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Identification of the Phosphocarrier Protein Enzyme III^{gut}: Essential Component of the Glucitol Phosphotransferase System in *Salmonella typhimurium*

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The phosphoenolpyruvate-dependent phosphorylation of glucitol has been shown to require four distinct proteins in *Salmonella typhimurium*: two general energy-coupling proteins, enzyme I and HPr, and two glucitol-specific proteins, enzyme II^{gut} and enzyme III^{gut}. The enzyme II^{gut} was solubilized from the membrane and purified about 100-fold, free of the other protein constituents of the phosphotransferase system. Enzyme III^{gut} was found in both the soluble and the membrane fractions. The soluble enzyme III^{gut} was purified to near homogeneity by gel filtration, hydroxylapatite chromatography, and hydrophobic chromatography on butylagarose. It was sensitive to parital inactivation by trypsin and *N*-ethylmaleimide, but was stable at 80°C. The protein had an approximate molecular weight of 15,000. It was phosphorylated in the presence of phosphoenolpyruvate, enzyme I, and HPr, and this phosphoprotein was dephosphorylated in the presence of enzyme II^{gut} and glucitol. Antibodies were raised against enzyme III^{gut}. Enzyme III^{glc} and enzyme III^{gut} exhibited no enzymatic or immunological cross-reactivity. Enzyme II^{gut}, enzyme III^{gut}, and glucitol phosphate dehydrogenase activities were specifically induced by growth in the presence of glucitol. These results serve to characterize the glucitol-specific proteins of the phosphotransferase system in *S. typhimurium*.

The phosphotransferase system (PTS) in bacteria is responsible for the phosphorylation and translocation of a number of carbohydrates across the bacterial cytoplasmic membrane (8, 18). Two of the PTS proteins, phosphoenolpyruvate (PEP):HPr phosphotransferase (EC 2.7.3.9) (enzyme I) and the small phosphorylcarrier protein of the PTS, HPr, correctly designated phospho-enzyme I: sugar-specific phosphoryl acceptor protein phosphotransferase, are general cytoplasmic proteins required for the phosphorylation of all PTS substrates. Enzyme I catalyzes the transfer of high-energy phosphate from PEP to HPr (18, 22). Phosphate is then transferred to sugar in reactions which may result in the intermediate phosphorylation of the sugar-specific enzymes III and II.

A number of enzymes III have been described including the enzyme III^{lac} and enzyme III^{mtl} of *Staphylococcus aureus* (3, 5, 23), the enzyme III^{glc} of *Escherichia coli* and *Salmonella typhimurium* (5, 7, 9, 16), and the enzyme III^{fru} of *Arthrobacter pyridinolis* (26). Evidence, however, for the obligatory involvement of an enzyme III in the phosphorylation of some sugars is lacking. Specifically, purified *E. coli* enzyme II^{mtl}, enzyme I, and HPr are sufficient to catalyze PEP-dependent mannitol phosphorylation in detergent micelles (6). Consistent with this observation, genetic studies have led to the suggestion that none of the hexitol phosphotransferases function with the participation of more than a single sugar-specific phosphotransferase protein (13, 14). In spite of this conclusion, we show in this report that glucitol phosphorylation requires the presence of two distinct sugar-specific proteins, an integral membrane enzyme II^{gut} (phospho-enzyme III^{gut}:glucitol phosphotransferase) and a peripheral membrane enzyme III^{gut} (phospho-HPr:enzyme II^{gut} phosphotransferase).

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MATERIALS AND METHODS

Materials. Hexylagarose and butylagarose were obtained from Miles Laboratories. DEAE-agarose, nitrocellulose paper, acrylamide, and bisacrylamide were from Bio-Rad Laboratories. Lubrol-PX, sodium dodecyl sulfate (SDS), deoxycholate, NAD^+, β-chlorolactic acid, and all sugars and sugar phosphates were purchased from Sigma Chemical Co. Sephadex G-75 (superfine) was a product of Pharmacia Fine Chemicals, Inc. Ultrogel-HA was obtained from LKB. D-[^{14}C]glucitol (302 mCi/mmol), D-[^{14}C]methyl-α-glucoside (279 mCi/mmol), and $^{32}P_iO_4$ (carrier-free) were products of Amersham Corp. Freund adjuvant was obtained from Difco Laboratories. All other reagents were obtained from commercial sources and were of the highest grade available.

Assays. PEP-dependent glucitol phosphorylation activity was assayed as follows. Phosphorylation was determined in a total volume of 0.1 ml containing 50 mM potassium phosphate (pH 7.5), 12.5 mM $MgCl_2$, 25 mM KF, 2.5 mM dithiothreitol (DTT), 5 mM PEP, and 0.01 mM [^{14}C]glucitol (5 μCi/μmol). When assaying for enzyme II^{gut}, excess enzyme I, enzyme III^{gut}, and HPr were provided by inclusion in the assay of either a membrane-free extract of glucitol-grown cells or partially purified enzyme I, enzyme III^{gut}, and HPr. When enzyme III^{gut} was assayed, excess partially purified enzyme I, enzyme II^{gut}, and HPr were used (see Table 2 for amounts and degrees of purity). In all assays, the protein to be assayed was present in rate-limiting amounts. That is, the activity was proportional to the amount of the measured component. Increasing the amounts of the other PTS components did not appreciably change the activities measured. In assays using solubilized enzyme II^{gut}, the assay mixture also included Lubrol (0.3%). The reactions were terminated by the addition of 3 ml of cold water. Sugar phosphate was determined by ion-exchange chromatography as described previously (25). Assays of enzyme III^{glc} activity were carried out as above with two modifications.

[^{14}C]methyl-α-glucoside (5 μCi/μmol) replaced glucitol, and saturating amounts of enzyme II^{glc}, enzyme I, and HPr were provided by addition of a crude extract from an *E. coli crrA* mutant. HPr and enzyme I were assayed similarly, using extracts of *ptsH* and *ptsI* mutants, respectively. Glucitol-6-phosphate dehydrogenase assays were performed in a total volume of 1 ml containing 0.1 M Tris-hydrochloride (pH 9.0), 1 mM NAD^+, 5 mM glucitol-6-phosphate, and the sample to be assayed. Absorbance at 340 nm was monitored spectrophotometrically, and the activity was determined from the linear increase in absorbance observed for 1 min at room temperature. Protein was determined according to Lowry et al., with bovine serum albumin as the protein standard (15).

Growth of bacteria and separation of membranes from soluble proteins. *S. typhimurium* strains LT-2 and LJ409 (*ppc-201 mtlA309*) were harvested in the late logarithmic phase of growth in buffered Luria broth containing 0.5% D-glucitol when used as sources of enzyme for purification procedures. For induction studies and immunoblotting, strain LT-2 was grown to late logarithmic phase in complex medium containing 0.5% glucitol, glucose, or mannitol. Cells grown by all methods were harvested after cooling to 4°C by centrifugation at 10,000 × *g* for 10 min. Cells were washed twice with buffer containing 20 mM Tris-hydrochloride (pH 7.5) and 1 mM DTT, suspended in the same buffer, and broken by passage through a French press at 10,000 lb/in^2 at 4°C. Unbroken cells and cell debris were removed by centrifugation at 20,000 × *g* for 20 min. Membranes were obtained by centrifugation of the supernatant for 2 h at 100,000 × *g* and were suspended in the same buffer. The supernatant was centrifuged again at 100,000 × *g* for 12 h at 4°C. The resulting supernatant, termed high-speed supernatant, was frozen in aliquots at −70°C.

Enzyme II^{gut} purification. Membranes from 3 g (wet weight) of *S. typhimurium* strain LJ409 (*mtlA309*) were solubilized with 10 ml of extraction buffer containing 20 mM Tris-hydrochloride (pH 8.1), 1 mM DTT, 0.5% deoxycholate, and 0.2 M NaCl. After the extraction mixture was left on ice for 15 min, the mixture was centrifuged for 90 min at 100,000 × *g* and 4°C. The supernatant was applied to a 10-ml hexylagarose column (poured in a 10-ml plastic syringe) at 4°C and washed with extraction buffer until a protein peak eluted and the absorbance at 280 nm returned to near the base-line value. Enzyme II^{gut} was eluted with 40 ml of extraction buffer containing 0.5% Lubrol, and 5-ml fractions were collected. The peak fractions were pooled and dialyzed overnight at 4°C against 40 volumes of 20 mM Tris-hydrochloride (pH 7.5)–1 mM DTT–0.5% Lubrol. The retentate was frozen in aliquots at −70°C. Enzyme II^{gut} was stable for several months if stored in this manner.

Enzyme III^{gut} purification. A 25-ml portion of high-speed supernatant from *S. typhimurium* strain LT-2 (0.5 to 1 g of protein) was applied to a Sephadex G-75 (superfine) column (3 by 50 cm) with a bed volume of 350 ml. The column was eluted with 75 mM potassium phosphate, pH 7.5, containing 1 mM DTT, and 5-ml fractions were collected. The active fractions (approximately 70% of the total peak) were pooled and dialyzed against 40 volumes of 2 mM potassium phosphate, pH 6.5, containing 1 mM DTT. The retentate was applied to a 100-ml Ultrogel-HA column (2.2 by 26 cm), and fractions containing appreciable enzyme III^{gut} activity were collected as flowthrough. The flowthrough was then applied to a 30-ml butylagarose column (1.5 by 17 cm), and a 0 to 0.3 M NaCl gradient in 2 mM potassium phosphate, pH 7.0, containing 1 mM DTT was started. The enzyme III^{gut} activity eluted at approximately 0.1 M NaCl. The active fractions representing approximately 70% of the total activity peak were pooled. The enzyme solution was dialyzed overnight against 20 mM Tris-hydrochloride, pH 7.5, containing 1 mM DTT and then divided into aliquots and frozen at −70°C. It was necessary to periodically replace both the Ultrogel-HA and butylagarose columns to maintain a high degree of purity of enzyme III^{gut}.

Enzyme I, HPr, and enzyme III^{glc} purifications. Enzyme I, HPr, and enzyme III^{glc} were purified by using modifications of the methods of Waygood and Steeves (25), Beyreuther et al. (1), and Osumi and Saier (17), respectively.

Protein phosphorylation. Proteins were phosphorylated by using [^{32}P]PEP synthesized by the original procedure (11) with the following modifications. Carrier-free $H_3{}^{32}PO_4$ (0.4 ml, 0.8 mCi) was evaporated (2 h) to dryness in a vacuum centrifuge, and 0.1 ml of 0.62% β-chlorolactic acid (Sigma) in acetonitrile was added. The solvent was evaporated as above, and drying was repeated twice after two additions of 0.1-ml volumes of acetonitrile. The reaction mixture was further dried (30 min) over P_2O_5 under vacuum, and then 50 μl of dimethyl sulfoxide, 5 μl of triethylamine, and 1 μl of trichloroacetonitrile were added. After a 2-h incubation period at 37°C the reaction was stopped by addition of 50 μl of water. Care was taken to use solvents which had been redistilled and stored over CaH_2. Production of PEP was verified by thin-layer chromatography on polyethyleneimine-cellulose (Machery-Nogel) with either 0.5 M KH_2PO_4 containing 5 mM EDTA or 0.25 M LiCl containing 1 M acetic acid as the solvent system. An approximate yield of 70% was usually obtained. Carrier-free [^{32}P]PEP was added to 40 μl of a buffer containing 50 mM Tris-hydrochloride (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM PEP, and the desired PTS components. The reactions were stopped after 10 min at 25°C by addition of 15 μl of cold SDS sample buffer (pH 7.0). The samples were loaded onto a 1-mm SDS-polyacrylamide gel cooled to 4°C, and electrophoresis was begun within 5 min. Electrophoresis was continued until the dye front (marked with bromphenol blue) eluted from the bottom of the gel (2 to 3 h). The gel was then washed three times (20 min each) with 100 ml of 0.1 N NaOH in 50% methanol. This step lowered the background radioactivity and stabilized the phosphohistidine proteins. The gel was covered with one thickness of Saran Wrap, and autoradiography with Kodak XAR-5 film was performed for 1 to 3 h. We had no difficulty observing the labile phosphohistidine proteins with this method. After autoradiography, the gels were stained and destained (10) and carefully swollen to the original size of the gel used for autoradiography so that the positions of enzyme I, HPr, and enzyme III^{gut} could be determined.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 1- or 2-mm-thick slabs, and gels were stained for protein with Coomassie blue as described previously (10). Because electrophoresis of phosphorylated proteins was performed at 4°C, the pH values of the stacking and separating gels and the running buffer were increased due to the temperature coefficient of the Tris buffer. Standards used for molecular weight determination were as follows: insulin (3,000), bovine trypsin inhibitor (6,200), cytochrome *c* (12,300), lysozyme (14,300), β-lactoglobulin (18,400), α-chymotrypsinogen (25,700), and ovalbumin (43,000).

Preparation of antibodies. Antibodies were raised against enzyme III^{gut}, using the method of Goudie et al. (4). Ouchterlony double-diffusion analysis was done as described before (2). Ouchterlony plates were stained for protein as

follows. The developed plates were washed overnight in 0.25 M NaCl and then washed overnight in distilled water. The plates were dried by placing filter paper on top of the washed plates. The plates were stained for protein and then destained as for polyacrylamide gels.

Immunoblotting. Electroblotting of the proteins from 1-mm-thick SDS-polyacrylamide gels was performed as described previously (24). Detection of antigens was performed with the Bio-Rad immunoblot (GAR-HRP) assay kit. We used a 1/40 dilution of primary antibody (enzyme III^{gut} immune serum) and a 1/1,000 dilution of secondary antibody (Bio-Rad goat anti-rabbit horseradish peroxidase conjugate). All other procedures were as suggested by Bio-Rad except that the blocking step with 3% gelatin was extended overnight, and the washing steps were extended 30 min each. To minimize the use of antibodies, the nitrocellulose paper was cut into thin strips after electroblotting, and each strip was incubated separately in small volumes.

RESULTS

Partial purification of enzyme II^{gut}. Enzyme II^{gut} was partially purified by using a procedure similar to that of Jacobson et al. for the purification of enzyme II^{mtl} (6). An *S. typhimurium* strain defective for the *mtlA* gene was used as the source of enzyme II^{gut} to prevent copurification with enzyme II^{mtl}. Enzyme II^{gut} was extracted from bacterial membranes with deoxycholate, applied to a hexylagarose column, and eluted with Lubrol. In this manner, enzyme II^{gut} was purified approximately 10- to 20-fold.

Partially purified enzyme II^{gut} catalyzed PEP-dependent glucitol phosphorylation when the other required PTS proteins were supplied by a high-speed supernatant. Purified enzyme I and HPr, however, could not substitute for the high-speed supernatant. When washed membranes from glucitol-grown *S. typhimurium* cells were used rather than partially purified enzyme II^{gut}, the addition of enzyme I and HPr was sufficient for glucitol phosphorylation. These observations provided the first evidence that a fourth protein, required for glucitol phosphorylation, was present in the soluble fraction of the extract and was also peripherally membrane bound.

Purification and properties of the enzyme III^{gut}. Table 1 summarizes the purification scheme for the new protein. After gel filtration chromatography, hydroxylapatite chromatography, and butylagarose chromatography, the specific activity was increased approximately 65-fold over that in the high-speed supernatant. The enzyme preparation contained no enzyme I, HPr, or enzyme III^{glc} activity, and SDS-PAGE indicated that the preparation consisted of a single major protein band (Fig. 1). In some cases, SDS-PAGE revealed that this band was actually a doublet consisting of a major and a minor band. In general, the doublet was very poorly resolved by SDS-PAGE. These two bands represented >95% of the protein on the gel. This estimate was made as follows. Increasing amounts of protein were applied to the gel, and after electrophoresis the gels were stained with Coomassie blue. With 10 μg of total protein applied to the gel, no protein other than that which was identified as enzyme III^{gut} could be detected. Since the method easily detects 0.5 μg of most proteins, it was presumed that the enzyme III^{gut} preparation was >95% pure. Only when this enzyme was added to an assay mixture containing saturating amounts of enzyme I, HPr, and enzyme II^{gut} could PEP-dependent glucitol phosphorylation be demonstrated (Table 2). Enzyme III^{glc} could not substitute for enzyme III^{gut} even though the enzyme III^{glc} used in this study was fully active for PEP-dependent methyl-α-glucoside phosphorylation.

Enzyme III^{gut} was found to have a molecular weight of approximately 17,000 by gel permeation chromatography. Enzyme III^{gut} eluted from a Sephadex G-75 column after enzyme III^{glc} (molecular weight, ~20,000) and before HPr (molecular weight, ~9,500). Protein and activity coeluted.

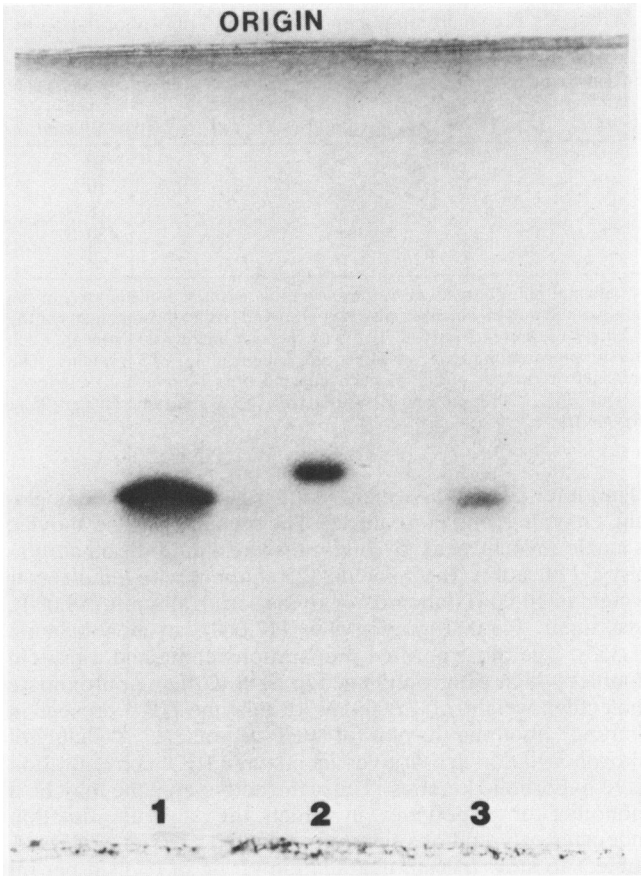

FIG. 1. SDS-polyacrylamide gel of enzyme III^{gut} after the butylagarose chromatography step. Electrophoresis was performed with a 15% separating gel (pH 8.8) and a 6% stacking gel (pH 6.8) at room temperature, 20 mA per slab. Lane 1, enzyme III^{gut} (10 μg); lane 2, myoglobin (4 μg), lane 3, enzyme III^{gut} (2 μg). The broadening of the enzyme III^{gut} band (lane 1) was often observed with pure preparations of enzyme whether dialyzed against Tris-hydrochloride or potassium phosphate.

TABLE 1. Enzyme III^{gut} purification summary[a]

Sample	Total protein (mg)	Total activity (μmol/30 min)	Sp act (μmol/30 min per mg)	Yield (%)	Purification factor
High-speed supernatant	852	200	0.23	100	1.0
Sephadex G-75	63	157	2.4	76	10
Ultrogel-HA	7	92	13.1	46	57
Butylagarose	4	60	15.0	30	65

[a] Activity refers to glucitol-6-phosphate formed by the PEP-dependent phosphorylation reaction. In all cases, enzyme III^{gut} was present in rate-limiting amounts. The amounts of the other PTS components were as follows: enzyme I, 4 μg; HPr, 10 μg; enzyme II^{gut}, 25 μg. The enzyme II^{gut} was partially purified through the hexylagarose chromatography step as described in the text. The purity of all components is given in Table 2.

TABLE 2. Enzyme requirements for glucitol phosphorylation by the PTS in *S. typhimurium*[a]

Enzyme II^{gut} (5 μg)	Enzyme I (5 μg)	HPr (10 μg)	Enzyme III^{gut} (10 μg)	Enzyme III^{glc} (15 μg)	Glucitol-6-phosphate formed (pmol/30 min)
−	+	+	+	−	10
+	−	−	−	−	10
+	+	+	−	−	8
+	+	+	+	−	450
−	+	+	−	+	9

[a] Glucitol phosphorylation was measured as described in the text. In the only permutation in which activity was observed, with all components of the glucitol PTS present, enzyme II^{gut} was the rate-limiting component. Each protein preparation used was free of contaminating PTS proteins. The estimated degree of purity of each enzyme preparation was as follows: enzyme II^{gut}, <20%; enzyme I, >80%; HPr, >95%; enzyme III^{gut}, >95%; enzyme III^{glc}, >90%.

(The other molecular weight standards used were hemoglobin, enzyme I, and peroxidase.) The preparation also showed a single protein peak by high-pressure liquid chromatography gel filtration (Bio-Sil TSK-125 column) with a molecular weight of 16,000 (standards—bovine serum albumin [68,000], ovalbumin [43,000], myoglobin [17,000], cyanocobalamin [1,355]. The most purified preparations contained a protein doublet which electrophoresed in SDS with an approximate molecular weight of 15,000. With enzyme III^{gut} present in limiting amounts, a plot of PEP-dependent [^{14}C]glucitol phosphorylation activity versus enzyme III^{gut} concentration gave hyperbolic kinetics. Thus, the active enzyme may be a monomer or a polymer in which the subunits function independently and are tightly associated. The enzyme was subject to slow trypsin degradation (2 mg of trypsin per ml caused a 50% loss in activity after 30 min at 23°C) and was relatively heat stable. Incubation of the enzyme at 80°C for 5 min caused no loss in the activity of enzyme III^{gut}, although 50% of the activity was lost after a 5-min incubation period at 95°C. Treatment of enzyme III^{gut} for 10 min with 5 mM *N*-ethylmaleimide also caused a 50% loss in activity. Treatment of enzyme II^{gut} in the same manner resulted in a 100% loss in enzyme activity.

Protein phosphorylation experiments indicated that both of the protein bands observed on SDS-polyacrylamide gels were probably enzyme III^{gut}. Because of the minimal resolution of the two bands on SDS-PAGE, however, it was difficult in all but a few experiments to identify two radioactive bands by autoradiography. In the presence of PEP, enzyme I, and HPr, protein was phosphorylated which comigrated with the two enzyme III^{gut} bands on SDS gels (Fig. 2, lane 3). Omission of enzyme I prevented this phosphorylation (Fig. 2, lane 1). The addition of enzyme II^{gut} and glucitol caused the dephosphorylation of all radioactive bands in this region of the gel (Fig. 2, lane 5). In the absence of glucitol, however, enzyme III^{gut} was not dephosphorylated (Fig. 2, lane 4). If enzyme II^{gut} and glucitol were added to an assay mixture containing PEP, enzyme I, and HPr, phospho-HPr was still generated, further indicating that phospho-HPr cannot donate its phosphoryl group to enzyme II^{gut} in the absence of enzyme III^{gut} (data not shown). These results suggest that phosphorylation occurs sequentially from enzyme I to HPr, then to enzyme III^{gut}, and finally to glucitol in the presence of enzyme II^{gut}.

Figure 3A shows an Ouchterlony double-diffusion analysis in which a precipitin band was formed with immune serum but not with preimmune serum. The immune serum against enzyme III^{gut} did not cross-react with HPr, enzyme I, or enzyme III^{glc}. Also, when immune serum and enzyme III^{gut} were mixed, a precipitate formed, and after centrifugation no enzyme III^{gut} activity remained in the supernatant. The supernatant also lacked the 15,000-dalton protein as determined by SDS-PAGE. In control experiments with preimmune serum, neither enzyme III^{gut} activity nor the 15,000-dalton protein could be precipitated.

The results of an immunoblotting experiment are shown in Fig. 3B. This experiment verified that a 15,000-dalton protein cross-reacted with enzyme III^{gut} antibodies (lane 1). When a crude extract of glucitol-grown LT-2 cells was blotted, only one band which corresponded to the 15,000-dalton protein was observed (lane 2). Protein staining showed, however, that complete transfer of high- and low-molecular-weight proteins had occurred. If a crude extract of mannitol-grown LT-2 cells replaced the glucitol-grown LT-2 extract, no bands of any size were observed under the conditions used. These results are consistent with the data shown in Table 3 which indicate that glucitol-grown LT-2 cells have 20-fold more enzyme III^{gut} activity than mannitol-grown LT-2 cells.

Induction of enzyme III^{gut} synthesis. *S. typhimurium* strain LT-2 grown in complex medium plus glucitol had about 20-fold more enzyme III^{gut} per milligram of protein than cells grown in complex medium plus either glucose or mannitol (Table 3). Glucitol-grown bacteria also exhibited induced levels of enzyme II^{gut} and glucitol-6-phosphate dehydrogenase compared with glucose- or mannitol-grown cells. The partial induction of enzyme II^{gut} activity by mannitol (Table 3) can be attributed to the cross substrate specificities of the glucitol and mannitol enzymes II (6). A mutant constitutive

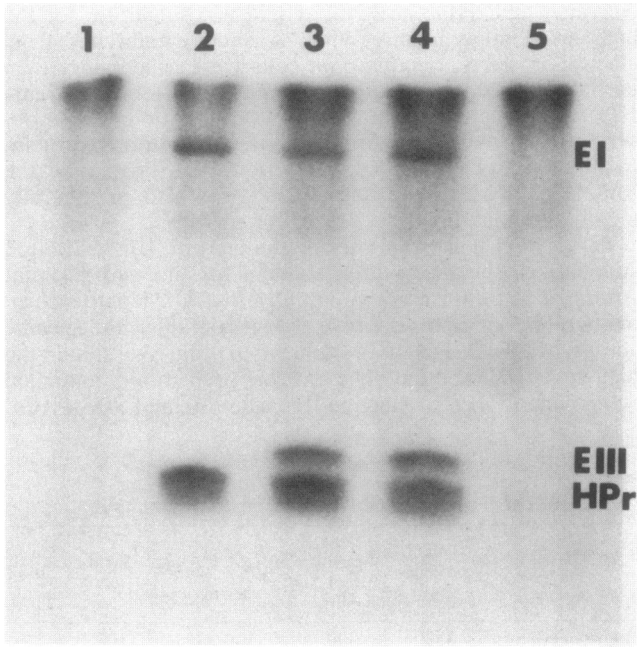

FIG. 2. Autoradiograph showing phosphorylation of enzyme III^{gut}. Experimental conditions used for the phosphorylation of the protein with [^{32}P]PEP are given in the text. SDS-polyacrylamide gels were run as in Fig. 1 but at 4°C and with a 16% separating gel. All lanes contained the PEP-generating system and 5 μg of HPr. In addition, the lanes contained: 1, enzyme III^{gut} (3 μg); 2, enzyme I (1 μg); 3, enzyme I (1 μg) and enzyme III^{gut} (3 μg); 4, as in lane 3 plus enzyme II^{gut} (40 μg); 5, as in lane 4 plus 200 μM glucitol. The marked positions of enzyme I, enzyme III^{gut}, and HPr were determined after protein staining of the gel used for autoradiography.

for the glucitol operon was shown to synthesize enzyme II^{gut}, enzyme III^{gut}, and glucitol-6-phosphate dehydrogenase at high constitutive levels, and these three enzyme activities were coordinately repressed by growth of this strain in the presence of glucitol (A. M. Chin, F. C. Grenier, and M. H. Saier, Jr., unpublished data).

DISCUSSION

The data presented in this report demonstrate that, in addition to enzyme I, HPr, and enzyme II^{gut}, a fourth protein is required for PEP-dependent glucitol phosphorylation in vitro. Enzyme III^{gut} is a glucitol-specific, glucitol-inducible component of the PTS as is enzyme II^{gut}. This protein also exhibits hydrophobic characteristics as demonstrated by its successful purification, using hydrophobic chromatography on butylagarose. These characteristics might be expected for a protein which must interface with the membrane-bound enzyme II^{gut}. Our experiments have shown that phospho-HPr can phosphorylate enzyme III^{gut} in vitro and suggest that enzyme III^{gut} can directly donate its phosphoryl group to enzyme II^{gut} for the sugar group translocation process. SDS-gel electrophoresis experiments indicate that the enzyme III^{gut} is highly purified and that two different species exist. Both species are apparently phosphorylated and then dephosphorylated by the addition of the appropriate PTS components, but the relative rates of these processes for each enzyme III^{gut} species are not known. Meadow and Roseman have reported that enzyme III^{glc} can exist as two species, one being the product of a protease cleavage reaction acting upon the other species (16). We have raised antibodies against the enzyme III^{gut} and have shown that enzyme III^{gut} activity and the 15,000-dalton protein can be coprecipitated by these antibodies. These results are consistent with the conclusion that the 15,000-dalton protein is enzyme III^{gut}.

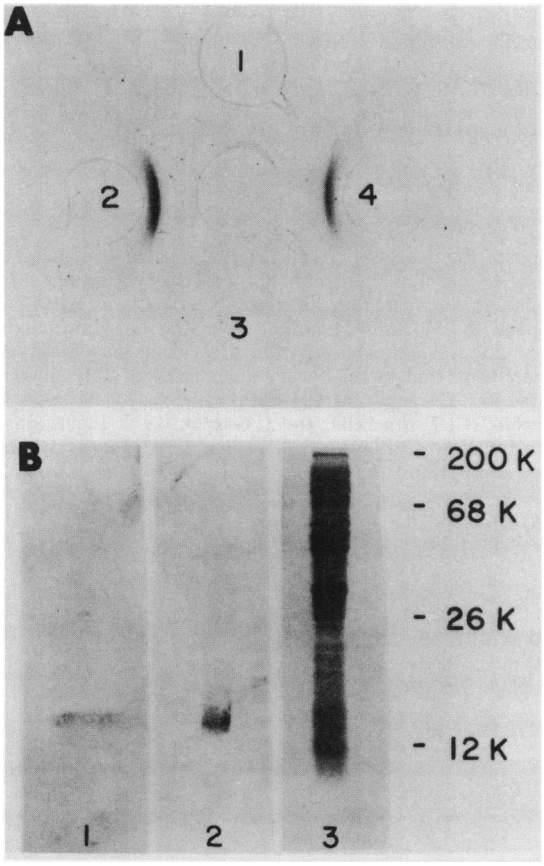

FIG. 3. Immunochemical characterization. (A) Ouchterlony double-diffusion analysis. Wells 1 and 3 contained 10 μl of preimmune serum, wells 2 and 4 contained 10 μl of immune serum, and the center well contained 10 μl of enzyme III^{gut} (0.65 mg/ml). (B) Immunoblotting. Lane 1 contained 4 μg of enzyme III^{gut} and lanes 2 and 3 contained 400 and 100 μg, respectively, of glucitol-grown LT-2 crude extract. Lanes 1 and 2 show nitrocellulose blots that were visualized with enzyme III^{gut} antibodies as described in the text. Lane 3 shows a stained gel (Coomassie blue) before electroblotting.

TABLE 3. Induced synthesis of the protein constituents of the glucitol catabolic enzyme system[a]

Growth of bacteria in medium containing:	Enzyme II^{gut}	Enzyme III^{gut}	Glucitol-6-phosphate dehydrogenase (μmol/min per mg of protein)
Glucitol	4.2	10.8	85
Mannitol	1.3	0.4	5
Glucose	0.3	1.0	10

[a] The component measured was always present in rate-limiting amounts. The membrane and supernatant fractions from the bacteria grown in media containing the different sugars were assayed for each enzyme activity under comparable conditions so that comparisons would be valid. Enzyme II^{gut} was assayed by using a high-speed supernatant (10 mg/ml) from an extract prepared from glucitol-grown *S. typhimurium* strain LT-2. Enzyme III^{gut} was assayed by using purified coupling enzymes as follows: 5 μg of enzyme I, 10 μg of HPr, 25 μg of enzyme II^{gut}. The degree of purity of each protein was as described in footnote *a*, Table 2.

Earlier genetic and physiological studies with *E. coli* K-12 did not reveal the presence of enzyme III activities for galactitol, glucitol, or mannitol (13, 14). Nevertheless, enzyme III^{gut} is present in glucitol-grown *S. typhimurium* and *E. coli* cells (F. C. Grenier and M. H. Saier, unpublished data), and it is required for the in vitro phosphorylation of this sugar. We have recently isolated mutants lacking enzyme III^{gut}, and these mutants, like enzyme II^{gut}-negative mutants, are unable to grow on or take up [^{14}C]glucitol (20). Thus, enzyme III^{gut} is an essential component of the glucitol PTS. Recent experiments suggest that a corresponding enzyme III specific for galactitol is not present in *E. coli* (E. B. Waygood, personal communication).

It has been suggested that the three hexitol operons arose from a common ancestral operon (13, 14, 19). Our recent experiments have shown that the major band in a 100-fold purified enzyme II^{gut} preparation has an apparent molecular weight of 45,000 as determined by SDS-PAGE (Grenier and Saier, unpublished data). Since the molecular weights of the enzyme II^{mtl} and enzyme III^{gut} are 60,000 and 15,000, respectively, by the same criteria, it is interesting to speculate that the enzyme II^{gut}-enzyme III^{gut} pair may have arisen from internal chain termination within a single enzyme II structural gene analogous to that which encodes enzyme II^{mtl}. The enzyme II^{mtl} gene has been sequenced, and the derived amino acid sequence has been evaluated by hydropathy analysis (12). It is clear that the NH_2-terminal half of enzyme II^{mtl} is predominantly hydrophobic and that the COOH-terminal half is predominantly hydrophilic. It was speculated that the hydrophilic portion of the molecule extends into the cytoplasm and contains a phospho-HPr binding site. This portion of the molecule would therefore be expected to resemble the enzyme III^{gut}. It is also possible, of course, that enzyme III^{gut} arises due to post-translational

modification of a larger enzyme II^{mtl}-like protein. The immunoblotting results, however, do not support this hypothesis. No protein other than the 15,000-dalton band cross-reacted with enzyme III^{gut} antibodies. This experiment does not completely rule out the possibility of post-translational modification, but we do not favor this hypothesis. Sequence analysis of the glucitol operon will be required to establish if the enzyme II^{gut} and the enzyme III^{gut} are encoded by separate genes. The evolutionary divergence of the hexitol operons will be interesting to trace.

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LITERATURE CITED

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