Hydrophilic C-Terminal Domain of the *Escherichia coli* Mannitol Permease: Phosphorylation, Functional Independence, and Evidence for Intersubunit Phosphotransfer†

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ABSTRACT: The mannitol-specific enzyme II (mannitol permease) of the *Escherichia coli* phosphotransferase system (PTS) catalyzes the concomitant transport and phosphorylation of D-mannitol. Previous studies have shown that the mannitol permease (637 amino acid residues) consists of 2 structural domains of roughly equal size: an N-terminal, hydrophobic, membrane-bound domain and a C-terminal, hydrophilic, cytoplasmic domain. The C-terminal domain can be released from the membrane by mild proteolysis of everted membrane vesicles [Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 8230–8234]. In this report, we show that phosphorylation of the intact permease by [^{32}P]HPr (a general phosphocarrier protein of the PTS) followed by tryptic separation of the two domains resulted in labeling of only the C-terminal domain. Phosphorylation of the C-terminal domain occurred even in the complete absence of the N-terminal domain, showing that the former contains most, if not all, of the critical residues comprising the interaction site for phospho-HPr. The phosphorylated C-terminal domain, however, could not transfer its phospho group to mannitol, suggesting that the N-terminal domain is necessary for mannitol binding and/or phosphotransfer from the enzyme to the sugar. The elution profile of the C-terminal domain after molecular sieve chromatography showed that the isolated domain is monomeric, unlike the native permease which is likely a dimer in the membrane. Experiments employing a deletion mutation of the *mtlA* gene, which encodes a protein lacking the first phosphorylation site in the C-terminal domain (His-554) but retaining the second phosphorylation site (Cys-384), demonstrated that a phospho group could be transferred from phospho-HPr to Cys-384 of the deletion protein, and then to mannitol, only in the presence of the full-length permease. These results establish that the C-terminal domain of the mannitol permease is functionally independent from the N-terminal, intramembrane domain and that productive intersubunit phosphotransfer can occur between the C-terminal domains of two different subunits in a permease dimer. They also suggest that the C-terminal domain may be comprised of at least two structural and functional subdomains.

The mannitol-specific enzyme II (EII^{mtl} or mannitol permease) of the *Escherichia coli* phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) is responsible for the coupled transport and phosphorylation of D-mannitol in reactions requiring PEP and the general phosphocarrier proteins of the PTS, enzyme I and HPr [for reviews, see Postma and Lengeler (1985), Saier (1985), and Robillard and Lolkema (1988)]:

$$\text{PEP} + \text{HPr} \overset{\text{enzyme I}}{\leftrightarrow} \text{HPr–P} + \text{pyruvate} \quad (1)$$

$$\text{HPr–P} + \text{mannitol}_{out} \xrightarrow{EII^{mtl}} \text{HPr} + \text{mannitol 1-phosphate}_{in} \quad (2)$$

Reaction 2 has been shown to proceed by way of one or more phosphointermediates of the mannitol permease. Experiments by Waygood et al. (1984) and Roossien and Robillard (1984b) first established the existence of phospho-EII^{mtl}, while recent results have demonstrated the existence of at least two phosphorylation sites on the protein (Pas et al., 1988) which have been identified as Cys-384 and His-554 (Pas & Robillard, 1988). Because the mannitol permease is comprised of a hydrophobic, membrane-bound, N-terminal half and a hydrophilic, cytoplasmic, C-terminal half (Jacobson et al., 1983a; Lee & Saier, 1983; Stephan & Jacobson, 1986b), it was of interest to investigate the sites of phosphorylation relative to these two domains, and whether one or both domains were necessary for phosphorylation to occur. In this report, we show that phosphorylation appears to be confined to the C-terminal, cytoplasmic domain, as predicted from the assignment of the two phosphorylation sites, and does not require the presence of the N-terminal domain. The latter domain, however, is necessary for phosphotransfer to the sugar substrate. Using a deletion protein lacking one of the phosphorylation sites, we further show that intersubunit phosphotransfer can occur between different sites in a permease heterodimer and can result in the phosphorylation of mannitol to mannitol 1-phosphate.

Materials and Methods

Chemicals. [^{32}P]ATP (3000 Ci/mmol), ^{125}I-labeled *Staphylococcus aureus* protein A (9.4 mCi/mg), [^{3}H]mannitol (19 Ci/mmol), and [^{14}C]mannitol (45 mCi/mmol) were purchased from Dupont/New England Nuclear Co. (Boston, MA). L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin, soybean trypsin inhibitor (SBTI), and other reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

[^{32}P]Phosphoenolpyruvate (PEP) was prepared from [^{32}P]ATP according to the method of Roossien et al. (1983). Labeled PEP was then separated from ATP by stepwise salt elution from a 2-mL AG1-X8–bicarbonate column as described by Mattoo and Waygood (1983), except that elution was performed with sodium bicarbonate rather than triethylammonium bicarbonate. Most (>95%) of the PEP was

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Table I: *E. coli* K12 Strains and Plasmids Used in This Study

strain/ plasmid	genotype	source
KL141	F⁻ *recA56 thi pyrE41 argG6* mtl⁺	D. Smith
LGS322	F⁻ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50* Δ(*mtlA'p*) *mtlD*ᶜ Δ(*gutR'-M D B A p - recA*)	Grisafi et al. (1989)
pGJ9	Cm *mtlAp* on pACYC184	Grisafi et al. (1989)
pGJ9-Δ137	pGJ9 Δ(*mtlA*) from stop codon to between codons 393 and 519	Grisafi et al. (1989)

recovered in two 1-mL fractions of the 0.6 M salt step, resulting in a final concentration of 25 μM (1460 Ci/mol).

Bacterial Strains, Plasmids, and Preparation of Membrane Vesicles. The *E. coli* K12 strains and plasmids used in this study are listed in Table I. Strain KL141 (wild-type) was grown, and everted membrane vesicles were prepared from it, as described previously (Jacobson et al., 1983a). Strain LGS322, containing a chromosomal deletion of *mtlA* and either plasmid pGJ9 (*mtlA*⁺) or plasmid pGJ9-Δ137 (encoding a truncated mannitol permease), was grown, and vesicles were prepared as described by Grisafi et al. (1989). For complementation experiments, membrane vesicle suspensions from strains LGS322/pGJ9 and LGS322/pGJ9-Δ137 were incubated with 0.25% deoxycholate (Khandekar & Jacobson, 1989) after incubation with 2 mM mannitol to convert endogenous $EII^{mtl}\sim P$ to EII^{mtl} (Roossien & Robillard, 1984b). These partially solubilized vesicles were then used for activity assays or in [^{32}P]PEP labeling experiments (see below). A soluble fraction from *Salmonella typhimurium* strain LJ144 was used as a source for enzyme I and HPr of the PTS, also as previously described (Begley et al., 1982). Protein concentrations of these fractions were estimated by the method of Lowry et al. (1951).

Trypsinolysis and Partial Purification of the Cytoplasmic Domain. Everted membrane vesicles (0.5–5.0 mg/mL) were treated with trypsin (0.5 μg/mL) at 20 °C for 15–30 min as described by Stephan and Jacobson (1986b). These conditions were found to result in optimum yields of the cytoplasmic domain. Proteolysis was terminated by the addition of SBTI to a final concentration of 0.5 mg/mL. After trypsinolysis, the membrane suspension was centrifuged twice at 100000*g* for 2 h at 4 °C to remove the bulk of the membrane vesicles. Further purification was achieved by chromatography of 2 mL of the resulting supernatant on a 30 × 1.8 cm column of Sephacryl S-200 (Pharmacia) equilibrated with 20 mM Tris-HCl and 1 mM dithiothreitol, pH 8.0, at 4 °C (also see Results).

Protein Labeling with [^{32}P]PEP. Membrane vesicles (0.5–5.0 mg/mL), the supernatant derived from them after trypsinolysis, or the supernatant derived from deoxycholate-extracted membranes was labeled in a mixture containing 1.3 mg/mL *Salmonella* cytoplasmic protein (as a source of enzyme I and HPr), 7.5 μM [^{32}P]PEP, 12 mM Tris-HCl, 50 mM NaH_2PO_4, 1 mM NaF, 0.5 mM $MgCl_2$, and 2 mM dithiothreitol (pH 7.5) for 5 min at 30 °C. The reaction was terminated by addition of electrophoresis sample buffer (see below).

Electrophoresis, Autoradiography, and Immunoblotting. ^{32}P-Labeled protein mixtures were extracted with buffer containing 62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.003% bromphenol blue, pH 8.8, for 30 min at 30 °C. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in slabs using the buffer systems of Weber and Osborn (1969). 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, wrapped in plastic, and exposed to Kodak XAR-5 X-ray film at −70 °C without staining. Electrophoretic blotting of proteins from gels onto nitrocellulose was carried out according to the method of Towbin et al. (1979) with the addition of 1% SDS to the transfer buffer. Visualization of the mannitol permease or its fragments using anti-permease antibodies and ^{125}I-labeled *S. aureus* protein A was carried out as described by Stephan and Jacobson (1986b).

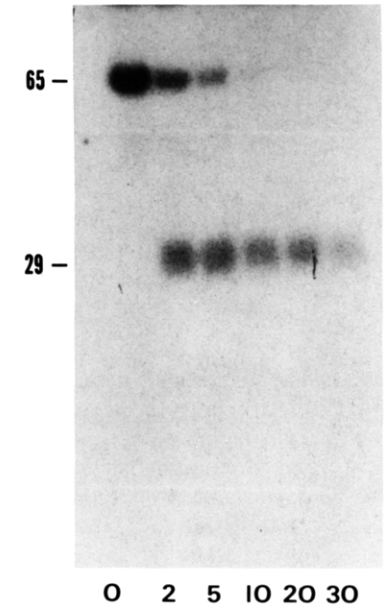

FIGURE 1: Trypsinization of ^{32}P-labeled mannitol permease in everted membrane vesicles. *E. coli* membrane vesicles containing the mannitol permease were prepared and labeled with [^{32}P]PEP as described under Materials and Methods. The everted vesicles were collected by centrifugation at 12800*g* for 15 min and resuspended at room temperature to a final protein concentration of 2.6 mg/mL. Trypsin (0.5 μg/mL) was added, and samples were withdrawn at the times (in minutes) indicated below the autoradiogram. Proteolysis was stopped in each sample by the addition of a 500× molar excess (over trypsin) of SBTI. Samples were electrophoresed, and the gel was exposed as described under Materials and Methods. Molecular masses of labeled bands (at left, in kilodaltons) were calculated from standard proteins run in parallel.

Activity Assays. Assays of mannitol 1-phosphate dependent and PEP-dependent phosphorylation of D-mannitol were carried out by using either ^{3}H- or ^{14}C-labeled mannitol as described previously (Jacobson et al., 1983a).

Results

The C-Terminal Domain of the Mannitol Permease Contains All Detectable Phosphorylation Sites. Mild trypsinolysis of everted membrane vesicles from mannitol-induced *E. coli* cleaves the mannitol permease (65 kDa in SDS–PAGE) into a membrane-bound, N-terminal fragment (34 kDa) and a soluble, C-terminal fragment (29 kDa) (Stephan & Jacobson, 1986b). In Figure 1, we show that if the mannitol permease was first phosphorylated with [^{32}P]PEP, enzyme I, and HPr and then treated with trypsin after removal of the soluble components by centrifugation, only a single radiolabeled band (apparent M_r 29K) appeared with time. Prolonged proteolysis eventually resulted in degradation of the 29-kDa band as well (Figure 1). Figure 2 shows that all of the radioactivity associated with the 29-kDa band was found in the soluble fraction. The insoluble fraction showed no labeling either of a 34-kDa band (the N-terminal domain) or of the residual

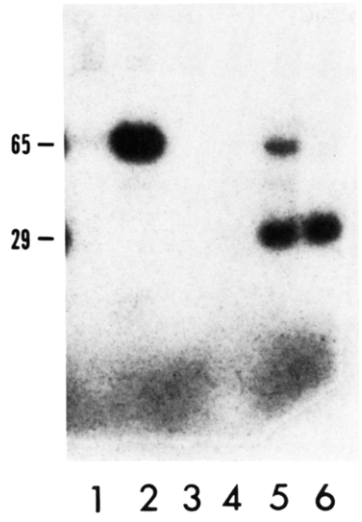

FIGURE 2: Identification of the 29-kDa fragment as the C-terminal domain. ^{32}P-Labeled everted membrane vesicles were trypsinized for 2 min as described in the legend to Figure 1. After trypsinization, the suspensions were centrifuged at 12800*g* for 15 min to separate the soluble and insoluble fractions; these fractions were electrophoresed and the gel was autoradiographed as described in Figure 1. Lanes 1–3 are untrypsinized (control) membranes: lane 1, insoluble fraction; lane 2, unfractionated mixture; lane 3, soluble fraction. Lanes 4–6 are trypsinized membranes: lane 4, insoluble fraction; lane 5, unfractionated mixture; lane 6, soluble fraction. Note that in lanes 1 and 4 no labeled 65-kDa band is observed, indicating that the phosphogroup(s) initially bound to the intact protein (lanes 2 and 5) must be hydrolyzed during the centrifugation step (also see text).

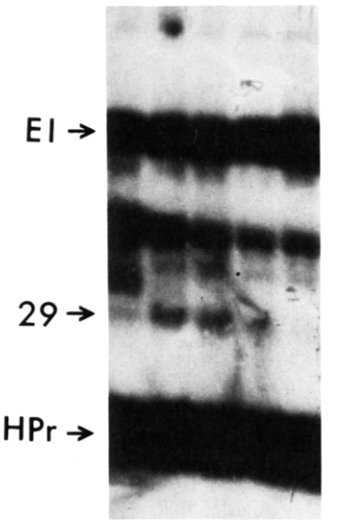

FIGURE 3: Partial purification and phosphorylation of the C-terminal domain. A trypsinized membrane suspension was centrifuged, and the supernatant was chromatographed on Sephacryl S-200 as described under Materials and Methods. Fractions were then subjected to SDS–PAGE after phosphorylation with [^{32}P]PEP, enzyme I, and HPr. Shown is an autoradiogram of part of this gel showing that the C-terminal domain eluted predominantly in fractions 17 and 18 (M_r 29 000), and was phosphorylated in the absence of the N-terminal domain (see text). Molecular masses of labeled bands on the ordinate were deduced from standards run in parallel. Labeled bands are enzyme I (69 kDa), C-terminal domain (29 kDa), and HPr (ca. 10 kDa). The identity of other labeled bands is not known.

intact permease (which had been labeled before the centrifugation; Figure 2). These results show that the 29-kDa band corresponds to the C-terminal domain and that the intact phosphopermease is relatively unstable. If the N-terminal domain is labeled under these conditions, then it must be sufficiently unstable to be observed by these techniques (also see Discussion).

Recently, Pas et al. (1988) have shown that the mannitol permease contains at least two sites of covalent phosphorylation, and these were subsequently identified as His-554 and Cys-384 (Pas & Robillard, 1988). His-554 is apparently directly phosphorylated by phospho-HPr while Cys-384 is apparently phosphorylated by an intramolecular transfer from His-554 and appears to be involved in the direct phosphorylation of mannitol (Pas et al., 1988; Pas & Robillard, 1988; Grisafi et al., 1989). Phosphorylation of His-554 is insensitive to pretreatment of the protein with *N*-ethylmaleimide (NEM), whereas phosphorylation of Cys-384 is abolished by pretreatment with this sulfhydryl reagent (Pas et al., 1988). Therefore, an experiment was carried out to compare the incorporation of ^{32}P from PEP in both intact permease and the C-terminal domain before and after treatment with NEM. Membrane samples were phosphorylated as in Figure 1 either with or without prior NEM treatment (0.2 mM for 5 min at 30 °C), trypsinized for 2 min as in Figure 2, electrophoresed, and autoradiographed. ^{32}P incorporation was reduced by 57% (±6%) in the residual intact permease and by 45% (±4%) in the C-terminal domain if the membranes were first treated with NEM (data not shown). Since NEM inhibited PEP-dependent phosphorylation of mannitol in intact membranes over 95% under these conditions, these results indicate that both the NEM-sensitive (Cys-384) and the NEM-insensitive (His-554) phosphorylation sites are located in the C-terminal domain, as expected.

Phosphorylation of the C-Terminal Domain Does Not Require the N-Terminal Domain. In order to determine whether the N-terminal domain is necessary for phosphorylation of the C-terminal domain by phospho-HPr, we partially purified the latter domain. Although centrifugation after trypsinolysis removes most of the vesicular fraction, immunoblotting experiments revealed that some residual membranes remained, even after extensive centrifugation. Therefore, the soluble fraction after trypsinolysis was subjected to molecular sieve chromatography on a column of Sephacryl S-200 to further purify the C-terminal domain. Figure 3 shows the results of an experiment in which fractions from such a column were first phosphorylated with [^{32}P]PEP, enzyme I, and HPr and then electrophoresed and autoradiographed. As can be seen, a phosphorylated band corresponding to the C-terminal domain could be observed that eluted predominantly in fractions 17 and 18. When a similar gel of unphosphorylated fractions was run and immunoblotted with anti-permease antibodies, a band of the same mobility was observed in the same fractions. This immunoblot also showed that neither residual intact permease nor a band corresponding to the N-terminal domain coeluted with the C-terminal domain on this column (data not shown). Thus, the N-terminal domain is not necessary for the PEP-dependent phosphorylation of the C-terminal domain.

In order to determine if the phospho group from the partially purified C-terminal domain could be transferred to mannitol, a reaction carried out by the intact permease (Waygood et al., 1984; Roossien & Robillard, 1984b), fractions from the Sephacryl S-200 column were first phosphorylated and then incubated with an excess (50 μM) of mannitol over the concentration of PEP used to phosphorylate the protein (7.5 μM). As shown in Figure 4, treatment with mannitol removed the phospho group from the intact permease as well as from the added enzyme I and HPr, but not from the C-terminal domain

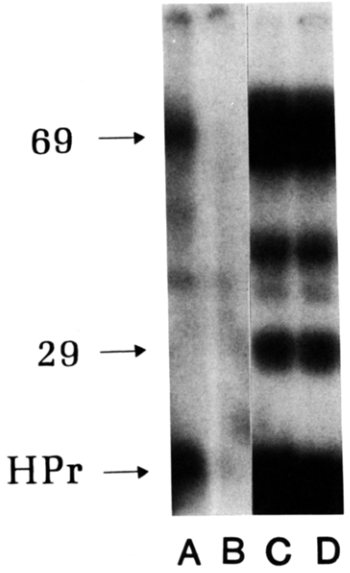

FIGURE 4: Effect of mannitol on the phosphorylated intact permease and the phosphorylated C-terminal domain. Phosphorylated untrypsinized membranes and phosphorylated C-terminal domain (fraction 18 from Figure 3) were incubated with an excess of mannitol (=50 μM) over the concentration of [^{32}P]PEP used in the phosphorylation reaction for 10 min at 30 °C. Samples were subjected to SDS–PAGE, and the gel was autoradiographed. Lane A, phosphorylated untrypsinized membranes incubated in the absence of mannitol; lane B, phosphorylated untrypsinized membranes incubated subsequently with mannitol; lane C, phosphorylated C-terminal domain incubated in the absence of mannitol; lane D, phosphorylated C-terminal domain incubated subsequently with mannitol. Labeled bands are enzyme I (69 kDa), C-terminal domain (29 kDa), and HPr (ca. 10 kDa). The intact mannitol permease in lane A is not well resolved from enzyme I. However, dephosphorylation of all bands in lane B shows that this concentration of mannitol is sufficient to drain all phosphate from PTS proteins in the presence of the intact permease. Identical results were obtained when the concentration of mannitol used was 5 mM (not shown).

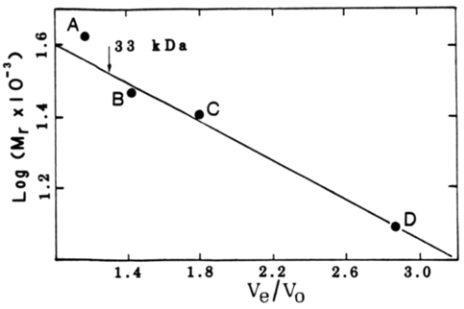

FIGURE 5: Apparent molecular mass of undenatured C-terminal domain. The Sephacryl S-200 column used to partially purify the C-terminal domain (Figure 3) was calibrated with the following standards: (A) ovalbumin (44 kDa); (B) carbonic anhydrase (29 kDa); (C) chymotrypsinogen A (25 kDa); (D) cytochrome *c* (12.4 kDa). The elution position of the C-terminal domain, determined as in Figure 3, indicated an apparent molecular mass of 33 kDa (arrow).

under the same conditions. The same results were obtained with even a vast excess (5 mM) of mannitol under these conditions (not shown). Thus, the N-terminal domain is necessary for the phosphotransfer reaction to the sugar (also see Discussion).

The Isolated C-Terminal Domain Is a Monomer. The column used for the partial purification of the C-terminal domain was standardized with molecular weight markers. Figure 5 shows that the elution profile of this domain, as determined by immunoblotting or phosphorylation of the fractions, was consistent with an apparent molecular mass of about 30 kDa, the same size as determined by SDS–PAGE. This result shows that this domain is a monomer after release from the membrane by trypsin.

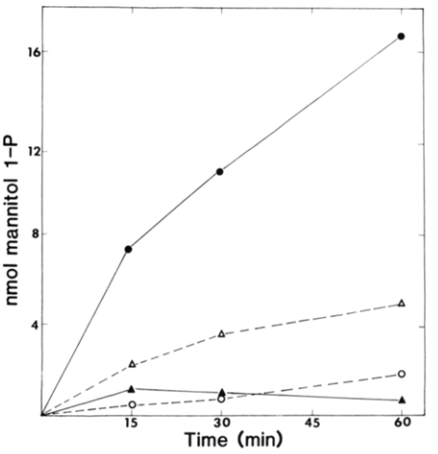

FIGURE 6: Complementation of PEP-dependent activity of NEM-inactivated mannitol permease by a truncated permease lacking His-554. Membranes from strain LGS322/pGJ9 containing intact permease and from strain LGS322/pGJ9-Δ137 encoding a truncated permease were treated with deoxycholate (see Materials and Methods) followed by 3 mM dithiothreitol for 30 min at 30 °C to insure complete reduction of Cys-384. Part of the LGS322/pGJ9 preparation was further treated with 10 mM NEM for 30 min at 30 °C, followed by addition of 27 mM dithiothreitol to stop the reaction. PEP-dependent phosphorylation of mannitol was measured at the indicated time intervals with these samples, either singly or in combination, as described under Materials and Methods. (●) LGS322/pGJ9 extract; (○) NEM-treated LGS322/pGJ9 extract; (▲) LGS322/pGJ9-Δ137 extract; (△) NEM-treated LGS322/pGJ9 extract plus LGS322/pGJ9-Δ137 extract.

Intersubunit Phosphotransfer in the Mannitol Permease. If phosphotransfer occurs between His-554 and Cys-384 in the C-terminal domain, then the question arises whether this occurs within the same polypeptide, or between subunits in a permease dimer. This is particularly pertinent in view of recent evidence suggesting that PEP-dependent phosphorylation of mannitol may require a dimeric form of the permease, at least transiently (Robillard & Blaauw, 1987; Pas et al., 1987; Khandekar & Jacobson, 1989). To determine if intersubunit phosphotransfer can occur in the mannitol permease, we used a strain of *E. coli* containing a chromosomal deletion of *mtlA* (LGS322; Table I) and harboring either a plasmid encoding the intact mannitol permease (pGJ9) or a derivative of this plasmid (pGJ9-Δ137) which encodes a truncated permease (M_r 51 000) that lacks His-554 but contains Cys-384 (Grisafi et al., 1989). Membranes were prepared from these strains and then treated with deoxycholate (see Materials and Methods). Part of the extract from strain LGS322/pGJ9 membranes was further treated with NEM to inactivate Cys-384. These extracts were then assayed for PEP-dependent phosphorylation of mannitol, either singly or in combination. As shown in Figure 6, the NEM-treated extract from strain LGS322/pGJ9 (○) had less than 10% of the activity of the untreated control (●), while the extract from the strain harboring the deletion plasmid (LGS322/pGJ9-Δ137) exhibited less than 5% of the control activity (▲). When mixed together, however, the NEM-treated and deletion extracts showed approximately 30% of the control activity (△). These results showed that the extract from LGS322/pGJ9-Δ137 could complement that from the strain synthesizing the intact permease that had been inactivated with NEM (i.e., that phosphotransfer could possibly occur from the latter protein to the former, followed by phosphorylation of mannitol).

To provide more direct evidence for intersubunit phosphotransfer, the same extracts were used in phosphorylation experiments with [^{32}P]PEP, enzyme I, and HPr. As shown in

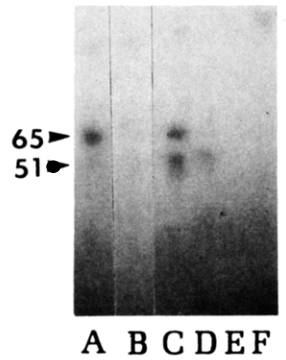

FIGURE 7: Intersubunit phosphotransfer between intact and truncated permeases. Deoxycholate-treated membrane preparations were prepared as described in the legend to Figure 6, and labeled, either singly or in combination, with [^{32}P]PEP as described under Materials and Methods. To half of each labeling mixture was then added mannitol (50 μM), and all samples were incubated for 15 min at 30 °C. Each sample was then centrifuged at 12800g for 15 min, and the pellets were resuspended in SDS sample buffer, followed by electrophoresis and autoradiography as described under Materials and Methods. Extracts: lane A, LGS322/pGJ9; lane B, LGS322/pGJ9-Δ137; lane C, LGS322/pGJ9 plus LGS322/pGJ9-Δ137; lane D, NEM-treated LGS322/pGJ9 plus LGS322/pGJ9-Δ137; lanes E and F, same as lanes C and D, respectively, after incubation with 50 μM mannitol. Molecular masses of labeled bands (left) are in kilodaltons and were deduced from standard proteins run in parallel.

Figure 7A, phosphorylation of the intact permease could readily be detected in the extract from strain LGS322/pGJ9, as had shown above for the membrane-bound protein in the absence of deoxycholate. No phosphorylation of the protein from strain LGS322/pGJ9-Δ137 was detectable under the same conditions, presumably because it lacks His-554 (Figure 7B). However, when the extracts from the two strains were mixed, phosphorylation of a 51-kDa deletion protein from strain LGS322/pGJ9-Δ137 occurred regardless of whether the extract from LGS322/pGJ9 was pretreated with NEM or not (Figure 7C,D). In either case, addition of an excess of mannitol removed the phospho groups from the truncated permease (Figure 7E,F). It should be noted that in the presence of deoxycholate, phosphorylation of the NEM-treated mannitol permease in LGS322/pGJ9 membranes could not be detected (compare lanes C and D in Figure 7). This was true even in the absence of extracted LGS322/pGJ9-Δ137 membranes, although a very weak band was occasionally observed (not shown). In contrast, NEM treatment of intact membranes reproducibly resulted in only about a 50% reduction in labeling (see above). The simplest explanation for these results is that (His-554)~P in the permease is much more labile to hydrolysis in the presence of deoxycholate than in its absence and thus that the phosphorylations shown in Figure 7 are primarily on Cys-384, which in the absence of mannitol acts as a dead-end sink for phosphate either on the active permease (Figure 7A) or on the truncated molecule (Figure 7C,D). In any case, these results suggest not only that phosphotransfer can occur between subunits of the mannitol permease but also that this transfer occurs between His-554 of one subunit (NEM-inactivated intact permease) and Cys-384 of the other (product of pGJ9-Δ137) with ultimate transfer to mannitol.

Discussion

The mannitol-specific enzyme II is one of the most extensively characterized permeases of the bacterial PTS. It has been purified (Jacobson et al., 1979), a number of its physicochemical and catalytic properties have been determined (Jacobson et al., 1983a,b; Roossien et al., 1984; Roossien & Robillard, 1984a,b; Stephan & Jacobson, 1986a,b; Pas et al., 1987; Robillard & Blaauw, 1987), and it has been shown to be covalently phosphorylated by phospho-HPr (Waygood et al., 1984; Roossien & Robillard, 1984b). The gene encoding the mannitol permease (*mtlA*) has been cloned, its primary amino acid sequence has been deduced (Lee & Saier, 1983), and the protein has been shown to consist of distinct membrane-bound and cytoplasmic domains corresponding to the N- and C-terminal halves of the protein, respectively (Stephan & Jacobson, 1986b). Very recent work has identified at least two covalent phosphorylation sites in the mannitol permease, His-554 and Cys-384, both of which appear to be necessary for PEP-dependent mannitol phosphorylation (Pas et al., 1988; Pas & Robillard, 1988; Grisafi et al., 1989).

The present study was undertaken to further clarify the structural and functional roles of the cytoplasmic, C-terminal domain of the mannitol permease. In particular, if His-554 and Cys-384 are the only phosphorylated residues of the mannitol permease, then only the C-terminal domain of the protein should be labeled with [^{32}P]PEP, enzyme I, and HPr. Our results showed that only the C-terminal domain was labeled in such experiments and that this domain contained at least one NEM-insensitive and one NEM-sensitive site, in agreement with the localization of both phosphorylation sites in the C-terminal domain. A variety of labeling conditions were attempted, but no labeling of the N-terminal domain was detected. Although these experiments do not rule out that a residue in the N-terminal domain of the permease can be phosphorylated, any such phospho intermediate would have to be too labile to have been detected either in our experiments or in those of others (Pas & Robillard, 1988).

Our experiments also provide evidence that the C-terminal half of the mannitol permease not only is an independent structural domain (Stephan & Jacobson, 1986b) but also is functionally independent. Thus, phosphorylation of the partially purified C-terminal domain by phospho-HPr occurred even in the complete absence of the N-terminal domain. Although the lability of phospho-enzyme II^{mtl} intermediates has prevented a detailed kinetic comparison of the phosphorylation rate of the intact permease with that of the isolated C-terminal domain, our results nonetheless show that this domain contains most, if not all, of the amino acid residues critical for phospho-HPr interaction. However, the inability of the phosphorylated C-terminal domain to transfer its phospho groups to mannitol in the absence of the N-terminal domain suggested that the latter domain plays a role in this final phosphotransfer reaction. Indeed, it has recently been shown that the N-terminal 60% of the enzyme II^{mtl} molecule contains the high-affinity mannitol-binding site of the protein (Grisafi et al., 1989). Finally, although there is considerable evidence that the intact permease may be at least partially associated as a dimer in the membrane (Roossien & Robillard, 1984b; Stephan & Jacobson, 1986a; Robillard & Blaauw, 1987; Pas et al., 1987; Khandekar & Jacobson, 1989), the isolated C-terminal domain eluted as a monomer on molecular sieve chromatography columns. This result is consistent with the hypothesis that the permease dimer is held together primarily by hydrophobic interactions between residues in the N-terminal domain (Roossien & Robillard, 1984b; Stephan & Jacobson, 1986a).

The roles of the two phosphorylation sites within the C-terminal domain of the mannitol permease have recently been investigated. Thus, NEM treatment of the intact permease allows phosphorylation of His-554, but not of Cys-384, by

phospho-HPr, and inactivates the mannitol phosphorylating activity of the permease (Pas et al., 1988; Pas & Robillard, 1988). Furthermore, C-terminal deletions of the permease removing His-554, but not Cys-384, inactivate PEP-dependent mannitol phosphorylation without affecting mannitol/mannitol 1-phosphate phospho exchange catalyzed by the permease (Grisafi et al., 1989). These results would indicate that His-554 is the direct phosphoacceptor from phospho-HPr but that subsequent phosphotransfer to Cys-384 is necessary for mannitol phosphorylation (i.e., Cys-384 is the direct phosphodonor to mannitol) (Pas & Robillard, 1988; Grisafi et al., 1989). With this in mind, and given the hypothesis that the mannitol permease may only be active as a dimer (see above), we asked the question in the present study whether phosphotransfer within the C-terminal domain could occur between permease subunits. We demonstrated that phosphotransfer could occur between NEM-inactivated intact permease (in which Cys-384, but not His-554, was modified) and a deletion protein lacking His-554 but retaining Cys-384. Moreover, we showed that the phospho group covalently bound to the deletion protein could be transferred to mannitol.

These results demonstrate that catalytically productive phosphotransfer can occur between the C-terminal domains of an at least transient permease dimer and that this phosphotransfer may occur between one site on one subunit (His-554) and a distinct site on the other (Cys-384). They also provide one possible explanation for the hypothesized obligatory involvement of a permease dimer in PEP-dependent mannitol phosphorylation (Pas et al., 1987; Khandekar & Jacobson, 1989). In such a permease dimer, His-554 of one subunit must at least transiently be very close to Cys-384 of the other. The fact that the deletion protein from plasmid pGJ9-Δ137, which lacks His-554 and has an apparent molecular weight of 51 000 (Grisafi et al., 1989), can accept a phospho group from an intact permease subunit in a heterodimer and transfer it to mannitol implies that Cys-384 in the deletion protein is in more or less the same position relative to the rest of the molecule as in the intact permease. This is consistent with our recent suggestion that residues 499–637 constitute a separate structural subdomain, comprising a "fused Enzyme III" that has little influence on the structure of the rest of the molecule (Grisafi et al., 1989). Our experiments, however, do not rule out that *intra*subunit phosphotransfer from His-554 to Cys-384 could also occur, nor the possibility that phosphorylation of Cys-384 of the deletion protein could occur directly from phospho-HPr bound to a full-length subunit in a heterodimer without the intermediate formation of (His-554)$\sim$P in the latter. Further experiments will be necessary to test these possibilities.

It is worth noting that it has recently been shown that the PTS enzymes II specific for *N*-acetylglucosamine and β-glucosides can complement enzyme II^{glucose} for the PEP-dependent phosphorylation of glucose or methyl α-glucoside in strains lacking enzyme III^{glucose}, which is homologous to the C-terminal domains of the first two proteins (Vogler et al., 1988). Since the glucose enzyme II contains a phosphorylation site obligatory for its function (Begley et al., 1982; Waygood et al., 1984; Erni, 1986), these experiments imply that heterodimers of enzyme II^{glucose} with these other enzymes II must at least transiently form, with catalytically productive phosphotransfer between the two subunits. Thus, intersubunit phosphotransfer between two different sites may be a general mechanism for those PTS enzymes II, such as those specific for mannitol, *N*-acetylglucosamine, and β-glucosides, which combine both an enzyme II and an enzyme III function in the same molecule. In the future, it will be interesting to relate this phosphotransfer mechanism to the conformational change(s) that must occur during mannitol permease function (Khandekar & Jacobson, 1989) and to the fact that the protein apparently contains only one high-affinity mannitol-binding site per permease dimer (Pas et al., 1988).

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