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The *Escherichia coli* Mannitol Permease as a Model for Transport via the Bacterial Phosphotransferase System

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The bacterial phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) consists of several proteins whose primary functions are to transport and phosphorylate their substrates. The complexity of the PTS undoubtedly reflects its additional roles in chemotaxis to PTS substrates and in regulation of other metabolic processes in the cell. The PTS permeases (Enzymes II) are the membrane-associated proteins of the PTS that sequentially recognize, transport, and phosphorylate their specific substrates in separate steps, and the *Escherichia coli* mannitol permease is one of the best studied of these proteins. It consists of two cytoplasmic domains (EIIA and EIIB) involved in mannitol phosphorylation and an integral membrane domain (EIIC) which is sufficient to bind mannitol, but which transports mannitol at a rate that is dependent on phosphorylation of the EIIA and EIIB domains. Recent results show that several residues in a hydrophilic, 85-residue segment of the EIIC domain are important for the binding, transport, and phosphorylation of mannitol. This segment may be at least partially exposed to the cytoplasm of the cell. A model is proposed in which this region of the EIIC domain is crucial in coupling phosphorylation of the EIIB domain to transport through the EIIC domain of the mannitol permease.

KEY WORDS: Mannitol; bacterial transport; phosphotransferase system; enzyme II; enzyme mechanism; membranes; chemotaxis.

INTRODUCTION

Transport of common carbohydrates that can serve as carbon and energy sources for heterotrophic bacteria is often carried out by the phosphoenolpyruvate (PEP)[2]-dependent carbohydrate phosphotransferase system (PTS) [for recent reviews, see Meadow *et al.*, 1990; Postma *et al.*, 1993)]. A number of different phosphotransfer proteins and protein domains participate in the transfer of the phospho group from PEP to the carbohydrate (Scheme 1).

EI and HPr are the general, soluble phosphotransfer proteins of the PTS. P-HPr, phosphorylated by P-EI, transfers its phospho group to a hydrophilic EIIA domain (or protein) which then transfers the phospho group to a second hydrophilic domain (or protein), EIIB. Following this, the carbohydrate is translocated by the integral-membrane IIC domain and is subsequently phosphorylated by phosphotransfer from the IIB domain. As illustrated in Fig. 1, the EII may consist of a single, multi-domain polypeptide (e.g., the mannitol permease) or as many as three separate proteins (e.g., the cellobiose permease), each protein in this case corresponding to one of the domains. Regardless of the PTS, however, the EII's are likely to transport and phosphorylate their substrates by similar mechanisms.

The complexity of the PTS undoubtedly reflects its additional roles in chemotaxis to PTS substrates (Lengeler and Vogler, 1989; Postma *et al.*, 1993) and

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[2] Abbreviations used: PEP, phosphoenolpyruvate; PTS, bacterial PEP-dependent, carbohydrate phosphotransferase system; EI, Enzyme I of the PTS; HPr, heat-stable, phospho-carrier protein of the PTS; EII, enzyme II of the PTS; EIIA, EIIB, and EIIC, domains or proteins comprising a specific EII that contain phosphorylation site 1, phosphorylation site 2, and the transmembrane region, respectively; MCP, methyl-accepting chemotaxis protein.

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(1) PEP + Enzyme I (EI) <----> P-EI + pyruvate

(2) P-EI + HPr <----> P-HPr + EI

(3) P-HPr + EIIA (domain or protein) <----> P-EIIA + HPr

(4) P-EIIA + EIIB (domain or protein) <----> P-EIIB + EIIA

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

Scheme 1.

in regulation of other metabolic processes in the cell (Saier, 1989; Postma *et al.*, 1993). In this minireview, we shall concentrate on recent studies of the mechanism of mannitol transport, phosphorylation, and chemotaxis carried out by the *E. coli* mannitol-specific EII, or mannitol permease, arguably the best understood PTS permease (for earlier reviews of this protein, see Robillard and Lolkema, 1988; Jacobson and Stephan, 1989).

MECHANISM OF PHOSPHORYLATION

Recent studies in which various domains of the mannitol permease have been subcloned and expressed (White and Jacobson, 1990; van Weeghel *et al.*, 1991a,b) and in which site-directed mutagenesis has been employed (van Weeghel *et al.*, 1991c; Weng *et al.*, 1992) are fully consistent with previous biochemical and biophysical studies (Pas and Robillard, 1988; Pas *et al.*, 1991) showing that His-554 in the IIA domain and Cys-384 in the IIB domain are the phosphorylated sites in the mannitol permease. His-554 is the phospho-acceptor from P-HPr and the phospho-donor to Cys-384, while Cys-384 is the phospho-donor to mannitol. That these sites are sequentially phosphorylated as part of the *bone fide* catalytic mechanism was also inferred from studies of the stereochemical course of mannitol phosphorylation by the permease. The mannitol PTS of *E. coli* was shown to catalyze the phosphorylation of mannitol in the presence of PEP (chiral at phosphorus), EI, HPr, and membranes containing the mannitol permease, with overall stereochemical inversion of the configuration of the phospho group compared to PEP (Mueller *et al.*, 1990). This result is consistent

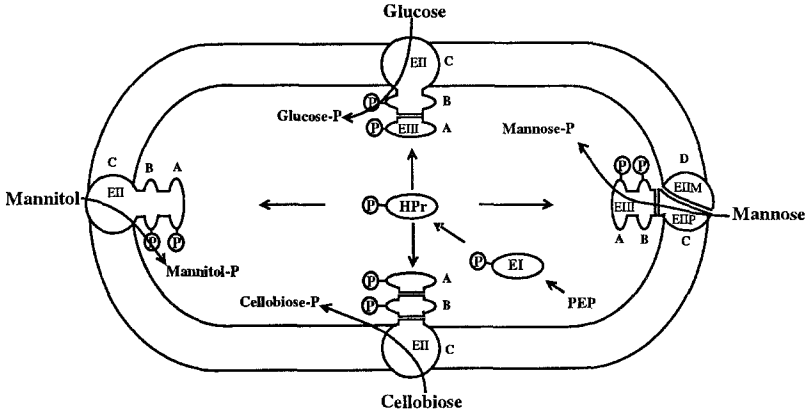

Fig. 1. Domain/Protein Organization for Four PTS Permeases of *E. coli*. The EIIA, EIIB and EIIC domains may all be fused into a single polypeptide (mannitol permease), may all be separate proteins (cellobiose permease), or may be fused in other combinations (glucose and mannose permeases) as shown. In addition to EIIC, the mannose permease also has a second transmembrane protein (EIID). Domain EIIA (also called Enzyme III or EIII if it is a separate protein) is the phospho-acceptor from P-HPr, and the phosphorylated residue in this domain is His in all PTS permeases for which this has been determined (His-554 in the mannitol permease). The EIIB domain is phosphorylated by the P-EIIA domain and the phosphorylated residue in this domain is Cys (at least in the mannitol permease). The substrate is translocated through the EIIC domain, and is phosphorylated by phospho-transfer from the EIIB domain. See Postma *et al.* (1993) for original references to the domain/protein organization of these PTS permeases.

with the odd number (5) of phosphotransfer steps shown in Scheme 1, and rules out a mechanism in which there is a single phospho-intermediate of the permease.

It has also been shown that phosphotransfer from His-554 to Cys-384 can occur between subunits of a permease dimer (Stephan *et al.*, 1989; van Weeghel *et al.*, 1991c; Weng *et al.*, 1992). In these studies, different inactive mutant proteins lacking an intact His-554 and an intact Cys-384, respectively, were either mixed *in vitro* or expressed in the same cell *in vivo*. In either case, PEP-dependent mannitol phosphorylation activity was restored, showing that intersubunit phosphotransfer is at least one mechanism for this reaction. Indeed, the dimer seems to be the major structure of the protein both *in vivo* and *in vitro* (reviewed in Jacobson, 1992; Postma *et al.*, 1993). As will be discussed below, while phosphorylation of the permease by P-HPr greatly accelerates mannitol transport by the IIC domain, phosphorylation of mannitol is not essential to the transport mechanism. Conversely, the purified permease (Jacobson *et al.*, 1979) and the membrane-bound permease (Lolkema *et al.*, 1991b) can phosphorylate mannitol independently of transport.

STRUCTURE AND FUNCTION OF THE IIC DOMAIN

Since the IIC domain is the integral-membrane portion of an EII, it is reasonable to presume that it is responsible for substrate binding and translocation. Indeed, a mutant of the mannitol permease in which the IIA and IIB domains had been deleted still bound mannitol with a similar affinity and stoichiometry as the wild-type protein (Grisafi *et al.*, 1989). Moreover, the IIC domain alone could carry out facilitated diffusion of mannitol at a slow rate (Elferink *et al.*, 1990; Lolkema *et al.*, 1991b), and it was shown that the phosphorylated form of the permease carries out the translocation of mannitol at a rate that is several orders of magnitude higher than the unphosphorylated form or than the IIC domain alone (Lolkema *et al.*, 1991b). Translocation of mannitol by the phosphorylated form of the permease was not strictly coupled to mannitol phosphorylation in these studies. Once translocated, over 50% of the mannitol molecules dissociated from the permease before they were phosphorylated. This free mannitol, however, could rapidly rebind to the "inward-facing" binding site, and subsequently be phosphorylated by the phospho-IIB domain (Lolkema *et al.*, 1991b). Thus, rapid translocation of mannitol by the permease is strictly coupled to phosphorylation of the EII (and presumably by a subsequent conformational change in the protein) rather than to phosphorylation of the substrate itself.

In order to understand how the IIC domain accomplishes mannitol binding and translocation, it will be necessary to know its structure in the membrane and the amino acid residues that participate in these functions. In the absence of a crystal structure, structure–function relationships in this "business end" of the mannitol permease have been probed primarily by molecular genetic and mutagenesis techniques. The current model of the secondary structure of the IIC domain of the mannitol permease, shown in Fig. 2, has been derived from a combination of structural prediction techniques (Jacobson and Stephan, 1989) and *'phoA* fusion analyses (Sugiyama *et al.*, 1991). It consists of six transmembrane helices (I-VI in Fig. 2) connected by five hydrophilic "loops" (2–6 in Fig. 2) comprising the N-terminal half of the permease (IIC domain; residues 1 to ca. 334). The periplasmic loops (2,4 and 6) are relatively small, while cytoplasmic loops 3 and 5 are relatively large. Residues ca. 335 to ca. 457 comprise the IIB domain including Cys-384, and residues ca. 458–637 (the C-terminus) comprise the IIA domain including His-554. Both of these latter domains are cytoplasmic.

MECHANISM OF TRANSLOCATION PROBED BY MUTAGENESIS OF *mtlA*

As mentioned earlier, site-directed mutagenesis of *mtlA* has been useful in understanding the mechanism of mannitol phosphorylation by the permease and further confirmed the direct involvement of His-554 and Cys-384 in the phosphotransfer mechanism. Mutagenesis of some amino acid residues in the IIC domain (circled and boxed residues in Fig. 2) has also given clues as to those residues in this part of the permease that are important for its activities (boxes) and those that are not (circles). Somewhat surprisingly, all of the residues in the IIC domain that have so far been identified as being important for mannitol binding, transport, and/or phosphorylation are located in an 85-residue segment comprising putative cytoplasmic loop #5 in the current model (Fig. 2). The mutations of these residues that have been studied, and the presumptive roles for these residues deduced from these studies, are listed in Table I.

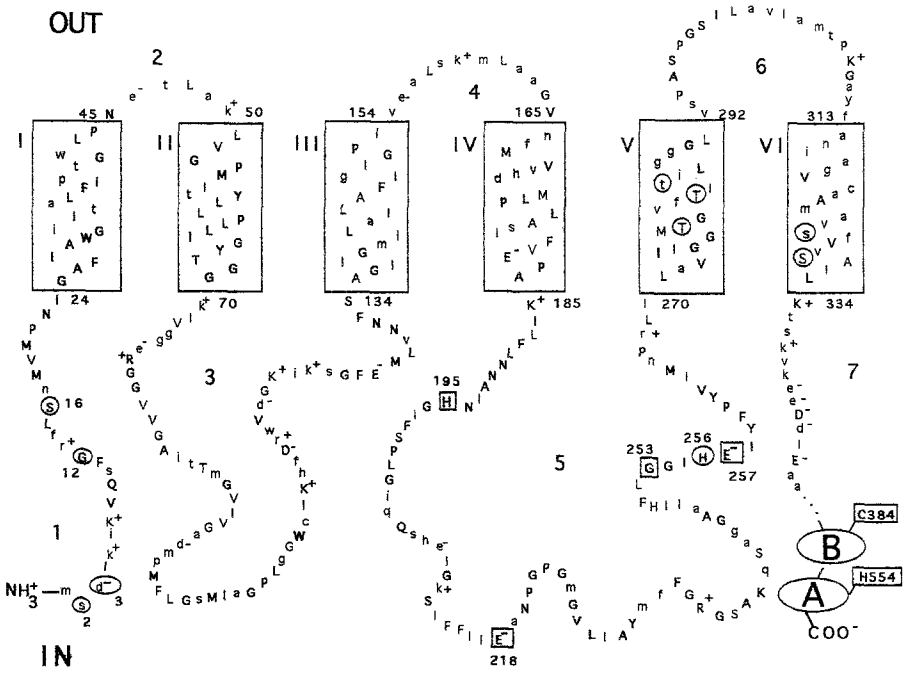

Fig. 2. Topological model for the mannitol permease of *E. coli* indicating residues that have been studied by mutagenesis. This model is, in part, derived from that reported in Sugiyama *et al.*, 1991. The amino acid sequence of the permease is that deduced from the sequence of the *mtlA* gene (Lee and Saier, 1983). Presumptive transmembrane helices (I-VI) are enclosed in rectangles. Loops connecting these helices are numbered 2 through 6 from the N-terminal region (#1). The sequences of the cytoplasmic domains A and B (#7) are not shown. Residues that are identical or are conservatively replaced in other bacterial PTS mannitol permeases that have been sequenced, including from *Staphylococcus carnosus* (Fischer and Hengstenberg, 1992) and *Streptococcus mutans* (A. Honeyman, personal communication) are shown in capital letters. Residues that are unconserved in these 3 proteins are shown as lower-case letters. Residues that have been mutated individually are boxed or circled. Boxes denote residues that, when mutated, significantly alter one or more functions of the permease (also see Table I). Circles denote residues that, when mutated, do not significantly alter its functions.

Table I. Important Amino Acid Residues in the Mannitol Permease as Determined by Mutagenesis Studies[a]

Residue	Domain	Mutations studied	Phenotype of mutant protein(s)	Role(s)
His-554	IIA	H554A, H554D	No phosphorylation by P-HPr	Phospho-acceptor from P-HPr
Cys-384	IIB	C384S, C384H, C384D	Cannot phosphorylate mannitol	Phospho-donor to mannitol
His-195	IIC	H195A, H195R	Lower affinity for mannitol; defective in mannitol phosphorylation	Mannitol binding and phosphorylation
Glu-218	IIC	E218A	Lower affinity for mannitol; facilitated diffusion of mannitol	Mannitol binding; coupling of permease phosphorylation to transport
Gly-253	IIC	G253E	Cannot transport but still phosphorylates mannitol (binding site locked in inward-facing state?)	Important for conformational change necessary for translocation (?)
Glu-257	IIC	G257A	Cannot bind mannitol (thus also cannot transport or phosphorylate mannitol)	Mannitol binding plus (?)

[a] References for each mutation listed are given in the text. The locations of these residues in the structural model of the permease are highlighted by boxes in Fig. 2.

Among the interesting mutant permeases isolated or constructed in loop 5 are: (1) G253E, which cannot transport but still can phosphorylate mannitol (Manayan *et al.*, 1988); (2) E218A which has a high K_m for mannitol but which can carry out facilitated diffusion of mannitol at a rate higher than the wild-type protein in its unphosphorylated form (J. Lengeler and H. Heuel, personal communication); (3) H195A and H195R which have a 40-fold higher K_D for mannitol that the wild-type protein ($K_D = 40$ nM) and are also defective in mannitol phosphorylation even at high mannitol concentrations (Weng and Jacobson, 1993); and (4) E257A which is normally expressed and inserted into the membrane but which does not detectably bind mannitol up to a concentration of at least 1 mM (C. Saraceni-Richards and G. Jacobson, unpublished data). Interestingly H195 and, especially, E257 are found in similar regions and sequence contexts in many EII's of very different substrate specificities (Lengeler, 1990; Saier *et al.*, 1992), suggesting that they may have fundamental roles in the mechanisms of these EII's.

Residues in the IIC domain that are not important for function on the basis of mutagenesis studies (circled in Fig. 2) include several at the extreme N-terminus (Q. P. Weng and G. Jacobson, unpublished data), His-256 (Weng *et al.*, 1992) which had previously been suggested as a phosphorylation site (Bramley and Kornberg, 1987; Saier *et al.*, 1988), and five potential H-bonding residues in helices V and VI (C. Briggs and G. Jacobson, unpublished data) at least some of which had previously been hypothesized to participate in mannitol binding (Briggs *et al.*, 1992).

The mutagenesis results show that regions in putative cytoplasmic loop #5 of the mannitol permease appear to be involved in mannitol binding, in mannitol translocation, and in mannitol phosphorylation, as summarized in Table I. Obviously, many more mutants will need to be studied to determine if residues in other parts of the IIC domain, including its transmembrane regions, are critical for these functions. If, however, loop 5 is indeed cytoplasmic, or at least does not completely span the membrane, then how does it participate in all of these steps leading to mannitol transport and phosphorylation? One model, consistent with the evidence, is that this region might act at the cytoplasmic surface of the protein as a "cap" or "gate" for a hydrophilic mannitol channel formed by amphipathic, transmembrane helices of the IIC domain (Postma *et al.*, 1993). Alternatively, this loop could project up into such a channel and act more as a "plug" (Lengeler, 1990). In either case, we hypothesize that this region comprises at least part of a mannitol-binding site at or near the end of the channel. Phosphorylation of the IIB domain of the permease could then favor a conformational change in the IIC domain involving this loop such that the binding site is now exposed to the cytoplasm allowing for mannitol translocation into the cell. Consistent with a conformational interaction between these domains, it has been shown that mutations at the second phosphorylation site (Cys-384) in the IIB domain effect the K_D for mannitol binding to the IIC domain (Lolkema *et al.*, 1991a; Weng and Jacobson, 1993). Clearly, however, much more work will be necessary to prove or disprove this model, and to define the precise role of this 85-residue region in mannitol transport and phosphorylation.

THE MANNITOL PERMEASE AS A PRIMARY CHEMOTACTIC RECEPTOR

Most flagellated bacteria that have been studied can swim up concentration gradients of certain nutrients. This behavior, chemotaxis, operates via a complex signal transduction pathway involving, in part, the phosphorylation and dephosphorylation of so-called Che proteins in the cytoplasm, which in turn influence flagellar rotation (reviewed in Bourret *et al.*, 1991). PTS permeases are one type of primary chemotactic receptor since elimination of a specific EII abolishes chemotaxis to its substrate (reviewed in Lengeler and Vogler, 1989; Postma *et al.*, 1993). Other types of chemotactic receptors are the methyl-accepting chemotaxis proteins (MCP's) which bind, but do not transport, their ligands. Binding of a ligand to an MCP influences the kinase activity of a protein, CheA, which modulates the phosphorylation of other Che proteins in the cell to elicit a chemotactic response (Bourret *et al.*, 1991).

Thus, it is also of interest to understand how the PTS permeases function as chemotactic receptors and feed information into the chemotactic signal transduction pathway. Recent evidence favors a mechanism in which the individual PTS permeases do not *directly* influence the phosphorylation states and activities of the Che proteins upon substrate binding as is the case for the MCP's. Rather, its appears to be the level of phosphorylation of one or more of the general PTS proteins, possibly HPr (Grübl *et al.*, 1990), that is the signal. This phosphorylation level, in turn, depends on whether PTS permeases are actively transporting (and

thus phosphorylating) their substrates (Postma *et al.*, 1993). In accord with this, it was recently shown by the use of site-directed mutants that the chemoreceptor activity of the mannitol permease correlated with its ability to transport and phosphorylate mannitol, and not simply with its ability to bind this ligand. Mutant proteins showing normal mannitol binding, but defective in transport and phosphorylation, invariably were unable to act as chemotactic receptors (Weng *et al.*, 1992). It is not yet known, however, exactly how the phosphorylation levels of PTS proteins influence the activities of the Che proteins. One obvious potential mechanism, phosphorylation of one of the Che proteins by a phospho-PTS protein, has yet to be demonstrated.

CONCLUSIONS AND PERSPECTIVES

The mannitol permease is arguably the best understood of the PTS permeases in terms of its structure and mechanism. Despite recent advances in our understanding of this protein, however, we are still largely ignorant of the precise molecular mechanism by which it carries out vectorial transport of its substrate. As has been true for the elucidation of mechanisms of soluble enzymes, this goal ultimately will require both a refined 3-dimensional structure of the protein and a continuation of biochemical, biophysical, and molecular genetic probes into structure–function relationships of this and other PTS permeases.

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