Site-specific Mutagenesis of Residues in the *Escherichia coli* Mannitol Permease That Have Been Suggested to Be Important for Its Phosphorylation and Chemoreception Functions*

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The *Escherichia coli* mannitol permease is an integral membrane protein that catalyzes the concomitant transport and phosphorylation of D-mannitol and also acts as the chemoreceptor for chemotaxis of *E. coli* to this hexitol. At least 4 aminoacyl residues in this protein have been suggested to be important in these activities: His-195, His-256, Cys-384, and His-554. Previous evidence has implicated His-554 and Cys-384 as residues that are covalently phosphorylated, in sequence, as intermediates in phosphotransfer to mannitol. We have constructed a number of site-specific mutants of the mannitol permease at these positions. The properties of proteins in which His-554 or Cys-384 has been changed are consistent with their essential roles in phosphorylation. We also used these mutants to show that intermolecular phosphotransfer between His-554 and Cys-384 can occur in vivo in membrane-bound heterodimers consisting of different mutant subunits. The properties of proteins with mutations at position 195 suggest an important role for this residue involving hydrogen bonding, while His-256 performs no significant function in the mannitol permease. Finally, the phosphorylation and chemoreception activities for each mutant protein were each roughly in the same proportion to these activities in the wild-type protein, showing that these functions of the mannitol permease are tightly coupled under normal physiological conditions.

The phosphoenolympyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) couples the transport and phosphorylation of many carbohydrates in a variety of bacterial species (reviewed in Ref. 1). The phosphotransfer reactions involved in this process are detailed below.

PEP + Enzyme I $\rightarrow$ P-Enzyme I + pyruvate
P-Enzyme I + HPr $\rightarrow$ P-HPr + Enzyme I

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††The abbreviations used are: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent carbohydrate phosphotransferase system; EI, Enzyme I of the PTS; HPr, heat-stable phosphocarrier protein of the PTS; EII, Enzyme III of the PTS; EII$, EII specific for D-mannitol; SDS, sodium dodecyl sulfate; MCP, methyl-accepting chemotaxis protein.

P-HPr + Enzyme III $\rightarrow$ P-Enzyme III + HPr
P-Enzyme III + Enzyme II $\rightarrow$ P-Enzyme II + Enzyme III
P-Enzyme II + carbohydrate$^-\text{ext}$ $\rightarrow$ Enzyme II + P-carbohydrate$^-\text{int}$

Enzyme I (EI) and HPr are general cytoplasmic phosphotransfer proteins of the PTS, while Enzyme III (EIII) and Enzyme II (EII) are carbohydrate-specific components. For some substrates (e.g. glucose in *Escherichia coli*), EIII is a soluble, cytoplasmic enzyme that phosphorylates the membrane-bound EII, while for others (e.g. mannitol in *E. coli*), the functions of EII and EIII are combined into a single EII polyepptide with an integral membrane (EII) domain and a cytoplasmic (EIII) domain (2). In addition to participating in PEP-dependent phosphorylation of its substrate, each EII also catalyzes a phosphoexchange reaction between its phosphorylated product and its substrate, reflective of the phospho-EII intermediate shown in the sequence of reactions above.

In addition to its carbohydrate transport and phosphorylation functions, the PTS also regulates the activities and expression of non-PTS transport systems (3), and many of the PTS EIIIs serve as primary chemotactic receptors for their cognate substrates, at least in the enteric bacteria (4).

One of the best characterized of the PTS EIIIs is the one that is specific for D-mannitol in *E. coli* (EII$^\text{mt}$ or mannitol permease). This 68-kDa integral membrane protein has been purified (5) and characterized (reviewed in Refs. 6 and 7), and its gene (mllA) has been cloned and sequenced (8). It consists of an N-terminal, membrane-bound domain (residues from 1 to about 334) that spans the membrane at least six times (9) and a hydrophobic, cytoplasmic domain (residues from about 335 to 637) that includes an EIII-like subdomain (10, 11). The N-terminal domain is responsible for mannitol binding and translocation, while the C-terminal domain carries out the phosphorylation functions of the mannitol permease (12). Recent experiments using biochemical (13), biophysical (14, 15), and molecular genetic (12, 16) approaches have provided strong evidence that His-554 in the EIII-like subdomain of the mannitol permease is the phosphoacceptor from P-HPr and that this phospho group is subsequently transferred to Cys-384, which then acts as the phosphoacceptor to mannitol as it enters the cell through the N-terminal domain. In addition to His-554, at least two other histidyl residues, His-195 and His-256, have been hypothesized to have roles in the activities of the mannitol permease (2, 17).

In this report, we have used site-specific mutagenesis to further investigate the roles of His-554, Cys-384, His-195, and His-256 in the various activities of the mannitol permease. Our results characterizing proteins with various mutations at positions 554 and 384 confirm and extend previous conclusions regarding both the roles of the residues found at these
positions in the wild-type protein and the mechanisms of the phosphotransfer reactions catalyzed by the mannitol permease. Studies on mutant proteins bearing substitutions at positions 195 and 256 suggest an important role for His-195, but not for His-256, in the wild-type protein. Furthermore, our results show that the phosphorylation and chemoreceptor functions of the mannitol permease are all tightly coupled under normal physiological conditions.

MATERIALS AND METHODS

Chemicals and Enzymes—[γ -32P]ATP (3000 Ci/mmol), d-[1-3H]mannitol (19 Ci/mmol), and d-[3-14C]mannitol (49 mCi/mmol) were purchased from Du Pont-New England Nuclear. Restriction enzymes were obtained from New England BioLabs (Beverly, MA); T4 DNA ligase, DNA polymerase (Klenow fragment), T3 polynucleotide kinase, and DNA sequencing reagents were from U. S. Biochemical Corp. All enzymic and sequencing reactions were carried out as recommended by the supplier. Goat anti-rabbit IgG-horseradish peroxidase conjugate was purchased from Bio-Rad. Oligonucleotides used for mutagenesis and sequencing were synthesized by Dr. Tolain (Boston University) using a Milligen (Bedford, MA) model 6500 instrument. A cytoplasmic fraction from Salmonella typhimurium, strain LT144 (see Table I), was prepared and used as a source for EI and HPr activities as previously described (21). Other chemicals were reagent grade and were purchased from Sigma.

Construction of Mutants—Site-specific mutagenesis of the mtlA gene was performed according to Kunkel (24). The mtlA gene was removed from plasmid pGJ9 (12) using the flanking SalI and BamHI restriction sites and was subcloned into these sites both in phagemid pUC119 yielding pUQW1 (see Table I) and in bacteriophage M13mp19. These were then used to transform E. coli RZ103 cells (30) to which the appropriate oligonucleotides had been cloned, and each was confirmed by DNA sequencing. Mutant portions of these genes were subcloned into the plasmid pGJ9 from which the corresponding part of the wild-type mtlA gene had been removed, and each was transformed into E. coli LGS322 (which contains a deletion in the chromosome mtlA gene). By these procedures, the following mutants in the mannitol permease were obtained: H554A, H554D, C384H, C384D, H195N, H195R, H195A, and H256A (also see Tables I and II).

To examine whether these mutant proteins were expressed and inserted into the membrane, membrane vesicles were prepared and subjected to Western blot analysis after electrophoresis on SDS gels. As shown in Fig. 1, the mutant proteins were detected at the same apparent molecular mass (65 kDa) but not for His-256, in the wild-type protein. Furthermore, our results show that the phosphorylation and chemoreceptor functions of the mannitol permease are all tightly coupled under normal physiological conditions.

RESULTS

Construction and Expression of Mutant mtlA Genes—As described under "Materials and Methods" and in Table II, site-specific mutants of mtlA were constructed using single-stranded DNA from pUC119 or M13mp19 into which mtlA had been cloned, and each was confirmed by DNA sequencing. Mutant portions of these genes were subcloned into the plasmid pGJ9 from which the corresponding part of the wild-type mtlA gene had been removed, and each was transformed into E. coli LGS322 (which contains a deletion in the chromosome mtlA gene). By these procedures, the following mutants in the mannitol permease were obtained: H554A, H554D, C384H, C384D, H195N, H195R, and H554A (also see Tables I and II).

To examine whether these mutant proteins were expressed and inserted into the membrane, membrane vesicles were prepared and subjected to Western blot analysis after electrophoresis on SDS gels. As shown in Fig. 1, the mutant proteins were detected at the same apparent molecular mass (65 kDa) and in comparable amounts with the wild-type protein, although some of the mutant proteins (e.g. H195A, H195N, H195R, and H554A) were slightly degraded, presumably by endogenous proteolytic activity.

Phenotypic Properties of Cells Containing EII<sup>mtl</sup> Mutant Proteins—For a qualitative assay of EII<sup>mtl</sup> activity, E. coli LGS322 cells harboring mutant plasmids were grown on MacConkey mannitol indicator plates. As with wild-type EII<sup>mtl</sup>, colonies producing mutant proteins H256A and H195N were red, indicating efficient transport and metabolism of mannitol. However, colonies producing mutant proteins H195R, H195A, C384H, C384D, H554A, and H554D were white, indicating that these mutant enzymes are defective in transport and/or phosphorylation of mannitol.

Phosphorylation of EII<sup>mtl</sup> Mutants—EII<sup>mtl</sup> is covalently phosphorylated by <sup>32</sup>P]PEP in the presence of EI and HPr (31, 32). Further evidence suggested that this protein contains two phosphorylation sites that are intermediates in phosphorylation of mannitol.

Site-specific mutants are denoted by the single letter code of the wild-type residue followed by position number in the primary sequence and the code for the replacement residue. Thus, H554A is the mutant protein in which histidine (H) at position 554 has been replaced by alanine (A).
phototransfer steps were proposed to be the acceptance of a phospho group from phospho-HPr by His-554, the transfer of this group from His-554 to Cys-384, and the transfer from Cys-384 to mannitol (6, 7, 10, 12-14, 16). If so, mutations in these two mutant proteins was observed even at much longer exposures of the same gel used for Fig. 2 (not shown). However, mutants H195A, H195N, H195R, H256A, and C384H were phosphorylated by [32P]PEP, as expected, although some quantitative differences were seen (Fig. 2; also see “Discussion”). This is consistent with the evidence that His-554 is the phosphoacceptor from phospho-HPr.

Effects of Mutations on Mannitol Phosphorylation Activities of EIIα—Everted membrane vesicles derived from different cells harboring the mutant proteins were used to measure both PEP-dependent mannitol phosphorylation and phosphoexchange activities. The results are presented in Table III. As expected, replacement of His-554 with either Ala or Asp resulted in undetectable PEP-dependent phosphorylation activity, but phosphoexchange activity was close to that of the wild-type protein. Similar results were obtained recently by van Weeghel et al. (16) for an H554A mutant enzyme. Substitution of His-195 with Asn led to a protein exhibiting nearly 100% of the PEP-dependent activity and 30% of the phosphoexchange activity of the wild-type protein, while substitution of His-256 with Ala had no significant effect on either activity. These results show that neither of these His residues is phosphorylated as an obligatory intermediate in mannitol phosphorylation as had been previously proposed (2, 17). However, both H195A and H195R mutant proteins exhibited very low activities in both assays. This could be explained by the possibility that His-195 has a role involving electrostatic interactions; this role could be supplied by His or Asn at this position, but not by Ala or Arg (also cf. “Discussion”). Mutants C384H and C384D were inactive in PEP-dependent phosphorylation of mannitol but still exhibited partial phosphoexchange activity, suggesting that both His and Asp at position 384 can also accept a phospho group from mannitol-1-P. These results are in contrast to the mutant enzyme C384S, which has neither PEP-dependent nor phosphoexchange activity (16).

In Vivo Complementation of PEP-dependent Phosphorylation Activity—Much in vitro evidence suggests that functions of EIIα require an oligomer of the protein, minimally a dimer (reviewed in Refs. 6, 7, and 33). This has been most directly confirmed by demonstration of phospho group transfer between His-554 and Cys-384 on different subunits of EIIα (16, 26). To further investigate this process in vivo, mutant genes encoding C384H, H554A, and H554D proteins were each subcloned from the pGJ9 derivatives (Cm', pACYC184-de-
rived) into the compatible plasmid pBR322 (Amp') bearing a different replication origin. Various combinations of mutant plasmids were then serially transformed into E. coli LGS322 followed by plating on MacConkey mannitol indicator plates containing both chloramphenicol and ampicillin. All of the colonies of cells bearing plasmids encoding C384H and H554A, as well as those bearing plasmids encoding C384H and H554D, were red, in contrast to the colonies with each single mutant gene alone, which were white. This shows that complementation occurs between Cys-384 and His-554 mutants in vivo. To ensure that both plasmids were present in the complemented strains and to rule out that homologous recombination of mutant plasmids had occurred (even though strain LGS322 is recA-), the plasmids from these transformants were prepared and subjected to restriction analysis. These experiments showed that no detectable recombination between plasmids had occurred in the red transformants (data not shown).

To quantitatively estimate the complementation activity between His-554 and Cys-384 mutants, permeabilized cells of the transformants were used for assays of PEP-dependent phosphorylation of mannitol. The results are shown in Fig. 3. The strains containing the genes encoding H554A, H554D, or C384H mutant proteins alone exhibited only background PEP-dependent phosphorylation activity (i.e. that exhibited by strain LGS322 containing no plasmids). However, cells harboring plasmids expressing both C384H and H554A proteins or both C384H and H554D proteins exhibited 25-40% of the PEP-dependent activity of the control strain harboring pGJ9 (wild-type rtiA). These results provide direct evidence for formation of mutant hetero-oligomers in vivo resulting in intermolecular phosphotransfer from His-554 on one subunit to Cys-384 on another, followed by phosphotransfer to mannitol.

Chemotactic Receptor Activities of EII* Mutants—The PTS EIIs are the primary chemotactic receptors for their cognate substrates in chemotaxis of E. coli and S. typhimurium to PTS substrates (reviewed in Ref. 4). Although an early report suggested that the transport/phosphorylation and chemotactic receptor functions of EII* could be at least partially dissected by mutation (34), subsequent attempts to
ods."

mannitol phosphorylation as described under "Materials and Meth-

pAQW8 (H554D)  
pBQW17 (C384H)  
plasmids pGJ9 (wild-type enzyme)  
phorylation activity by formation of membrane-bound het-

tants of EIImtl (12). Phosphopeptide analysis demonstrated that PEP-dependent phosphorylation could be verify this have failed (4). Moreover, there is recent evidence that it is the phosphorylation state of HPr that sends the signal to the Che proteins in taxis to PTS substrates rather than a direct interaction of the receptors (EIIs) with HPr as appears to be the case for MCP-mediated taxis (35). If this is true, then it should not be possible to selectively abolish either transport/phosphorylation or chemoreceptor activity in an EII; these activities should be obligatorily coupled. To test this, we measured chemotaxis activities of LGS3222 cells harboring plasmids containing wild-type and various mutant mtlA genes using a semiquantitative swarm plate assay (see "Materials and Methods"). Chemotactic responses to mannitol of cells containing various deletion mutants of mtlA or site-specific mutants were proportionally similar to the PEP-dependent mannitol phosphorylation activities of these proteins when compared with the wild-type protein, as shown in Table IV. In contrast, chemotaxis toward aspartate, which is MCP-dependent, was similar in all of these strains. Moreover, several of the mutant proteins tested still bind mannitol normally but were inactive as chemotactic receptors. These results suggest that phosphorylation activity is indeed obligatorily coupled to PTS chemotactic receptor activity, at least in EIIm1.

**DISCUSSION**

That EIIm1 should contain two catalytically important phosphorylation sites was first inferred from the fact that it contains an EII-like domain (reviewed in Ref. 2). EIIs are separate proteins for some PTSs (e.g. glucose) and are known in these cases to be phosphorylated on a histidyl residue by phospho-HPr. In the *E. coli* glucose PTS, EII specific for glucose is also phosphorylated covalently in a catalytically important fashion (36). For EIIm1, two phosphopeptides have been isolated from the purified protein that had been phosphorylated with PEP, E1, and HPr (13), and it was also demonstrated that PEP-dependent phosphorylation could be genetically dissected from phosphoexchange activity in certain deletion mutants of EIIm1 (12). Phosphopeptide analysis (13), and more recently 31P NMR analysis of purified, phos-
mutant proteins were detectable but very low (Table III). These results could be explained if the hydrogen-bonding ability of the residue at position 195 plays a role, either directly or indirectly, in catalysis. This requirement could be fulfilled by Asn but not by Ala at this position. The inactivity of the H195R mutant is not surprising given the very different chemical properties of imidazole and guanidino groups, and this fact coincided with the fact that the H195N mutant is active rules out that a positive charge at this position is necessary for activity. Further work will be necessary, however, to determine the exact role of His-195 in the activities of EIImt.

By subcloning mutant mtlA genes from the pACYC184-based pGJ9 derivatives into pBR322, we were able to construct strains in a λmtlA background expressing various combinations of proteins containing mutations at positions 384 and 554. In all cases in which an inactive mutant at position 384 was coexpressed with an inactive mutant at position 554, mannitol fermentation in vivo was restored in strain LGS322, and restoration of PEP-dependent mannitol phosphorylation was also observed in permeabilized whole cells of the same strains. These results provide direct evidence for in vivo hetero-oligomer formation of EIImt in strains expressing these mutant proteins and extend to the intramembrane situation the previous evidence obtained in detergent solution which indicated that intermolecular phosphotransfer can occur between His-554 and Cys-384 (16, 26). In fact, it seems likely that this is the major, if not the sole, route for phosphotransfer within the wild-type enzyme, since in vivo complementation activities (Fig. 3) are close to those expected on the basis of the presumed amount of homodimers relative to inactive homodimers in the membrane (50% of the total) and since dissociated EIImt has a very low PEP-dependent activity (40).

Finally, our results concerning chemotactic activities of cells expressing mutant EIImt proteins are consistent with the hypothesis that PTS EIIs do not interact directly with the Che signaling proteins (4, 35) as do the MCPs, which are the primary receptors for most other chemotactants (reviewed in Ref. 41). The chemotactic behavior of these cells toward mannitol as a chemoattractant parallels the activities of these mutant proteins in PEP-dependent mannitol phosphorylation but does not correlate with the ability of mutant proteins to bind mannitol (Table IV). Thus, unlike the MCPs, transport and phosphorylation of its ligand, rather than simply binding, is essential for a PTS EII to act as a chemoattractant (at least in the case of EIImt). Indeed, recent results suggest that the phosphorylation state of HPr (which in turn is determined by the combined activities of the PTS EIIIs) that communicates, directly or indirectly, with the Che proteins, which themselves are involved in regulating flagellar rotation (35).

**CONCLUSIONS**

From the results of this and previous work, we present the model shown in Fig. 4 for the various activities of EIImt. 1)
We have confirmed the results of van Weeghel et al. (16) concerning the roles of His-554 (phosphoacceptor from P-HPr) and Cys-384 (phosphodonor to mannitol) in EIImt. We have also extended these results by showing that mutations at position 554 completely abolish phosphorylation of the protein while mutations at position 384 do not. 2) Previous results (16, 26) concerning the ability of the phospho group to be transferred intermolecularly to Cys-384 on another subunit of EIImt have been confirmed. We have extended this observation of intermolecular phosphotransfer to the membrane-bound protein in vivo and have provided evidence that this is the predominant, if not the sole, route of phosphotransfer within the enzyme. 3) We have shown that although mutant proteins with His or Asp at position 384 are inactive in PEP-dependent phosphorylation, they still catalyze phosphoexchange (in contrast to the C384S mutant (16)).

4) We have shown that His-256 performs no important function in EIImt, while His-195 appears to play a role in its activities involving hydrogen bonding. 5) Finally, using these mutants, we have shown that mannitol transport and phosphorylation appear to be essential for EIImt to act as a chemotactic receptor. These observations are consistent with the hypothesis that it is the HPr/P-HPr ratio that is sensed by the general chemotaxis (Che) proteins during taxis to PTS substrates (35).

In the future, it will be interesting to use site-specific mutagenesis to define further the roles of individual aminoacetyl residues in EIImt activities, especially those in the membrane-bound domain that are probably responsible for the translocation event itself.

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