Role of a Conserved Histidine Residue, His-195, in the Activities of the *Escherichia coli* Mannitol Permease†

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ABSTRACT: The mannitol permease, an enzyme II of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) of *Escherichia coli*, carries out the transport and phosphorylation of D-mannitol in this organism. Previous studies have shown that His-554 and Cys-384 in the mannitol permease are sequentially phosphorylated in reactions necessary for the transport and phosphorylation of the substrate. These residues are located in a large cytoplasmic domain of the protein. Interaction of the permease with mannitol, and its membrane translocation, however, must involve the N-terminal, transmembrane domain (EIIC domain) of the protein. In this report, we use site-directed mutagenesis and mutant complementation to investigate the role of His-195 in the EIIC domain of the mannitol permease, a residue that is conserved in many PTS permeases. In a previous report [Weng, Q.-P., Elder, J., & Jacobson, G. R. (1992) *J. Biol. Chem. 267*, 19529–19535], we inferred a role for His-195 that involves its hydrogen-bonding ability. Here we show that His-195 plays a role in high-affinity mannitol binding. Moreover, mutant complementation studies show that a functional His-195 must be on the same subunit as a functional Cys-384 in a permease dimer for phosphotransfer to mannitol to occur. These results and kinetic studies of His-195 mutant proteins imply that His-195 also may play an important role in this phosphotransfer reaction. His-195 is predicted to be in a cytoplasmic "loop" in the EIIC domain of the mannitol permease, in which several other residues have been shown to have roles in mannitol permease activity.

The phosphoenolpyruvate (PEP)[1]-dependent carbohydrate phosphotransferase system (PTS) carries out the transport and phosphorylation of many carbohydrates in a variety of anaerobic and facultatively anaerobic bacteria [for reviews, see Meadow et al. (1990) and Postma et al. (1993)]. Regardless of the organism or substrate, the following reactions comprise the phosphotransfer and transport steps of all known carbohydrates transported by the PTS:

$$\text{P-enolpyruvate} + \text{enzyme I (EI)} \leftrightarrow \text{P–EI} + \text{pyruvate} \quad (1)$$

$$\text{P–EI} + \text{HPr} \leftrightarrow \text{P–HPr} + \text{EI} \quad (2)$$

$$\text{P–HPr} + \text{EIIA (domain or protein)} \leftrightarrow \text{P–EIIA} + \text{HPr} \quad (3)$$

$$\text{P–EIIA} + \text{EIIB (domain or protein)} \leftrightarrow \text{P–EIIB} + \text{EIIA} \quad (4)$$

$$\text{P–EIIB} + \text{carbohydrate}_{(\text{out})} \xrightarrow{\text{EIIC}} \text{EIIB} + \text{carbohydrate–P}_{(\text{in})} \quad (5)$$

EI and HPr are general, cytoplasmic phospho-carrier proteins of the PTS while the EII complexes are substrate-specific. All known EII's have at least three structural and functional regions, named A, B, and C (Saier & Reizer, 1992), which may comprise three separate proteins, two proteins (*e.g.*, the glucose permease consisting of $EIICB^{Glc}$ and the separate $EIIA^{Glc}$), or simply three domains in a single polypeptide as is the case for the mannitol permease.

As shown above, phosphotransfer from P–HPr to the carbohydrate occurs via two phospho intermediates in an EII: the phospho acceptor from P–HPr in the EIIA domain and the phospho donor to the carbohydrate in the EIIB domain (which is phosphorylated by the EIIA domain). In EII^{Mtl}, the phosphorylated residue in the EIIA domain is His-554 (Pas & Robillard, 1988), and that in the EIIB domain is Cys-384 (Pas & Robillard, 1988; Pas et al., 1991). In site-directed mutagenesis studies, both of these residues have been shown to be essential for transport and phosphorylation catalyzed by the mannitol permease (van Weeghel et al., 1991c; Weng et al., 1992), and analogous His and Cys residues with the same roles exist in $EIIA^{Glc}$ and $EIICB^{Glc}$ (Dörschug et al., 1984; Presper et al., 1989; Erni, 1992). EII^{Mtl} has been shown to consist of a single kind of polypeptide (Jacobson et al., 1979) containing all three domains (White & Jacobson, 1990; van Weeghel et al., 1991a,b). The A and B domains comprise the C-terminal half of the mannitol permease which is hydrophilic and exposed to the cytoplasm of the cell (Stephan & Jacobson, 1986), while the EIIC domain is membrane-bound (Stephan & Jacobson, 1986; Grisafi et al., 1989), probably traverses the membrane at least 6 times as α-helical regions (Sugiyama et al., 1991), and is responsible both for binding (Grisafi et al., 1989) and for translocation (Lolkema et al., 1990; Elferink et al., 1990) of mannitol. EII^{Mtl} is most likely an oligomer in the membrane, probably a dimer [reviewed in Jacobson (1992) and Postma et al. (1993)], and *inter*-subunit phosphotransfer between His-554 on one subunit

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[1] Abbreviations: PEP, phosphoenolpyruvate; PTS, bacterial PEP-dependent carbohydrate phosphotransferase system; HPr, heat-stable phospho carrier of the PTS; EI, enzyme I of the PTS; EII, enzyme II of the PTS; EII^{Mtl}, EII specific for mannitol (=mannitol permease); EII^{Glc}, EII complex specific for glucose; EIIA, domain or protein of an EII containing the phospho acceptor site from P-HPr; EIIB, domain of the EII containing the second phosphorylation site (phospho donor to the substrate); EIIC, transmembrane domain of an EII; DTT, dithiothreitol.

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Table I: Bacterial Strains and Plasmids Used

strain/ plasmid	genotype or phenotype	reference
E. coli LGS322	F⁻ *thi-1,hisG1,argG6,-metB1,tonA2,supE44,-rpsL104,lacY1,galT6,-gatR49,gatA50,*Δ(*mtlA'p*)-*mtlD*^{c},Δ(*gutR'MDBAp-recA*)	Grisafi et al. (1989)
pGJ9	Cm^r *mtlA* on pACYC184	Grisafi et al. (1989)
pAQW2	H195N mutation on pGJ9	Weng et al. (1992)
pAQW3	H195R mutation on pGJ9	Weng et al. (1992)
pAQW6	H195A mutation on pGJ9	Weng et al. (1992)
pAQW7	C384H mutation on pGJ9	Weng et al. (1992)
pAQW9	C384D mutation on pGJ9	Weng et al. (1992)
pAQW4	H554A mutation on pGJ9	Weng et al. (1992)
pAQW8	H554D mutation on pGJ9	Weng et al. (1992)
pBQW11	Amp^r *mtlA* on pBR322	Weng et al. (1992)
pBQW12	H195R mutation on pBQW11	this study
pBQW15	H195A mutation on pBQW11	this study

and Cys-384 on the other is at least one route of phosphogroup migration through the protein (van Weeghel et al., 1991c; Weng et al., 1992).

Recent studies strongly suggest that phosphorylation of the mannitol permease by P–HPr converts it from a conformation that can carry out mannitol transport only very slowly to a conformation that carries out rapid mannitol transport (Elferink et al., 1990; Lolkema et al., 1991b). Phosphorylation of mannitol by the permease is thus a separable step from translocation, and therefore can occur without transport [*e.g.*, from the inside of the cell (Lolkema et al., 1991b)]. To understand the transport mechanism of EII^{Mtl}, it therefore will be important to understand not only how the EIIC domain interacts with mannitol but also how it interacts with the EIIA and/or EIIB domains in both their unphosphorylated and their phosphorylated states. Recently, we reported that His-195 in the EIIC domain of the mannitol permease appears to have a role in the activities of this protein that possibly involves the ability of this residue to form hydrogen bonds (Weng et al., 1992). An analogous His residue that is present in similar sequence and structural contexts is found in most other PTS EII's as well (Saier et al., 1988; Lengeler et al., 1990). In this report, we use mannitol binding and *in vivo* complementation studies to further investigate the role of His-195 in EII^{Mtl}. Our results suggest that this residue is involved both in interaction with mannitol and in phosphotransfer from Cys-384 in the EIIB domain to mannitol.

MATERIALS AND METHODS

Chemicals and Enzymes. D-[^{14}C]Mannitol (49 mCi/mmol) was purchased from Dupont/New England Nuclear Co. (Boston, MA). Restriction endonucleases and T_4 DNA ligase were obtained from New England Biolabs (Beverly, MA). A cytoplasmic fraction from *Salmonella typhimurium*, strain LJ144, was used as a source for EI and HPr as previously described (Begley et al., 1982). Other chemicals were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed and described in Table I. Plasmids encoding site-directed mutants of the gene for the mannitol permease, *mtlA*, were constructed as previously described (Weng et al., 1992). Two new plasmids, pBQW12 and pBQW15, were constructed for this study. These plasmids were made from pBWQ11, a pBR322 derivative containing the wild-type *mtlA* gene (Weng et al., 1992). This gene was excised from pBWQ11 by use of the flanking *Sal*I and *Bam*HI restriction sites and was replaced with a mutant *mtlA* gene, excised with the same restriction enzymes, from either plasmid pAQW3 or plasmid pAQW6 (pACYC184 derivatives) encoding mannitol permeases in which His-195 was replaced either by Arg (H195R) or by Ala (H195A), respectively (Weng et al., 1992). The resulting plasmids, pBQW12 and pBQW15, could then be used to coexpress the H195R and H195A mutant proteins with other site-directed mutant proteins encoded by the compatible pACYC184 derivatives (Table I).

Cell Growth and Preparation of Everted Membrane Vesicles. E. coli, strain LGS322 (Δ*mtlA*; Table I), harboring vectors encoding wild-type or mutant mannitol permeases was grown and used for the preparation of everted membrane vesicles as previously described (Grisafi et al., 1989). Ampicillin (Amp) at 100 μg/mL and/or chloramphenicol (Cm) at 30 μg/mL were included if necessary for plasmid selection. The protein concentration in these vesicles was estimated as described by Lowry et al. (1951) using bovine serum albumin as the standard.

Assays of Mannitol Binding and Phosphorylation Activities of Mannitol Permease. High-affinity binding of [^{14}C]-mannitol to membrane vesicles was measured as follows. Membrane vesicles (200 μg of protein/mL) in 25 mM Tris-HCl, 5 mM DTT, and 5 mM $MgSO_4$ (pH 7.5) were incubated with radiolabeled mannitol (50–400 nM) at 25 °C for 20 min. Membranes were separated from the supernatant using Centricon-30 filters (Amicon, Inc., Beverly, MA) essentially as described by Pas et al. (1988). The concentrations of bound and free mannitol were determined at each mannitol concentration by counting samples of the membranes and supernatant, respectively. Nonspecific trapping of mannitol by the membranes was corrected for by carrying out parallel assays using membranes from *E. coli* strain LGS322 containing no plasmid, which do not specifically bind mannitol (Grisafi et al., 1989). Dissociation constants were determined by the method of Scatchard (1949).

PEP-dependent phosphorylation of mannitol was measured using either permeabilized whole cells (for *in vivo* complementation assays) or everted membrane vesicles (for kinetic assays) as previously described (Weng et al., 1992; Grisafi et al., 1989).

In Vivo Complementation between Mutant Mannitol Permeases. Plasmid pBQW12 or pBQW15 (each Amp^r) encoding the H195R or H195A mutant permeases, respectively, was transformed (Mandel & Higa, 1970) into *E. coli* LGS322 cells harboring compatible, pACYC184-derived plasmids encoding other mutant permeases (Table I) by selecting for both Amp and Cm resistances (Weng et al., 1992). Restriction analyses of plasmid DNA from cell lysates confirmed the presence of both plasmids in these doubly-transformed strains (data not shown). These transformants were qualitatively tested for mannitol utilization on MacConkey mannitol plates, and permeabilized whole cells were used in PEP-dependent mannitol phosphorylation assays as described earlier.

RESULTS

Mannitol Binding by Position-195 Mutants of the Mannitol Permease. Previous studies using site-directed mutants of the mannitol permease were fully consistent with the evidence that His-554 and Cys-384 are the phosphorylation sites in the permease leading to transport and phosphorylation of mannitol

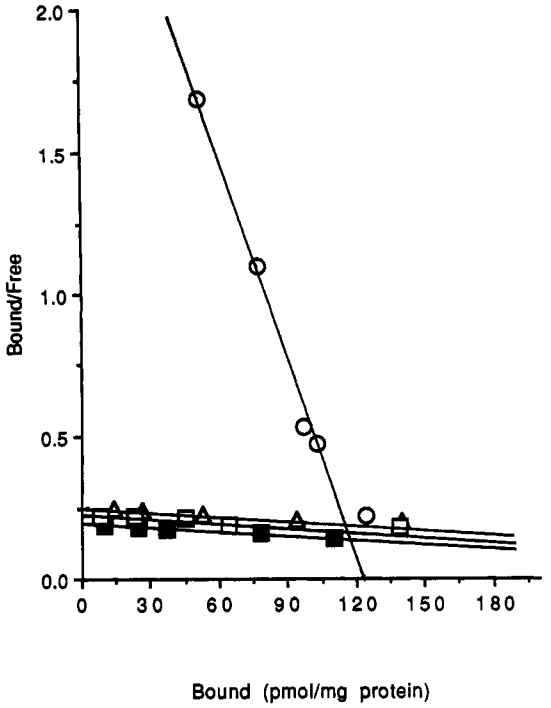


FIGURE 1: Scatchard plot of mannitol binding to the mannitol permease and mutant proteins at position 195. Membranes from strain LGS322 (Δ*mtlA*) containing various plasmids were assayed for binding of [^{14}C]mannitol as described under Materials and Methods. (○) Membranes containing the wild-type permease (pGJ9); (△) membranes containing the H195N mutant permease (pAQW2); (■) membranes containing the H195A mutant permease (pAQW6); (□) membranes containing the H195R mutant permease (pAQW3).

(van Weeghel et al., 1991c; Weng et al., 1992). We also proposed a role for His-195 in these activities based on the properties of H195A, H195N, and H195R mutant proteins (Weng et al., 1992). The H195A and H195R mutants had very low PEP-dependent phosphorylation and phospho-exchange activities, while the H195N mutant had a nearly wild-type level of PEP-dependent phosphorylation activity (at 100 μM mannitol) and about 30% of the wild-type activity in phospho exchange. From this, we inferred that the hydrogen-bonding ability of His-195 may be important for this role, which could be satisfied also by Asn at this position, but not Ala or Arg.

To determine whether the role of His-195 could involve the binding of mannitol, we measured high-affinity mannitol binding to membranes containing the wild-type permease and each of the three mutants at position 195 as described under Materials and Methods. The results, in the form of a Scatchard plot, are shown in Figure 1. All three mutant proteins exhibited a considerably higher K_D for mannitol than that for the wild-type permease. As shown in Table II, the K_D values for the mutants were about 70–80-fold higher than the value observed for the wild-type protein (K_D = 42 nM).[2] Since mutation of His-195 to Asn had an effect on mannitol binding similar to those of the H195A and H195R substitutions, these increases in the K_D for mannitol cannot by themselves account for the low activities of the H195A and H195R mutants compared to the H195N permease.

Table II: Dissociation Constants (K_D) for Mannitol Binding to Wild-Type and Mutant Mannitol Permeases[a]

permease[b]	K_D (μM)
wild-type (pGJ9)	0.042
H195N (pAQW2)	2.7
H195A (pAQW6)	3.4
H195R (pAQW3)	3.0
H554A (pAQW4)	0.043
H554D (pAQW8)	0.092
C384H (pAQW7)	0.096
C384D (pAQW9)	0.082

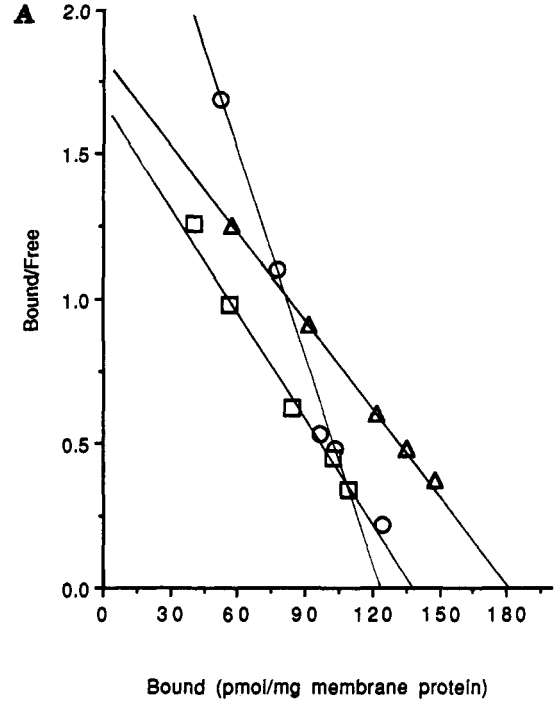
[a] Determined as described under Materials and Methods. [b] In parentheses are given the plasmids used to express the indicated mutant protein.

Mannitol Binding by Phosphorylation-Site Mutants. To determine if mutations at either of the phosphorylation sites of the mannitol permease, His-554 and Cys-384, affected mannitol binding, we conducted binding assays to these mutant proteins as shown in Figure 2A,B and also summarized in Table II. Substitution of His-554 by Ala had no effect on the K_D, while the H554D, C384H, and C384D mutants had K_D values for mannitol binding that were about 2-fold higher than that for the wild-type protein (Table II). Thus, in contrast to the His-195 mutations, these substitutions do not have a large effect on the mannitol binding affinity of the protein.

In Vivo Complementation between His-195 Mutant Proteins and Phosphorylation-Site Mutants. When expressed together in the same cell, mutant proteins at positions 554 and 384 can functionally complement one another in mannitol transport and phosphorylation (Weng et al., 1992). This presumably occurs via formation of an active heterodimer in the membrane in which intersubunit phosphotransfer between the "good" His-554 and Cys-384 residues on each mutant subunit can occur. It was therefore of interest to determine if either of the inactive mutant proteins at position 195 could be complemented by any of the phosphorylation-site mutants. To determine this, the inactive H195R and H195A proteins were expressed in the same cells as inactive mutants at either position 544 or position 384 (see Materials and Methods). As a qualitative measure of complementation, cells expressing these pairwise combinations of inactive mutants were scored on MacConkey mannitol fermentation plates. The results of this are presented in Table III. As shown, inactive mutant proteins at position 554 (H554A or H554D) could complement either the H195A or the H195R mutant protein for mannitol fermentation in these cells, but the inactive C384H mutant protein could not complement either the H195A or the H195R mutation.

For a quantitative estimate of complementation, we conducted PEP-dependent mannitol phosphorylation assays using permeabilized whole cells of strains containing the H195A mutant protein and each of the phosphorylation-site mutants. The results of these studies are presented in Figure 3 and Table III. As shown, cells containing the H195A protein and either the H554A or the H554D mutant exhibited about 10% of the mannitol phosphorylating rate of cells containing only the wild-type protein. In contrast, no significant activity was detected in strains expressing both the H195A and C384H mutant proteins, in agreement with the fermentation results. These results indicate that an unmutated His-195 and an unmutated Cys-384 must be on the same subunit for mannitol phosphorylation activity (see also Discussion).

[2] It is apparent from Figure 1 that the lines for all three mutant permeases intersect the abscissa at a value higher than that for high-affinity binding to the wild-type protein. This is likely due to the fact that a second, lower-affinity binding site for mannitol has been observed in the 1–10-μM range (Pas et al., 1988; Grisafi et al., 1989). Thus, the K_D values given in Table II for the His-195 mutant permeases are likely to represent binding to both the high-affinity site (with a reduced affinity in the mutants) and the lower-affinity site. We were unable to distinguish these two sites in the binding studies with the mutants (Figure 1), suggesting that these mutations affect the high-affinity site much more than the lower-affinity site.



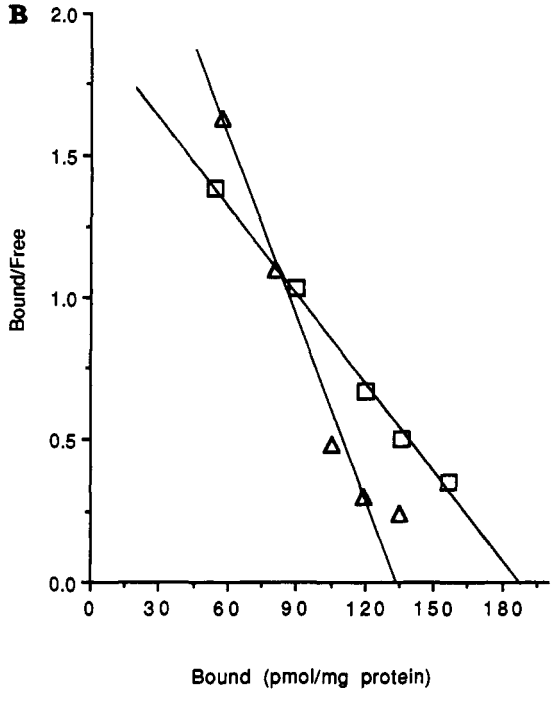


FIGURE 2: Scatchard plot of mannitol binding to phosphorylation-site mutants of the mannitol permease. Membranes from strain LGS322 (Δ*mtlA*) containing various plasmids were assayed for binding of [^{14}C]mannitol as described under Materials and Methods. (A) Mutants at position 384: (○) membranes containing the wild-type permease (pGJ9; the data are reproduced from Figure 1); (△) membranes containing the C384H mutant permease (pAQW7); (□) membranes containing the C384D mutant permease (pAQW9). (B) Mutants at position 554: (△) membranes containing the H554A mutant permease (pAQW4); (□) membranes containing the H554D mutant permease (pAQW8).

Kinetic Analyses of Position-195 Mutant Permeases. The results presented above suggest that, in addition to participating in mannitol binding, His-195 could also have a role in catalyzing phosphotransfer from Cys-384 on the same subunit to mannitol. To investigate this further, we measured the initial rates of PEP-dependent mannitol phosphorylation at varying mannitol concentrations using membranes derived from wild-type cells and cells expressing the H195A, H195R, or H195N mutant proteins. While the H195A and H195R mutant proteins do catalyze this activity at very low, but significant, levels at 100 μM mannitol (Weng et al., 1992), we found that increasing the mannitol concentration above this value (to as high as 1 mM) did not significantly increase the initial rate for either of these mutant proteins (not shown). The activities of both of these mutant proteins at all mannitol concentrations tested were too low to obtain reliable estimates

Table III: Fermentation Phenotypes and PEP-Dependent Mannitol Phosphorylation Activities of Cells Containing Pairwise Combinations of Inactive Mannitol Permease Mutants[a]

cells containing plasmid(s)	phenotype[b]	PEP-dependent mannitol phosphorylation[c]
pGJ9 (wild-type)	red	26
pBQW15/pAQW4 (H195A/H554A)	red	3
pBQW15/pAQW8 (H195A/H554D)	red	2.5
pBQW12/pAQW4 (H195R/H554A)	red	nd[e]
pBQW12/pAQW8 (H195R/H554D)	red	nd
pBQW15/pAQW7 (H195A/C384H)	white	<0.2[d]
pBQW12/pAQW7 (H195R/C384H)	white	nd

[a] LGS322 cells were transformed with the indicated plasmid(s) encoding the mutant proteins in parentheses. [b] Color of colonies on MacConkey mannitol plates after a 24-h incubation at 37 °C. [c] Assays were carried out with permeabilized cells as described under Materials and Methods. Units are nanomoles of mannitol-1-P formed per minute per milligram of protein calculated from the linear regions of the plots in Figure 3. [d] Beneath the limits of detectability in this assay. [e] nd = not determined.

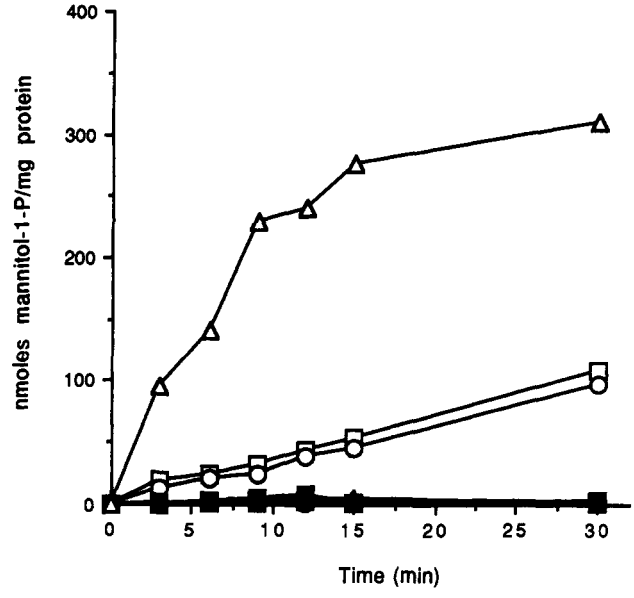


FIGURE 3: In vivo complementation of PEP-dependent mannitol phosphorylation activity in LGS322 cells expressing both the H195A mutant permease and various phosphorylation-site mutants. PEP-dependent phosphorylation was measured in permeabilized whole cells containing various plasmids as described under Materials and Methods. (△) Cells expressing the wild-type permease only (pGJ9); (□) cells expressing the H195A and H554A permeases (pBQW15/pAQW4); (○) cells expressing the H195A and H554D permeases (pBQW15/pAQW8); (■) cells expressing the H195A and C384H mutant permeases (pBQW15/pAQW7). Cells expressing either the H195A (▲), H554A (×), H554D (●), or C384H (+) mutant alone did not show significant activity under these conditions, and these symbols are not well-resolved from the solid squares in this figure.

Table IV: Kinetic Constants for PEP-Dependent Mannitol Phosphorylation by Wild-Type Mannitol Permease and Site-Directed Mutants at Position 195[a]

membranes from LGS322 cells containing plasmid[b]	K_M (μM)	V_{Max}[c]
pGJ9 (wild-type)	28	50
pAQW2 (H195N)	31	25
pAQW3 (H195R)	nd[d]	≤3[e]
pAQW6 (H195A)	nd[d]	≤3[e]

[a] Determined with membranes as described under Materials and Methods. [b] The mutant protein encoded by the indicated plasmid is given in parentheses. [c] Units are nanomoles of mannitol-1-P formed per minute per milligram of membrane protein. [d] Not determined (see text). [e] Estimated from the data in Weng et al. (1992) and from assays in this study conducted at mannitol concentrations between 0.1 and 1 mM (see text).

for K_M values for mannitol. However, from these results, it can be concluded that the H195A and H195R mutations do appear to drastically affect at least the V_{max} for this reaction [to about 5–6% of the value for the wild-type permease (Weng et al., 1992)].

In contrast to the H195A and H195R mutations, the H195N mutant protein has high PEP-dependent mannitol phosphorylation activity, at least at 100 μM mannitol (Weng et al., 1992). However, since it binds mannitol with a lower affinity than the wild-type protein (Table II), it was of interest to determine the initial-rate kinetic constants for this protein. As shown in Table IV, however, the K_M value for mannitol in PEP-dependent phosphorylation assays for the H195N mutant did not differ significantly from that determined for the wild-type protein, although the V_{Max} value for this same activity in the mutant was about half that of the wild-type protein.

DISCUSSION

In this report, we have investigated potential roles of His-195 in the mannitol permease of *E. coli*. This residue lies in a putative cytoplasmic loop between transmembrane helices 4 and 5 (from the N-terminus) (Sugiyama et al., 1991) and is found in similar primary sequence and structural contexts in other EII's of the PTS (Saier et al., 1988; Lengeler et al., 1990). On the basis of site-directed mutagenesis studies, we had earlier postulated a role for His-195 that involves its potential hydrogen-bonding ability because the H195A and H195R mutants had very low activities in PEP-dependent phosphorylation of mannitol while the H195N mutant had a nearly wild-type activity (Weng et al., 1992). In the present study, the mannitol binding affinities of these mutant proteins were determined. All three mutant proteins exhibited a higher K_D for mannitol, suggesting a role in mannitol binding for His at this position. However, whatever this role, it is clear that even Asn at position 195 cannot substitute for His in high-affinity mannitol binding, and that His at this position is not absolutely essential for the activity of the permease. The exact role of His-195 in mannitol binding, specifically whether it directly interacts with the substrate or it indirectly contributes to maintaining the optimal conformation of the binding site, remains to be determined.

We also studied the binding affinities of mutant permeases at the two phosphorylation sites in the protein, His-554 and Cys-384. These sites are in the hydrophilic, cytoplasm-facing domains EIIA and EIIB, respectively. Substitution of His-554 by Ala had no significant effect on the K_D for mannitol, in agreement with an earlier report (Lolkema et al., 1991a). However, the C384H and C384D mutant proteins both

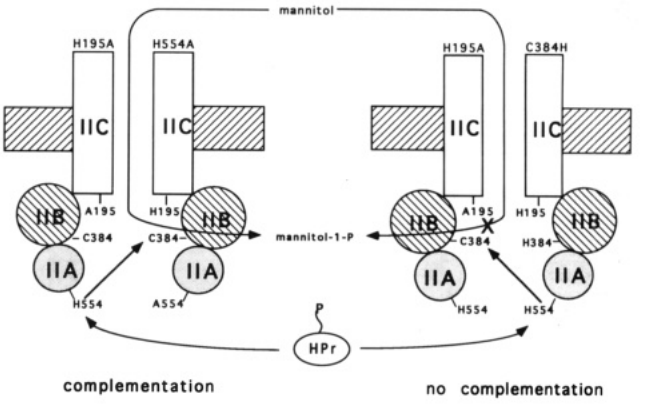


FIGURE 4: Schematic illustrating the ability of an H554 mutant subunit and an H195 mutant subunit to complement one another in a heterodimer (left) and the lack of complementation between a C384 mutant subunit and an H195 mutant subunit (right). It is proposed that a "good" H195 and a "good" C384 must be on the same subunit in order for PEP-dependent phosphorylation to occur, implying some interaction (direct or indirect) between these residues within a permease monomer that is important for this activity (also see the text).

exhibited an approximately 2-fold higher K_D for mannitol than the wild-type protein. A C384S mutant protein has also been shown to exhibit a higher K_D for mannitol (Lolkema et al., 1991a). Thus, as has been suggested previously (Lolkema et al., 1991a), there appears to be some interaction between the EIIB and EIIC domains such that the nature of the residue at position 384 affects the mannitol binding site. This is not surprising in view of the fact that Cys-384 is the direct phospho donor to mannitol. Substitution of His at position 554, the first phosphorylation site in the EIIA domain, by Asp had a similar effect on mannitol binding as the Cys-384 mutations. Possibly, the introduction of a negative charge at this site causes a conformational change that similarly affects the EIIC domain.

In order to further investigate the defect in the H195A and H195R mutant permeases compared to the H195N mutant, we carried out protein complementation assays. Previously, it had been shown that both *in vitro* (van Weeghel et al., 1991c) and *in vivo* (Weng et al., 1992) inactive mutant proteins at positions 554 and 384 can complement one another in PEP-dependent mannitol phosphorylation and transport. This was attributed to the formation of active heterodimers of position 554 and position 384 mutant monomers, in which intersubunit phosphotransfer could occur between the His-554 on the position 384 mutant monomer and the Cys-384 on the position 554 monomer. We found that both the H195A and H195R mutant proteins could be complemented for mannitol fermentation by either the H554A or the H554D mutant proteins *in vivo* when expressed in the same cell. In contrast, the C384H mutant protein could not complement either the H195A or the H195R mutant protein in mannitol fermentation *in vivo*, nor could the C384H protein complement the H195A protein in PEP-dependent phosphorylation *in vitro*. The simplest explanation for these results is that both His at position 195 and Cys at position 384 must be present on the same subunit of a permease dimer in order for PEP-dependent phosphorylation to occur at a high rate. Thus, mutants at position 554 could complement those at position 195, but mutants at position 384 could not, as shown schematically in Figure 4.

If both His-195 and Cys-384 must be on the same subunit for an active permease, then it is possible that these residues are in close proximity in a permease monomer. We have previously shown that not only PEP-dependent mannitol

phosphorylation but also mannitol·mannitol-1-P phospho exchange is drastically reduced in H195A and H195R mutant proteins (Weng et al., 1992). The exchange activity requires only Cys-384 and not His-554 (Grisafi et al., 1989; van Weeghel et al., 1991c; Weng et al., 1992), further implying an interaction between His-195 and Cys-384. The nature of this interaction, if it exists, and the exact role of His-195 in transport and catalysis remain to be determined.

Finally, to further define the role of His-195 in the mannitol permease, we carried out kinetic analyses of the PEP-dependent mannitol phosphorylation activities of the wild-type protein and the three mutant proteins at position 195. Although this activity is very low in the H195A and H195R mutant proteins, it is significantly greater than zero (Weng et al., 1992). These very low activities, even at high mannitol concentrations, precluded an accurate determination of K_M and V_{Max} values for this activity in the H195A and H195R mutants. However, since the rate of the reaction did not significantly increase between 0.1 and 1 mM mannitol in these mutants, we conclude that at least the V_{Max} for these mutant proteins must be much lower than that for the wild-type protein. This is fully in accord with the evidence discussed above that the most important role of His-195 is in catalysis of phosphotransfer and not in substrate binding, although it apparently contributes to the latter as well. Furthermore, we observed that the K_M for mannitol of the active H195N mutant was not significantly different from that of the wild-type protein, even though the K_D of this mutant for high-affinity mannitol binding was about 70-fold higher than that of the wild-type. This suggests either that the high-affinity binding site is not important for the phosphorylation reaction or that the kinetic rate constant for formation of mannitol-1-P from the P–EIIB·mannitol complex is much higher than that for the dissociation of free mannitol from this complex, even in the mutants.

The results presented here add further to the growing body of evidence for the functional importance of a region of the EIIC domain of PTS permeases that has been suggested to form a cytoplasmic "loop" between transmembrane helices [helices 4 and 5 in the model of this domain in the mannitol permease (Sugiyama et al., 1991)]. His-195 is in the N-proximal region of this loop in the mannitol permease. It has recently been shown that a nearby mutation (E218A) in this protein both greatly increases the K_M for mannitol and leads to a protein that carries out facilitated diffusion (J. W. Lengeler and H. Heuel, personal communication). In the more C-proximal region of this loop, a mutation has been isolated (G253E) resulting in a protein that can phosphorylate, but not transport, mannitol (Manayan et al., 1988). Moreover, the nearby Glu-257 residue, when mutated to Ala, results in a protein that can no longer detectably bind mannitol (C. Saraceni and G. Jacobson, in preparation). Glu-257 is in a highly conserved motif (GIXE) found in most PTS permeases (Lengeler et al., 1990), including the glucose permease. Interestingly, in this protein, a mutation in this motif has also been isolated (I296N) resulting in a glucose permease that has a high K_M for glucose and catalyzes facilitated diffusion (Ruijter et al., 1992). Thus, this putative loop appears to be important not only for substrate binding but also for translocation and phosphorylation as well. Either it could project up into a hydrophilic channel formed by the transmembrane regions of the EIIC domain (Lengeler, 1990) or, alternatively, it could form a "cap" or "gate" for such a channel on the cytoplasmic side of the membrane that would also comprise at least part of the substrate binding site of PTS permeases.

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