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Molecular Cloning of the C-Terminal Domain of *Escherichia coli* D-Mannitol Permease: Expression, Phosphorylation, and Complementation with C-Terminal Permease Deletion Proteins

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We have subcloned a portion of the *Escherichia coli mtlA* gene encoding the hydrophilic, C-terminal domain of the mannitol-specific enzyme II (mannitol permease; molecular mass, 68 kilodaltons [kDa]) of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system. This *mtlA* fragment, encoding residues 379 to 637 (residue 637 = C terminus), was cloned in frame into the expression vector pCQV2 immediately downstream from the λ p_r promoter of the vector, which also encodes a temperature-sensitive λ repressor. *E. coli* cells carrying a chromosomal deletion in *mtlA* (strain LGS322) and harboring this recombinant plasmid, pDW1, expressed a 28-kDa protein cross-reacting with antipermease antibody when grown at 42°C but not when grown at 32°C. This protein was relatively stable and could be phosphorylated in vitro by the general phospho-carrier protein of the phosphotransferase system, phospho-HPr. Thus, this fragment of the permease, when expressed in the absence of the hydrophobic, membrane-bound N-terminal domain, can apparently fold into a conformation resembling that of the C-terminal domain of the intact permease. When transformed into LGS322 cells harboring plasmid pGJ9-Δ137, which encodes a C-terminally truncated and inactive permease (residues 1 to ca. 480; molecular mass, 51 kDa), pDW1 conferred a mannitol-positive phenotype to this strain when grown at 42°C but not when grown at 32°C. This strain also exhibited phosphoenolpyruvate-dependent mannitol phosphorylation activity only when grown at the higher temperature. In contrast, pDW1 could not complement a plasmid encoding the complementary N-terminal part of the permease (residues 1 to 377). The pathway of phosphorylation of mannitol by the combined protein products of pGJ9-Δ137 and pDW1 was also investigated by using *N*-ethylmaleimide to inactivate the second phosphorylation sites of these permease fragments (proposed to be Cys-384). These results are discussed with respect to the domain structure of the permease and its mechanism of transport and phosphorylation.

The mannitol-specific enzyme II (EII^{Mtl} or mannitol permease) of the *Escherichia coli* phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is a 68-kilodalton (kDa), membrane-bound protein that catalyzes the concomitant transport and phosphorylation of D-mannitol in this organism. In addition to PEP as the original phospho donor, these reactions require the soluble PTS proteins enzyme I (EI) and HPr, which participate in the following cascade of phosphotransfer reactions (reviewed in references 17, 20, and 24):

$$\text{EI} + \text{PEP} \leftrightarrow \text{phospho-EI} + \text{pyruvate} \quad (1)$$

$$\text{Phospho-EI} + \text{HPr} \leftrightarrow \text{phospho-HPr} + \text{EI} \quad (2)$$

$$\text{Phospho-HPr} + EII^{Mtl} \leftrightarrow \text{phospho-}EII^{Mtl} + \text{HPr} \quad (3)$$

$$\text{Phospho-}EII^{Mtl} + \text{mannitol}_{(out)} \rightarrow EII^{Mtl} + \text{mannitol-1-phosphate}_{(in)} \quad (4)$$

Reactions 1 and 2, involving the general PTS phospho-carrier proteins EI and HPr, take place in the cytoplasm of the cell, reaction 3 takes place on the inner surface of the cytoplasmic membrane, and reaction 4 occurs on and within the membrane, leading to the vectorial phosphorylation and transport of mannitol into the cell.

EII^{Mtl} has been purified (4) and characterized (reviewed in references 5 and 20), and its gene, *mtlA*, has been cloned and sequenced (8). Hydropathy analysis of the deduced amino acid sequence of EII^{Mtl} suggested that it consists of at least two domains: a hydrophobic, N-terminal domain (residues 1 to 334) largely embedded in the membrane and a hydrophilic, C-terminal domain (residues 335 to 637) exposed at the membrane surface. Subsequently, it was shown directly that the C-terminal domain was exposed at the cytoplasmic surface of the membrane and could be released from the membrane by mild trypsinolysis of everted membrane vesicles (27).

Recent experiments have provided evidence for both the structural and functional independence of these two domains of EII^{Mtl}. The C-terminal domain is important for the phosphorylation function of the protein and contains at least two sites of covalent phosphorylation: His-554, the phospho acceptor from phospho-HPr, and Cys-384, which has been suggested to be the direct phospho donor to mannitol (3, 14–16). When partially purified after trypsinolysis of everted membrane vesicles, this domain can be phosphorylated at both sites by phospho-HPr in the complete absence of the N-terminal domain (28). On the other hand, the N-terminal domain of EII^{Mtl} contains a high-affinity binding site for mannitol that is independent of the C-terminal domain and appears to be properly inserted into the membrane in the complete absence of the C-terminal domain. Both domains, however, are necessary for the transport and phosphorylation of mannitol by the protein (3).

In this report, we describe the construction of a temperature-inducible expression vector for the synthesis of most of the C-terminal domain of EII^{Mtl} and describe some properties of the protein expressed in a strain of *E. coli* containing a chromosomal deletion of *mtlA*. These experiments not only provide further evidence for the structural and func-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
Strains		
E. coli K-12 LGS322	F⁻ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50 gutPO49 gutA50* Δ(*mtlA'p*) *mtlD*(Con) Δ(*gutR'MDBAp-recA*)	3
S. typhimurium LJ144	*cpd-401 cysA1150*(F'198) (*pts*⁺ on *cysA*⁺)	M. Saier
Plasmids		
pGJ9	Cm *mtlAp* on pACYC184	3
pGJ9-ΔSnaBI	pGJ9 Δ(*mtlA*) from stop codon to codon 378	3
pGJ9-Δ(number)	pGJ9 Δ(*mtlA*)[a]	3
pCQV2	Ap	18
pDW1	Ap *mtlA* (codons 379–637) on pCQV2	This study

[a] See reference 3.

tional independence of this domain but also show that it can functionally complement a deletion protein lacking the first phosphorylation site of EII^{Mtl} (His-554) but not a deletion protein lacking both phosphorylation sites. These results are discussed with regard to both the structure and phosphorylation mechanism of EII^{Mtl}.

MATERIALS AND METHODS

Chemicals and enzymes. [γ-^{32}P]ATP (3,000 Ci/mmol), ^{125}I-labeled *Staphylococcus aureus* protein A (9.4 mCi/mg), and [^{14}C]mannitol (45 mCi/mmol) were obtained from Dupont, NEN Research Products (Boston, Mass.). Phosphorylated BamHI linkers (10-mer), T4 DNA ligase, and restriction enzymes were products of New England BioLabs, Inc. (Beverly, Mass.). Calf intestinal phosphatase was a product of Pharmacia, Inc. (Piscataway, N.J.). All other chemicals used were reagent grade.

Bacterial strains, cell growth, and preparation of cell-free fractions. The bacterial strains used are listed in Table 1. *E. coli* K-12 strain LGS322 was grown in liquid culture or on solid media as described previously (3). Antibiotics, when present, were added to media to a final concentration of 50 mg/liter. For ^{32}P-labeling studies, 1-liter cultures were harvested at mid-logarithmic phase, after the addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM, by centrifugation at 10,000 × *g* for 10 min at 4°C. The cells were suspended in 5 ml of TDM buffer (50 mM Tris hydrochloride [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol), followed in some cases by incubation with 2 mM mannitol or 2 mM glucose for 60 min at 30°C to dephosphorylate EII^{Mtl} or its deletion fragments. Cells were lysed in a French pressure cell at 10,000 lb/in^2, the extract was centrifuged at 10,000 × *g* for 5 min to remove unbroken cells, and the supernatant from the low-speed centrifugation was centrifuged at 100,000 × *g* for 90 min at 4°C. The supernatant (cytoplasmic fraction) was recentrifuged to remove residual membranes, concentrated to ca. 5 mg of protein per ml in a Centricon microconcentrator (Amicon Corp., Danvers, Mass.), and stored at −20°C. The pellet from the first high-speed spin (membrane fraction) was suspended in 1 ml of TDM buffer and also stored at −20°C. Cell-free fractions for in vitro reconstitution of mannitol PTS activity were prepared in the same manner except that cells from 2-liter cultures were suspended in 2 ml of buffer before lysis and the cytoplasmic fractions were not further concentrated.

For immunoblotting (see below), mid-logarithmic-phase cultures were treated with EDTA and phenylmethylsulfonyl fluoride to final concentrations of 1 mM each. Samples of 1.0 ml were removed and spun at 15,000 × *g* for 5 min at 4°C, and the cell pellets were extracted with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (see below) for 3 min at 100°C before electrophoresis. *S. typhimurium* LJ144 (used as a source of EI and HPr) was grown and harvested, and cell-free fractions were prepared as described previously (1).

Plasmids and cloning techniques. The plasmids used are listed in Table 1. Plasmid DNA was purified from *E. coli* lysates by equilibrium density centrifugation in cesium chloride-ethidium bromide gradients (12). Transformations were carried out by the procedure of Davis et al. (2). Miniprep analysis was performed by the modified alkaline lysis method as described by Maniatis et al. (12). Restriction endonucleases and other enzymes were used as recommended by the supplier. DNA fragments were analyzed by electrophoresis in 0.8% agarose gels, using phage λ DNA restriction fragments as molecular weight markers. DNA was purified from agarose by the NaI-glass powder procedure (29). Ligations were performed at 15°C for 12 h, using a 5- to 10-fold molar excess of linker or insert in a volume of 20 μl.

In vitro labeling of proteins with [^{32}P]PEP. [^{32}P]PEP was prepared from [^{32}P]ATP according to the method of Roossien et al. (22) and was purified as described by Stephan et al. (28) (final concentration, 25 μM; 1,460 Ci/mol). Cell-free fractions (2.5 mg of protein per ml) were radiolabeled in a mixture containing 1.3 mg of *Salmonella* cytoplasmic protein per ml (as a source of EI and HPr), 0.5 μM [^{32}P]PEP, 12 mM Tris hydrochloride (pH 7.5), 2 mM dithiothreitol, 50 mM NaH_2PO_4 (pH 7.5), 1 mM NaF, and 0.5 mM $MgCl_2$. The reaction was terminated by the addition of SDS-PAGE sample buffer (see below).

Chemical modification with NEM. Membrane (10 mg of protein per ml) and cytoplasmic (10 mg of protein per ml) fractions were treated with 30 mM *N*-ethylmaleimide (NEM) for 45 min at 30°C to inactivate Cys-384 of intact EII^{Mtl} or its deletion fragments (14–16). This was followed by addition of 60 mM dithiothreitol and a further incubation for 30 min at 30°C to remove unreacted NEM. These treated fractions and untreated controls were used in in vitro assays of PEP-dependent mannitol phosphorylation as detailed below.

Electrophoresis, autoradiography, and immunoblotting. ^{32}P-labeled protein mixtures were extracted with buffer containing 62.5 mM Tris hydrochloride (pH 8.8), 2% SDS, 10% glycerol, and 0.003% bromphenol blue for 30 min at 30°C. For electrophoresis and immunoblotting of unlabeled proteins, the sample buffer used was identical except that pH 6.8 was used and the samples were heated to 100°C for 10 min. SDS-PAGE was carried out in 10% polyacrylamide slabs, using the buffer system of Weber and Osborn (30). Lanes containing molecular weight markers were cut off and stained with Coomassie brilliant blue, whereas lanes containing ^{32}P-labeled proteins were washed for 1 h in 0.1 M NaOH–50% methanol, dried, and exposed to Kodak XAR-5 X-ray film at −70°C without staining. Electrophoretic blotting of proteins from gels onto nitrocellulose and visualization of proteins cross-reacting with anti-EII^{Mtl} antibody, using ^{125}I-labeled *S. aureus* protein A, were carried out as described by Stephan and Jacobson (27).

Assays. For whole-cell PTS assays, mid-logarithmic-phase

cells were harvested at 15,000 × *g* for 10 min at 4°C and washed twice with TDM buffer. Cells in 1.0 ml of this suspension were permeabilized by addition of 15 μl of toluene, followed by vigorous agitation for 45 s and a 20-min incubation at 37°C. PEP-dependent mannitol phosphorylation was determined in 1.0-ml mixtures containing 25 mM Tris hydrochloride (pH 8.0), 1 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM KF, 0.1 mM [^{14}C]mannitol (4.5 mCi/mmol), and 0.5 ml of the permeabilized cells in both the presence and absence of 1 mM PEP. In vitro assays using membrane and cytoplasmic fractions were conducted identically except that the permeabilized cells were replaced by the appropriate membrane and cytoplasmic fractions (1.0 and 3.5 mg of protein per ml, respectively), and the *Salmonella* cytoplasmic fraction (0.25 mg of protein per ml) was added to ensure saturating levels of EI and HPr. In all cases, assay mixtures were incubated at 37°C, and 100-μl samples were withdrawn at appropriate intervals, added to 1 ml of ice-cold H_2O, and filtered through DEAE-cellulose filter disks (DE81; Whatman, Inc., Clifton, N.J.). After three washes with 5 ml of ice-cold H_2O, the filters were air dried and counted in standard toluene-based scintillation fluid. PTS activity was calculated as the difference between mixtures containing and lacking PEP in all cases.

Protein concentrations were determined by the method of Lowry et al. (11), with bovine serum albumin as the standard.

RESULTS

Construction of plasmid pDW1 and expression of the C-terminal domain of EII^{Mtl}. The experimental protocol for the construction of plasmid pDW1 encoding most of the C-terminal domain of EII^{Mtl} (outlined in Fig. 1) involved the following. Plasmid pGJ9, which contains the *mtlA* gene, was digested at its unique *Sna*BI site, which lies within codon 378 of *mtlA* (3). Phosphorylated *Bam*HI linkers (10-mer) were blunt end ligated onto this linearized molecule, followed by digestion of this mixture with *Bam*HI. This digestion cuts at the *Bam*HI sites of the incorporated linkers as well as at a unique *Bam*HI site of pGJ9 located 68 nucleotides downstream from the *mtlA* stop codon. This latter site was inadvertently created in the original construction of pGJ9 (data not shown). The products of this digestion were electrophoretically separated, and the 0.8-kilobase-pair fragment coding for the C-terminal portion of the permease was isolated and purified. This fragment was ligated to *Bam*HI-digested, calf intestinal phosphatase-treated plasmid pCQV2, an expression vector that uses bacteriophage λ regulatory elements for temperature-inducible protein synthesis (18). This vector contains the λ p_r promoter under the control of a vector-encoded, temperature-sensitive repressor and a downstream *Bam*HI site into which DNA can be cloned for temperature-inducible expression (Fig. 1). The products of this ligation were transformed into *E. coli* LGS322 containing a chromosomal deletion of *mtlA* (Table 1), and ampicillin-resistant transformants were selected. Restriction analysis of plasmid DNA identified a number of transformants harboring recombinant molecules containing two *Bam*HI sites and internal sequences of the insert (data not shown).

To identify those constructs carrying the insert in both the correct orientation and the proper coding frame, immunoblotting was performed on whole-cell extracts, using polyclonal antibody against EII^{Mtl} (7). This resulted in the identification of a cell line producing an immunoreactive

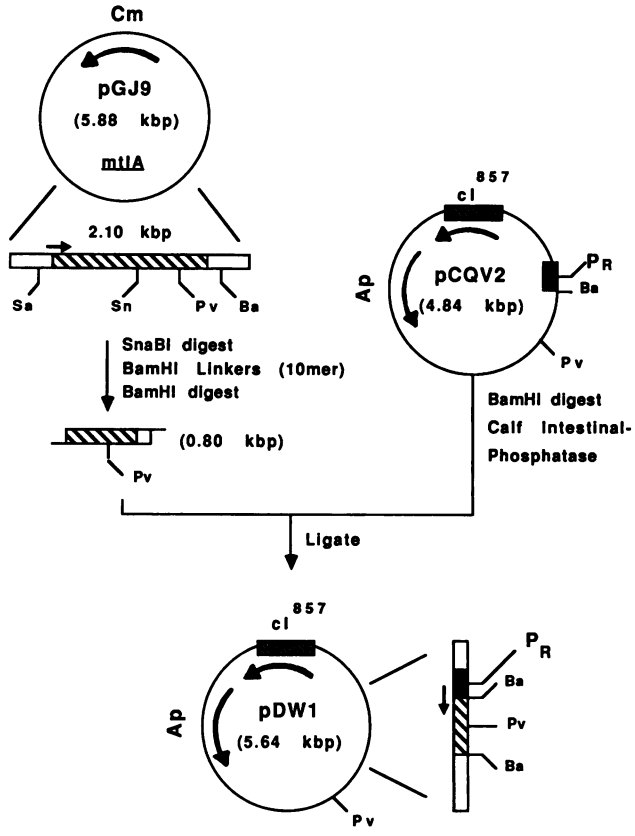

FIG. 1. Construction of pDW1. Plasmid pGJ9, which contains the *mtlA* gene (3), was digested at its unique *Sna*BI restriction site within codon 378 in *mtlA*. Phosphorylated *Bam*HI linkers (10-mer) were blunt end ligated to this molecule, followed by digestion with *Bam*HI. The products of this digestion were separated by agarose gel electrophoresis, and the 0.80-kilobase-pair (kbp) fragment coding for residues 379 to 637 of EII^{Mtl} was cut from the gel and purified as described in Materials and Methods. This fragment was then ligated to *Bam*HI-digested, calf intestinal phosphatase-treated plasmid pCQV2. The desired construct, pDW1, was identified by restriction mapping and immunological detection of the protein product (see text). The *mtlA* gene (▨), the polymerase recognition sequence from bacteriophage λ (p_r) (■), and the gene encoding the temperature-sensitive repressor of this regulatory region (cI857) (▩) are indicated. These regions are not necessarily drawn to scale. Arrows denote transcription directions. Abbreviations for restriction sites: Ba, *Bam*HI; Pv, *Pvu*II; Sa, *Sal*I; Sn, *Sna*BI.

protein with a molecular mass of 28 kDa when cells were grown under induced conditions (42°C [Fig. 2, lane 5]) that was absent in cells grown at 32°C (lane 4). Since cell extracts of strain LGS322 alone (lane 1) or LGS322 harboring pCQV2 (lane 2), both grown at 42°C, were immunonegative, it was concluded that a recombinant plasmid had been identified that expresses the C-terminal domain of EII^{Mtl}. This plasmid, pDW1 (Table 1 and Fig. 1), is predicted to encode a 263-amino-acid polypeptide that has a sequence of NH_2-Met-Asp-Pro-Cys (from the start codon of pCQV2 and the sequence of the *Bam*HI linker), followed by residues 379 to 637 of the native permease. The molecular weight of the protein produced (Fig. 2) is in excellent agreement with its predicted size. Only a single immunoreactive band was detected in strain LGS322(pDW1) grown at 42°C, and no proteolytic breakdown products were observed. This was true even in cells that had been induced at 42°C for a period

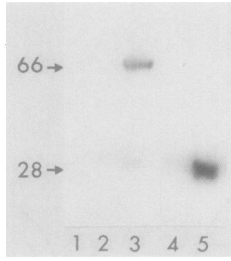

FIG. 2. Immunodetection of the product of pDW1. Cultures of *E. coli* LGS322 containing various plasmids were grown to mid-logarithmic phase at 32 or 42°C, harvested, extracted with SDS-PAGE sample buffer, electrophoresed, and immunoblotted as described in Materials and Methods. Lanes: 1, LGS322 (no plasmid) grown at 42°C; 2, LGS322(pCQV2) grown at 42°C; 3, LGS322(pGJ9) grown at 37°C; 4, LGS322(pDW1) grown at 32°C; 5, LGS322(pDW1) grown at 42°C. Molecular sizes were determined from protein standards run concurrently and are shown in kilodaltons on the left.

of several hours (data not shown), suggesting that the product of pDW1 is relatively stable to proteolysis.

In vitro phosphorylation of the product of pDW1. To determine whether the product of pDW1 could serve as a phosphorylation substrate for phospho-HPr, [^{32}P]PEP-dependent phosphorylation experiments were performed. Mid-logarithmic-phase cultures of strain LGS322(pDW1) grown at both 32 and 42°C were harvested, and cytoplasmic fractions were prepared as described in Materials and Methods. These samples were then incubated with [^{32}P]PEP, EI, and HPr, followed by SDS-PAGE and autoradiography. In the absence of LGS322(pDW1) cytoplasm, only two bands at 70 and 15 kDa were observed, corresponding to EI and HPr, respectively (Fig. 3, lane 1). This was also true for LGS322 cytoplasm from cells lacking pDW1 and grown at 42°C and for cytoplasm from LGS322(pDW1) cells grown at 32°C (Fig. 3, lanes 2 and 3, respectively). However, a ^{32}P-labeled band was observed at 28 kDa with cytoplasm from LGS322 (pDW1) cells grown at 42°C (Fig. 3, lane 4), showing that the product of this plasmid can be phosphorylated by phospho-HPr.

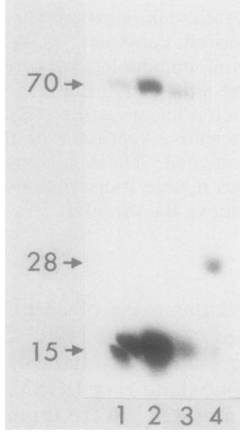

FIG. 3. In vitro phosphorylation of the product of pDW1. Cytoplasmic fractions from *E. coli* LGS322 with and without plasmid pDW1 were prepared and labeled with [^{32}P]PEP, EI, and HPr from an *S. typhimurium* extract as described in Materials and Methods. After labeling, the samples were electrophoresed and autoradiographed, also as described in Materials and Methods. Lanes: 1, *S. typhimurium* extract alone showing labeled EI and HPr at ca. 70 and 15 kDa, respectively; 2, same as lane 1 plus the cytoplasmic fraction from LGS322 (no plasmid) grown at 42°C; 3, same as lane 1 plus the cytoplasmic fraction from LGS322(pDW1) grown at 32°C; 4, same as lane 1 plus the cytoplasmic fraction from strain LGS322(pDW1) grown at 42°C. Molecular sizes were determined from protein standards run concurrently and are shown in kilodaltons on the left.

TABLE 2. Fermentation properties of strain LGS322 containing various plasmids

LGS322 carrying plasmid(s):	Size of protein product (kDa)[a]	Temp (°C)	Mannitol fermentation[b]
pGJ9	65	32 or 42	+
pDW1		32 or 42	−
pDW1 plus:			
pGJ9-Δ124	63	32	−
		42	+
pGJ9-Δ104	60	32	−
		42	+
pGJ9-Δ130	56	32	−
		42	+
pGJ9-Δ117	52	32	−
		42	+
pGJ9-Δ137	51	32	−
		42	+
pGJ9-ΔSnaBI	34	32 or 42	−
pGJ9-Δ21	33	32 or 42	−

[a] Refers to the membrane-bound product of the pGJ9 derivative (3).
[b] Determined after 24 h on MacConkey-mannitol plates. LGS322 cells containing all pGJ9 deletions listed were negative for mannitol fermentation at both 32 and 42°C in the absence of plasmid pDW1 in the same strain.

Functional complementation between the product of pDW1 and C-terminal deletion mutants of EII^{Mtl}. To determine whether the protein product of pDW1 could functionally complement deletion mutants of EII^{Mtl} lacking part or all of the C-terminal domain, pDW1 was transformed into strain LGS322 harboring a number of deletion plasmids that we have constructed from pGJ9 (3). These deletion plasmids encode C-terminally truncated EII^{Mtl} molecules that are totally inactive in PEP-dependent mannitol phosphorylation, and LGS322 cells containing these plasmids therefore form white colonies on MacConkey-mannitol plates (3). Transformants were selected on Luria broth agar plates containing both chloramphenicol (to select for pGJ9 derivatives) and ampicillin (to select for pDW1). DNA miniprep analysis confirmed the presence of both plasmids in the respective transformants, and the expression of both protein products in some of these cells grown at 42°C also was verified by immunoblotting of crude cell extracts (not shown). These experiments also revealed that no recombination had occurred between the two plasmids in these strains to yield an intact *mtlA* gene, since no immunoreactive protein with a size of 65 to 68 kDa was detected. This result was expected, since strain LGS322 is *recA* (Table 1).

To determine whether any of these cells containing C-terminal deletion plasmids and pDW1 could ferment mannitol, transformants were plated onto MacConkey-mannitol plates and incubated at either 32 or 42°C (Table 2). All C-terminal deletion plasmids that produced proteins of ≥51 kDa (e.g., pGJ9-Δ137) were complemented for mannitol fermentation by pDW1 in cells grown at the higher temperature but not in cells grown at 32°C. In contrast, deletion proteins with sizes of ≤34 kDa were not complemented in cells grown at 42°C by the product of pDW1. The latter included the product of pGJ9-ΔSnaBI, which encodes a protein that contains all of the amino acids missing in the product of pDW1 (residues 1 to 377) except for one (Arg-378) (3). Thus, some amino acid sequence overlap between the

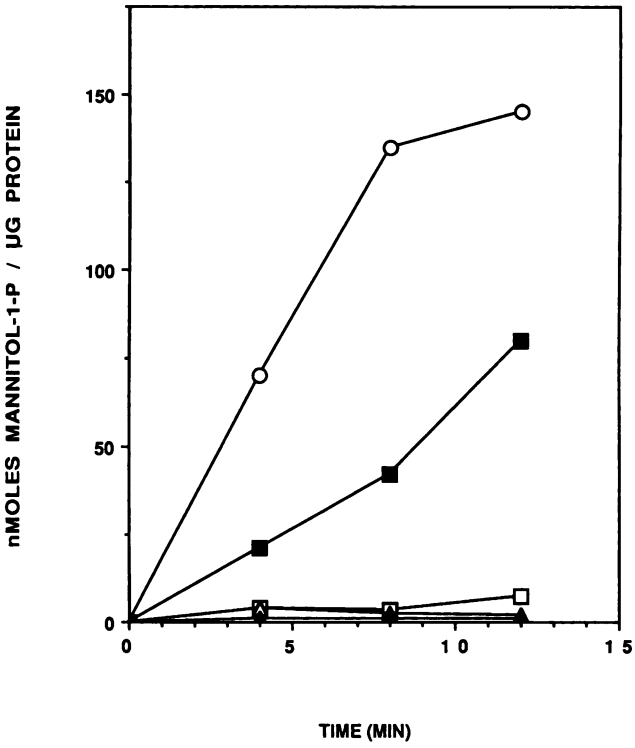

FIG. 4. Complementation of C-terminal deletions of EII^{Mtl} by the product of pDW1 in PEP-dependent mannitol phosphorylation, using permeabilized whole cells. Cells were grown, harvested, permeabilized with toluene, and assayed as described in Materials and Methods. Symbols: ○, strain LGS322(pGJ9) (intact EII^{Mtl} control) grown at 37°C; □, strain LGS322(pDW1, pGJ9-Δ137) grown at 32°C; ■, strain LGS322(pDW1, pGJ9-Δ137) grown at 42°C; △, strain LGS322(pDW1, pGJ9-ΔSnaBI) grown at 32°C; ▲, strain LGS322(pDW1, pGJ9-ΔSnaBI) grown at 42°C.

C-terminal portion of the deletion proteins and the N-terminal portion of the product of pDW1 is apparently necessary for complementation to occur. The extent of this overlap sufficient for complementation could not be further determined, since no deletion plasmids have yet been identified that produce stable protein products with sizes of between 34 and 51 kDa (3; also see Discussion).

To provide further evidence that the Mtl^+ phenotypes observed were due to reconstitution of EII^{Mtl} activity, whole cells of strains LGS322(pDW1, pGJ9-Δ137) and LGS322 (pDW1, pGJ9-ΔSnaBI) were grown at 32 and 42°C, permeabilized with toluene, and assayed for PEP-dependent mannitol phosphorylation activity. As shown in Fig. 4, strain LGS322(pDW1, pGJ9-Δ137), when grown at the higher temperature, exhibited approximately 45% of the activity of a control strain containing the intact EII^{Mtl} [LGS322(pGJ9)]. The former strain when grown at 32°C and strain LGS322(pDW1, pGJ9-ΔSnaBI) grown at either temperature exhibited no significant activity above background, in agreement with the fermentation results.

In vitro protein complementation and the mechanism of phosphotransfer. To further investigate the mechanism of protein complementation, cells of strains LGS322(pDW1) and LGS322(pGJ9-Δ137) were grown (the former at 42°C and the latter at 37°C) and harvested, and membrane and cytoplasmic fractions were prepared from each (see Materials and Methods). The membrane fraction of LGS322(pGJ9-Δ137) contains the product of pGJ9-Δ137 (3), whereas the cytoplasmic fraction of LGS322(pDW1) contains the product of pDW1 (see above). Significant PEP-dependent mannitol phosphorylation activity could be observed only in assay mixtures containing both membranes of the former strain and cytoplasm from the latter strain (Table 3), and this activity was comparable to that observed in assay mixtures containing the intact permease [membranes from LGS322 (pGJ9); not shown]. Thus, complementation could be observed in vitro as well as in permeabilized whole cells.

TABLE 3. Effects of NEM on in vitro reconstitution of PEP-dependent mannitol phosphorylation, using cell-free fractions from strains LGS322(pDW1) and LGS322(pGJ9-Δ137)

Fraction(s)[a]	PEP-dependent mannitol phosphorylation[b] (nmol/min per mg of protein)
Cytoplasm from LGS322(pDW1)	<0.01
Membranes from LGS322(pGJ9-Δ137)	<0.01
Membranes + cytoplasm	0.35
Membranes (NEM)[c] + cytoplasm	0.01
Membranes + cytoplasm (NEM)	0.20
Membranes (NEM) + cytoplasm (NEM)	<0.01

[a] Cell-free fractions were prepared from 2-liter, mid-logarithmic-phase cultures of strains LGS322(pDW1) and LGS322(pGJ9-Δ137) as described in Materials and Methods.

[b] Determined as described in Materials and Methods. Specific activities are based on the amount of membrane protein present except for the first entry, which is based on the amount of cytoplasmic protein present.

[c] (NEM), Treated with NEM as described in Materials and Methods.

The protein product of pGJ9-Δ137 contains the second phosphorylation site of EII^{Mtl} (proposed to be Cys-384 [15]) but not the first (His-554), whereas the product of pDW1 has both phosphorylation sites. Therefore, the question arises as to whether it is Cys-384 of the C-terminal deletion protein or the comparable cysteine in the product of pDW1 that is necessary for the complementation to occur. To determine this, we treated portions of the membrane and cytoplasmic fractions from these strains with NEM, which reacts with, and inactivates, Cys-384 (14–16). Various mixtures of NEM-treated and untreated fractions were then assayed for PEP-dependent mannitol phosphorylation activity in the in vitro assay (Table 3). Prior NEM treatment of membranes from LGS322(pGJ9-Δ137) completely inactivated reconstitution of activity regardless of whether the cytoplasmic fraction from LGS322(pDW1) was treated with NEM. In contrast, treatment of the cytoplasmic fraction from LGS322(pDW1) with NEM inhibited the reconstituted activity with untreated LGS322(pGJ9-Δ137) membranes less than 50% (Table 3). These results would suggest that Cys-384 of the product of pGJ9-Δ137, but not of the product of pDW1, is required for the complementation activity of these proteins.

DISCUSSION

In an ongoing effort to understand in detail the catalytic mechanism of sugar translocation by EII^{Mtl} of the bacterial PTS, we have constructed an expression vector for the inducible synthesis of most of the C-terminal domain of this protein (residues 379 to 637). This plasmid, pDW1, when transformed into an *E. coli* strain with a chromosomal deletion in *mtlA*, directed the synthesis of a stable, 28-kDa polypeptide that cross-reacted with anti-EII^{Mtl} antibody when cells were grown under induction conditions (42°C). Moreover, this polypeptide could be phosphorylated in vitro by [^{32}P]PEP in the presence of EI and HPr, demonstrating that it folds into a conformation similar to that of the C-terminal domain of intact EII^{Mtl}, at least as far as the

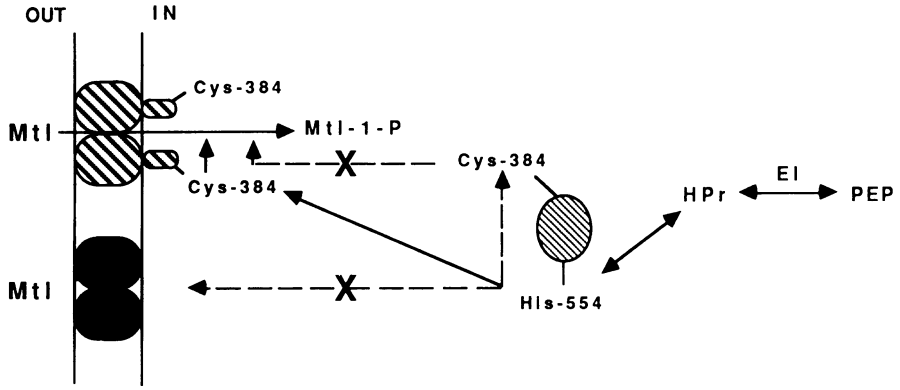

FIG. 5. Reconstitution of EII^{Mtl} activity in cells expressing a C-terminal deletion plasmid and pDW1. The product of pDW1 could complement that of pGJ9-Δ137, but not of pGJ9-ΔSnaBI, in PEP-dependent phosphorylation of mannitol. This finding suggests that some sequence overlap is necessary for this complementation (also see text). Experiments using NEM inactivation of cell-free fractions from strains LGS322(pDW1) and LGS322(pGJ9-Δ137) suggested that phosphotransfer between the two protein products and to mannitol proceeds by the pathway depicted schematically. Symbols: ▨, product of pDW1; ▨, product of pGJ9-Δ137; ▨, product of pGJ9-ΔSnaBI.

interaction site with phospho-HPr is concerned. These results show that none of the N-terminal 60% of EII^{Mtl} (residues 1 to 378) is necessary for the C-terminal domain to adopt this conformation, further demonstrating the structural autonomy of this domain (see also reference 28).

Experiments using C-terminally truncated deletion mutants of EII^{Mtl} showed that the product of pDW1 could functionally complement deletion proteins lacking up to at least ca. 150 amino acid residues of the C terminus of EII^{Mtl} (e.g., the product of pGJ9-Δ137). In contrast, the cloned C-terminal domain could not functionally complement a deletion protein containing all but one of the amino acid residues lacking in the product of pDW1 (residues 1 to 377; product of pGJ9-ΔSnaBI). These results suggest either that some overlap in amino acid sequence is necessary for complementation to occur or that the products of pDW1 and pGJ9-ΔSnaBI cannot form a functional complex for some other reason (e.g., because the N terminus of the product of pDW1 is very near the proposed active-site cysteine, Cys-384 of the intact protein). If overlap is necessary, this may reflect the proposed need for an at least transient dimer of EII^{Mtl} during its transport and phosphorylation functions (6, 10, 13, 19, 21, 26). Unfortunately, the extent of sequence overlap necessary to observe complementation with the product of pDW1 could not be further determined because none of the deletion plasmids producing C-terminally truncated EII^{Mtl} molecules that we have tested produce stable proteins in the size range between 34 and 51 kDa (3).

Experiments using NEM to modify Cys-384 in the product of pGJ9-Δ137, or the corresponding cysteine in the product of pDW1, provided further information regarding the mechanism of complementation. The results showed that Cys-384 of the former protein, but not that of the latter, was essential for reconstitution of mannitol phosphorylating activity. Thus, phosphotransfer must proceed from phospho-HPr to the histidyl residue of the product of pDW1 corresponding to His-554 of the native permease, then to Cys-384 of the product of pGJ9-Δ137, and finally to mannitol, as depicted schematically in Fig. 5. Apparently, therefore, the cysteinyl residue of the product of pDW1 corresponding to Cys-384 of native EII^{Mtl} cannot transfer a phospho group directly to mannitol bound to the product of pGJ9-Δ137 (Fig. 5), even though these activity-linked cysteines are close to one another in a dimer of native EII^{Mtl} (23). We have recently demonstrated intersubunit phosphotransfer between the two phosphorylation sites in an EII^{Mtl} heterodimer consisting of an intact, NEM-inactivated subunit and the product of pGJ9-Δ137 (28). It is therefore possible that such intersubunit phosphotransfer between these two different residues is essential to the activity of native EII^{Mtl}. If so, and if Cys-384 must be present on the same subunit that contains the mannitol-binding site (the N-terminal domain; 3) in order for phosphotransfer to mannitol to occur, then overlap between the product of pDW1 and a membrane-bound C-terminal deletion protein may be necessary for complementation simply because Cys-384 is required in the latter protein. Thus, the question remains open as to whether this overlap is necessary only for this reason or also for interaction between the two proteins as postulated above.

While the assignment of Cys-384 as the direct phospho donor to mannitol is supported by the available evidence (3, 14–16), absolute proof for this assignment has not yet been obtained. However, that EII^{Mtl} contains two different essential phosphorylation sites now seems certain on the basis of recent experiments investigating the stereochemical course of the phosphotransfer reactions catalyzed by EII^{Mtl} (E. Mueller, S. S. Khandekar, J. R. Knowles, and G. R. Jacobson, manuscript in preparation). It is also clear that NEM treatment of EII^{Mtl} results in reaction with Cys-384, with concomitant inactivation of the second phosphorylation site (14–16).

The expression vector coding for the inducible synthesis of the C-terminal domain of EII^{Mtl}, described in this report, should be useful for further investigations on the structure and mechanism of this protein and also for more general studies of the bacterial PTS and its multiple roles in the cell. Strains overproducing this domain could be used to purify it for physical and structural studies. Moreover, overproduction of this protein in cells could have effects on the pools of PEP and of phosphorylated PTS proteins, especially under conditions of energy limitation, since this domain could act as a dead-end sink for phosphate from PEP. Since the concentrations of phosphorylated PTS proteins regulate other cellular processes (reviewed in reference 25) and also are likely to be involved in signaling during chemotaxis of *E. coli* toward PTS sugars (9), the effects of expression of this domain on these processes could provide further insight to the physiological and regulatory roles of the PTS. Experiments designed to test these possibilities are now in progress in our laboratory.

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