkstra, R. H. ten Hoeve-Duurkens, and G. T. Robillard.** 1990. The membrane-bound domain of the phosphotransferase enzyme II^{Mtl} of *Escherichia coli* constitutes a mannitol translocation unit. Biochemistry **29:**10659–10663.
13. **Lolkema, J. S., D. Swaving-Dijkstra, and G. T. Robillard.** 1992. Mechanics of solute translocation catalyzed by enzyme II^{Mtl} of the phosphoenolpyruvate-dependent phosphotransferase system of *Escherichia coli*. Biochemistry **31:**5514–5521.
14. **Lolkema, J. S., R. H. ten Hoeve-Duurkens, D. Swaving-Dijkstra, and G. T. Robillard.** 1991. Interaction between the cytoplasmic and membrane-bound domains of enzyme II^{Mtl} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system. Biochemistry **30:**6721–6726.
15. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
16. **Meadow, N. D., D. K. Fox, and S. Roseman.** 1990. The bacterial phosphoenolpyruvate:glycose phosphotransferase system. Annu. Rev. Biochem. **59:**497–542.
17. **Pas, H. H., and G. T. Robillard.** 1988. *S*-phosphocysteine and phosphohistidine are intermediates in the phosphoenolpyruvate-dependent mannitol transport catalyzed by *Escherichia coli* EII^{Mtl}. Biochemistry **27:**5835–5839.
18. **Pas, H. H., R. H. ten Hoeve-Duurkens, and G. T. Robillard.** 1988. Bacterial

phosphoenolpyruvate-dependent phosphotransferase system: mannitol-specific EII contains two phosphoryl binding sites per monomer and one high-affinity binding site per dimer. Biochemistry **27:**5520–5525.

19. **Pas, H. H., G. H. Meyer, W. H. Kruizinga, K. S. Tamminga, R. P. van Weeghel, and G. T. Robillard.** 1991. ^{31}Phospho-NMR demonstration of phosphocysteine as a catalytic intermediate on the *Escherichia coli* phosphotransferase system EII^{Mtl}. J. Biol. Chem. **266:**6690–6692.
20. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
21. **Saier, M. H., Jr., and J. Reizer.** 1992. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate: sugar phosphotransferase system. J. Bacteriol. **174:**1433–1438.
22. **Saier, M. H., Jr., A. Reizer, G. M. Pao, L.-F. Wu, J. Reizer, and A. H. Romano.** 1992. Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system, section II. Molecular considerations, p. 171–204. *In* Mortlock, R. P. (ed.), Evolution of metabolic function. CRC Press, Inc., Boca Raton, Fla.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Saraceni-Richards, C., and G. R. Jacobson.** 1997. A conserved glutamate residue, Glu-257, is important for substrate binding and transport by the *Escherichia coli* mannitol permease. J. Bacteriol. **179:**1135–1142.
25. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51:**660–672.
26. **Stephan, M. M., and G. R. Jacobson.** 1986. Membrane disposition of the *Escherichia coli* mannitol permease: identification of membrane-bound and cytoplasmic domains. Biochemistry **25:**4046–4051.
27. **Sugiyama, J. E., S. Mahmoodian, and G. R. Jacobson.** 1991. Membrane topology analysis of the *Escherichia coli* mannitol permease by using a nested deletion method to create *mtlA-phoA* fusions. Proc. Natl. Acad. Sci. USA **88:**9603–9607.
28. **van Weeghel, R. P., Y. Y. van der Hoek, H. H. Pas, M. Elferink, W. Keck, and G. T. Robillard.** 1991. Details of mannitol transport in *Escherichia coli* elucidated by site-specific mutagenesis and complementation of phosphorylation site mutants of the phosphoenolpyruvate-dependent mannitol-specific phosphotransferase system. Biochemistry **30:**1768–1773.
29. **Weng, Q.-P., J. Elder, and G. R. Jacobson.** 1992. Site-specific mutagenesis of residues in the *Escherichia coli* mannitol permease that have been suggested to be important for its phosphorylation and chemoreception functions. J. Biol. Chem. **267:**19529–19535.
30. **Weng, Q.-P., and G. R. Jacobson.** 1993. Role of a conserved histidine residue, His-195, in the activities of the *Escherichia coli* mannitol permease. Biochemistry **32:**11211–11216.
31. **White, D. W., and G. R. Jacobson.** 1990. Molecular cloning of the C-terminal domain of *Escherichia coli* D-mannitol permease: expression, phosphorylation, and complementation with C-terminal permease deletion mutants. J. Bacteriol. **172:**1509–1515.