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Glucose Phosphoenolpyruvate-Dependent Phosphotransferase System of *Streptococcus mutans* GS5 Studied by Using Cell-Free Extracts†

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The glucose phosphotransferase system (PTS) of *Streptococcus mutans* GS5 has been partially characterized, using fractions derived from cells treated with the muramidase mutanolysin. Membranes retained functional PTS enzymes for the phosphoenolpyruvate-dependent phosphorylation of glucose, fructose, and mannose. This was confirmed by assaying membranes directly for enzyme I (EI) and enzyme II^{glc} (EII^{glc}) by employing specific phosphoryl-exchange reactions for each factor. Membranes prepared from glucose PTS^- mutants, however, were either deficient in glucose phosphorylation or reflected the "leakiness" displayed by whole cells. Mutant membranes were unable to catalyze the glucose:glucose 6-phosphate transphosphorylation reaction, indicating a defective EII^{glc} in these fractions. Although total cellular EI activities in the mutant clones were about the same as that measured for the wild-type strain by employing the pyruvate:phosphoenolpyruvate phosphoryl-exchange reaction, mutant membranes were found to possess less than 10% of the specific EI activity of wild-type membranes. The cytoplasmic fractions of mutants, however, displayed markedly increased specific activities for this enzyme when compared with wild-type extracts. These results strongly suggest a molecular association of EI with a normal membrane protein, perhaps EII^{glc}, that is absent in mutants. This would explain the absence of fructose PTS activity in glucose PTS^- mutant membranes despite the fact that whole cells of these clones are normal for this transport function.

The molecular details of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) have been well defined in a number of genera, as reviewed by Saier (10) and Dills et al. (1). The model system which has evolved from these studies involves a multi-component system allowing for sequential transfer of a phosphoryl group to an incoming sugar and can be summarized as follows:

$$HPr + PEP \xrightleftharpoons{EI} HPr - P + pyruvate \quad (1)$$

$$HPr - P + sugar \xrightleftharpoons{EII/EIII} sugar - P + HPr \quad (2)$$

In reaction 1, enzyme I (EI) hydrolyzes PEP and translocates the phosphoryl group to a low-molecular-weight, heat-stable protein (HPr). EI itself is a phosphorylated intermediate in this reaction. Reaction 2 involves the phosphorylation and concomitant translocation of the sugar moiety across the cytoplasmic membrane. Phosphoryl group transfer is through enzyme III (EIII), which also becomes a phosphorylated intermediate in the reaction sequence, whereas sugar transport is carried out by the membrane-bound permease enzyme II (EII). In the majority of systems studied, EI, HPr, and EIII are soluble proteins, an exception being the so-called low-affinity glucose system of *Escherichia coli*, wherein EIII is complexed in the membrane with EII. In other genera, certain other variations in components have been reported. For example, the spirochete *Spirochaeta aurantia* has only three components, EI, HPr, and EII, to transport (phosphorylate) mannitol (12). The fructose PTS (frcPTS) in *Pseudomonas aeruginosa* is composed of only two components, a soluble EI-like protein and a membrane-bound carrier (3).

All major sugars, including disaccharides, catabolized by *Streptococcus mutans* are transported into the cell by PEP-linked PTS (5, 6). The glucose PTS (glcPTS) of *S. mutans* GS5 appears to be relatively nonspecific in its substrate recognition properties and transports glucose, mannose, and 2-deoxyglucose (8a, 14). $glcPTS^-$ mutants are unable to transport each of these sugars but are still able to phosphorylate fructose by an independent frcPTS unaffected by the genetic lesion(s). Furthermore, the glcPTS appears to play a regulatory role in the transport of other sugar substrates into this species (2, 5, 8, 18). Thus, when strain GS5 is grown in mixtures of glucose and lactose typical diauxie can be observed as genes encoding the lactose PTS (lacPTS) are repressed until glucose is completely exhausted from the medium. $glcPTS^-$ mutants do not display diauxie, suggesting a prominent role for this system in transport regulation (8).

To this point, however, there have been no documented attempts to better define the *S. mutans* glcPTS at the molecular level. The present report describes the fractionation of muramidase (mutanolysin)-treated cells after osmotic lysis. Cellular extracts (cytoplasm and membranes) were assayed directly for the presumably soluble EI and the membrane-bound glucose permease EII^{glc}, as well as for total PTS activity. By using this approach, we have been able to detect a major lesion in $glcPTS^-$ mutants and to arrive at preliminary conclusions concerning the localization of key PTS components in *S. mutans* GS5.

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MATERIALS AND METHODS

Cultures and cell growth. *S. mutans* GS5 was routinely maintained in a tryptone-yeast extract (TYE) broth (5). This medium was composed of 10 g of tryptone per liter (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract per liter (Difco), 3 g of dibasic potassium phosphate per liter, and 20 mM glucose. The defined medium (DM) of Terleckyj et al. (19), supplemented with 20 mM glucose, was employed as indicated.

Membrane preparation. Cell-free membranes were prepared by a modification of the methods of Siegel et al. (15). Log-phase cells, grown in 200 ml of either TYE broth or DM, were washed twice in 0.9% NaCl, twice in 5 mM EDTA, and finally in a lysis buffer consisting of 20 mM Tris-hydrochloride (pH 6.8), 5 mM 2-mercaptoethanol, and 10 mM $MgCl_2$. Cells then were suspended in 50 ml of lysis buffer and lysed with 5 mg of purified mutanolysin (a gift from K. Yokagawa, Dainippon Chemical Co., Tokyo, Japan). After 60 min at 37°C, complete lysis had occurred as indicated by measurements of absorbance at 660 nm and the complete loss of viability upon plating on TYE agar. The lysate was incubated with 2.5 mg of RNase and 2.5 mg of DNase (Sigma Chemical Co., St. Louis, Mo.) for an additional 60 min at 37°C with stirring. One millimolar phenylmethylsulfonyl fluoride (Sigma Chemical Co.) was included in the lysis buffer to inhibit proteases known to exist in mutanolysin and DNase.

Membranes were collected by centrifugation at 30,900 × *g* for 60 min and washed once with 100 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. These two supernatants were pooled and stored at −70°C. This was referred to as the cytoplasmic fraction. The membrane pellet was resuspended in the same buffer and then centrifuged at 1,075 × *g* for 5 min to remove particulate debris. The resulting supernatant was then pelleted at 30,900 × *g* for 60 min. This pellet contained purified, cell-free membranes completely devoid of contamination with cell wall components as determined by gas-liquid chromatography (15). The product was suspended in 2.5 ml of buffer and stored at −70°C.

Assay for PEP-dependent PTS activity. PEP-dependent PTS activity was determined by phosphorylation of radiolabeled sugars, using a modification of techniques previously described (8, 16). Membranes, at protein concentrations shown in the table footnotes, were incubated in 80 mM PB (pH 7.0), 3.0 mM $MgCl_2$, 10 mM sodium PEP (Sigma), 10 mM sodium fluoride (Sigma), 4 mM 2-mercaptoethanol, and the labeled substrates at the concentrations shown in the table footnotes. Unless otherwise indicated, 100 μM unlabeled sugar also was included. The assay mixture was incubated for 30 min at 37°C in a volume of 200 μl. The reaction was terminated by the addition of 1% unlabeled sugar to a final volume of 2.0 ml with rapid cooling in an ice bath.

Membranes and phosphorylated sugar derivatives then were separated from unincorporated labeled substrates by filtration under vacuum through DE-81 anion-exchange filters (Whatman, Clifton, N.J.) which had been prewashed with a 1% solution of the sugar substrate. After loaded filters were washed once with 1% sugar solution and four times with distilled water, they were counted directly in a Beckman LS8000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) in 5 ml of scintillation fluor (Aquasol; New England Nuclear Corp., Boston, Mass.). Controls lacking PEP always were run to determine the amount of radioactivity on filters due to inadequate washing, simple adherence to membranes, or alternate transport mechanisms. In no case were control counts ever greater than 10% of the experimental samples. After correction for control counts, results were expressed as picomoles of sugar phosphate per microgram of membrane protein.

Assay for membrane-associated transphosphorylation. To assay directly for the EII^{glc}, the [^{14}C]glucose:glucose 6-phosphate exchange reaction of Saier et al. (11) was modified as follows: 70 mM phosphate buffer (pH 6.0), 3.5 mM $MgCl_2$, 3.5 mM 2-mercaptoethanol, 50 mM glucose 6-phosphate (Sigma), 10 mM NaF, and 50 μM D-[*U*-^{14}C]glucose (4 μCi/μmol) were reacted with membranes at the protein concentrations noted in the table footnotes. The total reaction volume was 200 μl. The mixture minus the labeled glucose was prewarmed to 37°C, and the reaction was begun upon the addition of D-[*U*-^{14}C]glucose. After 30 min at 37°C, the reaction was terminated by the addition of 2.0 ml of cold water with rapid cooling in ice. The product of the exchange reaction, D-[*U*-^{14}C]glucose 6-phosphate, was separated from other reactants by passage of the mixture through a Dowex AG1-X8 (Cl^-) column (0.7 by 4.0 cm; Bio-Rad Laboratories, Richmond, Calif.). The column was washed with ca. 3 column volumes of water, and labeled glucose 6-phosphate was eluted in 5 ml of 1 M $LiCl_2$ directly into a scintillation vial. Aquasol (10 ml) was added to the vial, and it was then counted in the Beckman LS8000 scintillation counter. Results were expressed as picomoles of [^{14}C]glucose 6-phosphate formed per microgram of membrane protein.

Assay for EI activity. EI was quantified by the pyruvate:[^{14}C]PEP phosphoryl-exchange reaction of Saier et al. (13). The reaction mixture was composed of 40 mM Tris-hydrochloride(pH 7.5), 2 mM 2-mercaptoethanol, 8 mM $MgCl_2$, 10 mM NaF, 2 mM sodium pyruvate, 0.2 mM phosphoenol[1-^{14}C]pyruvic acid cyclohexylammonium salt (10.6 μCi/μmol) plus the cellular fraction at protein concentrations listed below. The total reaction volume was 100 μl. All components except the radioactive substrate were incubated at 37°C, and the reaction was begun with the addition of [^{14}C]PEP. The reaction was carried out for 60 min, after which time 0.4 ml of Sigma color reagent (stock no. 505-2; 20 mg of 2,4-dinitrophenylhydrazine in 100 ml of 1 N HCl) was added. The tubes were mixed vigorously and incubated for another 10 min at 37°C. The derived osazone of pyruvate then was separated by the addition of 1 ml of ethyl acetate. After vigorous mixing, a sample (600 μl) was removed from the nonaqueous phase, added to 5 ml of Aquasol, and counted in the Beckman LS8000 liquid scintillation counter. Results were calculated per 600 μl of product and expressed as picomoles of [^{14}C]pyruvate formed per microgram of protein or picomoles of [^{14}C]pyruvate formed per 600 μl.

Mutagenesis and mutant selection. The process of mutagenesis and selection of $glcPTS^-$ mutants has been described previously (8).

Protein determination. The method of Lowry et al. (9) was used for protein determinations. Membranes were solubilized in 3% sodium dodecyl sulfate (Sigma) before assay.

Chemicals. PEP, ATP, glucose, and mannitol were purchased from Sigma Chemical Co. Mannose and fructose were obtained from Calbiochem-Behring, LaJolla, Calif. The purity of the sugars was determined by gas-liquid chromatography (4).

Radioisotopes. The following radioisotopes were purchased from New England Nuclear Corp.: D-[*U*-^{14}C]glucose (4.0 μCi/μmol), D-[*U*-^{14}C]fructose (359 μCi/μmol) and D-[1-^{14}C]mannose (48.6 μCi/μmol). Phosphoenol[1−^{14}C]

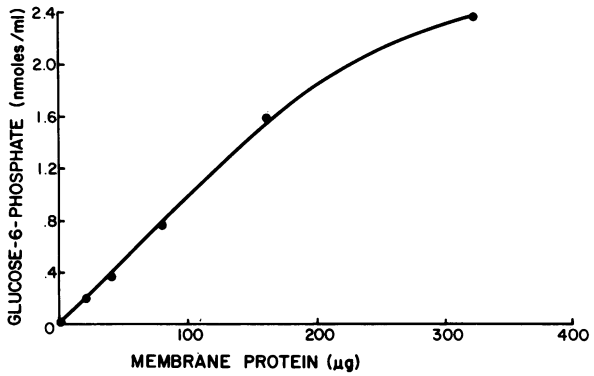

FIG. 1. Phosphorylation of glucose by cell-free membranes. Cell-free membranes, obtained after mutanolysin-induced lysis of *S. mutans* GS5, were assayed at various protein concentrations for PEP-dependent glucose phosphorylation (expressed as glucose 6-phosphate). Reaction mixture included 175 μM D-[*U*-^{14}C]glucose (4.0 μCi/μmol) plus the standard components described in the text for the PTS assay.

pyruvic acid (10.6 μCi/μmol) was obtained from Amersham Corp., Arlington Heights, Ill.

RESULTS

glcPTS activity in cell-free membranes of *S. mutans* GS5. PTSs are characterized by reactions catalyzed both by soluble (cytoplasmic) enzymes and by membrane-localized factors functioning in a coordinated manner. It was of interest to determine whether *S. mutans* membranes, prepared by using the muramidase mutanolysin, could phosphorylate glucose in the absence of added cytoplasmic extracts. Figure 1 shows the formation of phosphorylated glucose at levels proportional to the amounts of membrane protein in the reaction mixture up to 160 μg. Beyond 160 μg of membrane protein, one of the reaction components appears to become rate limiting for the entire system. When cytoplasmic extracts were added to these reaction mixtures at protein concentrations up to five times greater than that of the membrane protein, there was no increased trapping of radiolabeled glucose (data not shown).

Table 1 presents data that support the conclusion that membranes are capable of carrying out a PEP-dependent glucose phosphorylation. There is an absolute requirement for PEP; ATP does not substitute as the phosphoryl donor. The omission of NaF yields a slightly higher concentration of product than when it is included. Also, some residual activity is obtained in the absence of added PEP and NaF.

TABLE 1. PEP-dependent phosphorylation of glucose by mutanolysin-derived membranes of *S. mutans* GS5[a]

Conditions	pmol of glucose phosphate formed/μg of membrane protein[b]
+PEP, +NaF	7.3
+PEP, −NaF	8.6
−PEP, −NaF	1.1
+ATP, +NaF	0.0

[a] Cells were grown in DM plus 20 mM glucose, and membranes were prepared with mutanolysin (see the text).

[b] Membranes (40 μg of protien) were assayed for PTS activity, using 175 μM D-[*U*-^{14}C]glucose (4.0 μCi/μmol) as described in the text. Omissions in the reaction mixture were made as indicated above. ATP (10 mM) was substituted for PEP as indicated.

TABLE 2. Comparative PTS activities of mutanolysin-derived membranes of *S. mutans* GS5[a]

Substrate	Sugar phosphates formed[b] (pmol/μg of membrane protein)
Glucose	12.2
Mannose	12.2
Fructose	12.6

[a] Membranes were prepared from glucose-grown cells by the mutanolysin procedure (see the text).

[b] Membranes (48 μg of protein) were assayed for PTS activity as described in the text. The sugar concentrations were: 175 μM D-[*U*-^{14}C]glucose (4 μCi/μmol); 100 μM D-mannose plus 10 μM D-[1-^{14}C]mannose (48.6 μCi/μmol); or 100 μM D-fructose plus 1 μM D-[*U*-^{14}C]fructose (359 μCi/μmol).

These data strongly indicate the presence of an energy reserve associated with these membrane preparations. The absence of detectable activity when NaF is included without added PEP (Table 1) suggests that this reserve may be in the form of 2-phosphoglycerate, with enolase converting it to PEP. If these suppositions are correct, it would suggest some cytoplasmic incorporation into these membrane preparations, possibly due to the formation of membrane vesicles. The existence of such vesicles would explain PTS activity in the absence of added HPr or EI, although data presented below suggest a more stable membrane association for EI than previously reported.

PTS activity of membranes with various sugar substrates. Previous studies (8a; Liberman, Ph.D. thesis, University of Florida, Gainesville, 1982) have indicated that glucose-grown *S. mutans* GS5 cells carry out constitutive PEP-dependent phosphorylations of glucose, mannose, and fructose. Mutant studies and competition experiments with heterologous sugars indicate that a single PTS transports both glucose and mannose, whereas fructose is phosphorylated by an independent system. Table 2 demonstrates that membranes derived from glucose-grown cells also phosphorylate all three sugars, thereby reflecting the constitutiveness of these PTSs as found in the parent cell.

When competition experiments with excess amounts (10 mM) of unlabeled sugars are carried out, inhibition patterns for membranes are obtained that are quite similar to those obtained with decryptified cells (Table 3). As with whole cells, glucose and mannose block the phosphorylation of D-[*U*-^{14}C]glucose, indicating that a single PTS may serve for the uptake of these hexoses. Fructose is without inhibitory

TABLE 3. Inhibitory effects of competing sugars on the phosphorylation of D-[*U*-^{14}C]glucose by the PEP-dependent PTS of *S. mutans* GS5 membranes[a]

Competing sugar	% Inhibition[b]
None	0
Glucose	100
Mannose	93
Fructose	7
Mannitol	0

[a] Membranes were prepared by mutanolysin-induced lysis of glucose-grown cells (see the text).

[b] Membranes (50 μg of protein) were reacted in the standard PTS reaction mixture (see the text) containing 125 μM D-[*U*-^{14}C]glucose (4.0 μCi/μmol) and either no competing sugar or 10 mM unlabeled sugar.

effect, again suggesting the existence of a distinct fructose EII (EII^{frc}) in this organism. Of concern in experiments of this type is the possible confusion caused by trace glucose contamination of the large amounts of heterologous sugars employed. Mannitol, with 0.184% glucose, served as a negative control and showed that this small amount of contaminating glucose is without inhibitory affect. Mannose contained 0.063% glucose, which therefore was considered negligible.

Detection of EI and EII^{glc} in cell-free membranes. A convenient method for assaying the permease EII^{glc} is by the transphosphorylation reaction of Saier et al. (11). This membrane-bound factor catalyzes a phosphoryl exchange between radiolabeled glucose and glucose 6-phosphate without the need for other PTS components. The generation, therefore, of [^{14}C]glucose 6-phosphate is an indicator of EII^{glc} activity. This reaction was employed, using strain GS5 membranes, to determine the presence of an active permease in these preparations.

The transphosphorylation reaction is very sensitive to substrate inhibition and requires a phosphoryl donor-to-acceptor ratio of 1,000:1 to provide an optimum reaction (11). Table 4 shows the optimization of the reaction. With the donor-to-acceptor ratio held at 1,000:1, the maximum product yield was obtained with 50 mM glucose 6-phosphate and 50 μM D-[*U*-^{14}C]glucose. At higher concentrations of reactants, there was considerable inhibition of phosphoryl exchange. This reaction was proportional to the amount of membrane protein employed up to at least 195 μg (data not shown). These experiments, therefore, demonstrated the localization and activity of the EII^{glc} in strain GS5 membranes.

The primary observation that membranes alone catalyze PEP-dependent PTS reactions (Table 1) indicated that EI and HPr, presumably soluble factors, are either trapped within membrane vesicles or are directly associated with the membrane. To test for the presence of EI in cell-free membrane preparations, the pyruvate:[^{14}C]PEP phosphoryl-exchange reaction of Saier et al. (13) was employed. The generation of [^{14}C]pyruvate, obtained as its osazone derivative (see above), served as the indicator of EI activity. It is evident from Fig. 2 that strain GS5 membrane preparations catalyze this reaction in the absence of added cytoplasmic constituents. Furthermore, this reaction is proportional to the amount of membrane employed and is detected with as little as 2 μg of membrane protein.

Since EI is generally considered to be of cytoplasmic origin, it was of interest to determine its distribution in fractionated cells. Cytoplasm prepared as described above plus the first membrane wash collectively accounted for 65% of total cellular EI activity; the membrane fraction contained the remaining 35% of total activity. Subsequent buffer washings of membranes yielded no further activity or measurable protein. If indeed closed membrane vesicles exist in such preparations, they are resistant to leakage by soluble EI.

TABLE 4. Glucose:glucose 6-phosphate transphosphorylation by *S. mutans* GS5 membranes as a function of reactant concentrations[a]

Reactant or product	Concn				
Reactant[b]					
[^{14}C]glucose (μM)	12.5	25	50	75	100
Glucose 6-phosphate (mM)	12.5	25	50	75	100
Product					
[^{14}C]glucose 6-phosphate (pmol/μg of membrane protein)	1.07	3.81	7.86	5.36	4.40

[a] Membranes were prepared from cells grown in DM plus 5 mM glucose, using mutanolysin (see the text).

[b] Membranes (168 μg) were assayed by the method of Saier et al. (11) as described in the text. Concentrations of reactants were varied as indicated above.

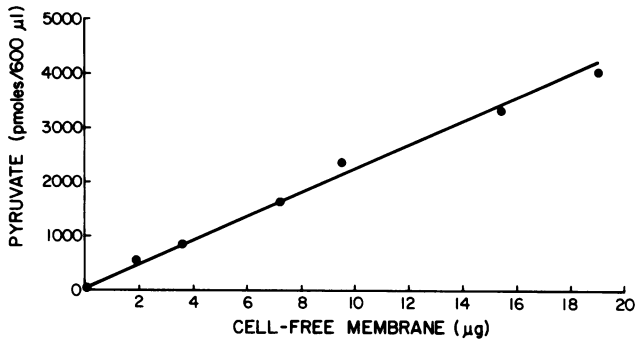

FIG. 2. Pyruvate:phosphoenol[1-^{14}C]pyruvate phosphoryl-exchange reaction by cell-free membranes. Membranes prepared from cells grown in DM plus 20 mM glucose were assayed for EI as described in the text, using a variety of membrane protein concentrations. Labeled pyruvate formed during the reaction was converted to the osazone derivative before separation and counting and is expressed as picomoles per 600 μl.

During our studies of EI, it became evident that this factor is quite labile in its soluble form. For example, the level of EI activity determined by the pyruvate:[^{14}C]PEP phosphoryl-exchange reaction decreased by 51 and 86% in 2 and 7 days, respectively, when cytoplasmic extracts were assayed with intermittent freeze-thawings. This assay with strain GS5 membranes revealed only a 27% decrease in activity in 5 days with daily freeze-thawings. Membrane association, therefore, may protect EI from denaturation. Indeed, the EI of *Staphylococcus aureus* has been shown to be labile in its purified form, but relatively stable to freeze-thawing when membrane associated (16).

PTS activities in membranes derived from glcPTS$^-$ mutants. glcPTS$^-$ mutants are unable to transport glucose, mannose, and 2-deoxyglucose but retain their transport function for fructose (8a). These findings supported the premise that the frcPTS in *S. mutans* is separate and distinct from a transport system able to phosphorylate glucose, mannose, and 2-deoxyglucose. It was of importance, therefore, to determine whether membranes derived from mutants reflected the altered physiology of the whole cell.

Table 5 summarizes PEP-dependent PTS reactions involving glucose and fructose employing membranes derived from wild-type cells and three mutant clones. Strains 3A and 4B were "tight" glcPTS$^-$ mutants, whereas strain 8B was slightly "leaky" with 13% residual glcPTS activity when compared with the wild-type parent strain (8). Membranes from the tight mutants were devoid of glcPTS activity, whereas strain 8B membranes still showed low (10%) levels of activity (Table 5). Thus, membranes of mutants reflect the whole cell in terms of glcPTS activity. frcPTS activity in these membranes, however, deviated significantly from activities measured in whole cells of these mutant clones. Although decryptified cells demonstrated normal or near-normal frcPTS activities, their membranes show only low levels of fructose phosphorylation. This unexpected finding was reproducible from batch to batch of mutant membranes

TABLE 5. Relative glcPTS and frcPTS activities in *S. mutans* GS5 membranes prepared from glcPTS$^-$ mutant and wild-type cells[a]

Strain	glcPTS[b]		frcPTS[b]	
	pmol of glucose phosphate/μg membrane protein	% wild type	pmol of fructose phosphate/μg of membrane protein	% wild type
Wild type	12.20	100	12.50	100
3A	0	0	0.56	4.5
4B	0	0	0.57	4.6
8B	1.20	10	0.47	3.9

[a] Membranes were prepared from cells grown in TYE broth supplemented with 20 mM mannitol as described in the text.

[b] Membranes (90 to 180 μg of protein) were reacted with either 125 μM D-[*U*-^{14}C]glucose (4.0 μCi/μmol) or 100 μM D-fructose plus 1 μM D-[*U*-^{14}C]fructose (359 μCi/μmol) in the standard PTS reaction mixture (see the text).

TABLE 7. Specific activities of EI in membranes and cytoplasmic fractions derived from wild-type and glcPTS$^-$ mutants of *S. mutans* GS5[a]

Strain	Sp act (pmol of [^{14}C]pyruvate/μg of protein)	
	Membranes[b]	Cytoplasmic fraction[b]
Wild type	690.0	205.8
3A	31.2	532.3
4B	22.8	708.0
8B	57.8	382.5

[a] Cell-free extracts were prepared by mutanolysin-induced lysis of cells grown to log phase in TYE broth supplemented with 20 mM mannitol. The procedure followed is outlined in the text.

[b] Membranes (5 to 45 μg of protein) and cytoplasmic fractions (2 to 12 μg of protein) were assayed for EI by the method of Saier et al. (13) as described in the text. Each preparation was assayed at several protein concentrations to obtain data in the linear ranges of activity.

and may reflect the altered relationship between membranes and EI (see data below).

To better define the site(s) of genetic lesion in these mutants, the phosphoryl-exchanage reactions were again employed as probes for EII^{glc} and EI. Table 6 clearly demonstrates the absence of a measurable EII^{glc} in membranes derived from two mutant clones, including the leaky strain 8B. Wild-type membranes isolated from mannitol-grown cells possessed about one-half the transphosphorylation activity when compared with membranes from glucose-grown cells (Table 4). Assays for total cellular EI activity, using the pyruvate:PEP phosphoryl-exchange reaction (13), indicated that wild-type and glcPTS$^-$ mutant clones were quite similar (data not shown). The striking difference is in the distribution within the cell of this enzyme (Table 7). The specific activity of EI in the membranes of mannitol-grown cells of the wild type was three times the value determined for glucose-grown cells (Fig. 2). glcPTS$^-$ mutants, however, showed markedly diminished membrane activities and markedly increased cytoplasmic specific activities for this key PTS factor (Table 7). Less than 10% of the wild-type EI specific activity was found in mutant membranes; the highest (8%) was measured in membranes of the leaky clone 8B. It is apparent, therefore, that EI is primarily a cytoplasmic constituent in glcPTS$^-$ mutants.

DISCUSSION

From the data presented in this report, it is clear that membranes prepared after the osmotic lysis of muramidase-treated *S. mutans* cells are able to catalyze PTS-mediated phosphorylations without the addition of soluble factors (Tables 1 and 2 and Fig. 1). That these reactions accurately reflect those occurring in decryptified cells is indicated by competition experiments employing heterologous sugars (Table 3) (8a). It appears, therefore, that cell-free membranes prepared by these methods contain a full complement of PTS proteins either bound to the membrane or trapped within vesicles. The apparent presence of an endogenous energy reserve (Table 1) in these well-washed preparations indicates that the latter is a definite possibility.

We chose to focus our present investigations on EI and EII^{glc} by employing the convenient phosphoryl-exchange reactions of Saier and colleagues (11, 13). *S. mutans* membranes possess a typical EII^{glc} that functions in PEP-dependent glucose phosphorylation (Table 1) and in the glucose:glucose 6-phosphate transphosphorylation reaction (Table 4). The latter reaction is similar to that described for *E. coli* (11) in that it is subject to severe substrate inhibition. glcPTS$^-$ mutants, however, are unable to carry out transphosphorylation, indicating a major lesion in these transport mutants is in the permease EII^{glc} (Table 6). In a previous report (8), we described the loss of regulation of lactose transport in glcPTS$^-$ mutants and proposed that the altered PTS component(s) could be pivotal in the regulatory mechanism. The identification of a genetic lesion in EII^{glc} in these regulation-deficient mutants suggests that this enzyme may be the key PTS component functioning in normal cells to regulate uptake of lactose and possibly other sugars (2). Proof of this suggestion awaits further molecular characterization of the entire glucose-specific PTS of this species. For example, Jacobson et al. (G. R. Jacobson, C. S. Mimura, P. J. Scott, and L. B. Eisenberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K114, p. 196) have reported a soluble EIII active in the PTS-mediated uptake of sucrose. Genetic lesions affecting EIII also could explain data reported above.

The pyruvate:phosphoenol[1-^{14}C]pyruvate phosphoryl-exchange reaction clearly demonstrated that much EI activity is closely associated with the cell membrane (Fig. 2). This was unexpected since EI normally is considered to be of cytoplasmic origin, although membrane vesicles prepared from *E. coli* by osmotic lysis (Kaback vesicles) contain low levels of both EI and PEP (7). Fractionation of glucose-grown strain GS5 cells indeed revealed EI to be mostly (65%) in the soluble extract, but the remaining 35% of EI activity was tightly bound to membranes. Elution of enzyme activity from membranes by repeated buffer washings was

TABLE 6. Glucose:glucose 6-phosphate transphosphorylation as a measure of EII^{glc} in membranes of *S. mutans* GS5 mutant and wild-type cells[a]

Strain	pmol of [^{14}C]glucose 6-phosphate formed/μg of membrane protein[b]	% Wild type
Wild type	3.3	100
4B	0	0
8B	0	0

[a] Membranes were prepared as described in the text, using cells grown in TYE broth with 20 mM mannitol.

[b] Transphosphorylation was carried out in the reaction mixture described in the text with 50 mM gluose 6-phosphate and 50 μM D-[*U*-^{14}C]glucose (4.0 μCi/μmol). To assure the validity of the negative results indicated above, two membrane concentrations were assayed for each strian: 180 to 240 μg and 455 to 475 μg of membrane protein.

unsuccessful, perhaps due to the trapping of EI (and presumably HPr) in closed vesicles. Such an association could be the result of nonspecific events involving the simple closure of membranes in the presence of residual cytoplasm, or it may be the result of a specific binding of EI to a membrane receptor such as EII^{glc}. The latter possibility would classify EI as an extrinsic membrane protein. Singer and Nicholson (17) define extrinsic membrane proteins as those which fractionate with membranes but can be removed by relatively gentle means such as changes in ionic strength or pH.

Support for the possibility that EI indeed is an extrinsic membrane protein is provided by three observations. The first is based on the lability of this enzyme in its soluble form as opposed to the membrane-bound state. Our own experiments and the work of Simoni et al. (16) clearly indicate that membrane association protects this enzyme from oxidative denaturation or other causes of denaturation. The second observation is the repeated measurement of substantial EI activity in membranes derived from both glucose- and mannitol-grown cells. The possibility remains, however, that some of the soluble EI was denatured during extraction, causing calculations to be skewed in favor of the membrane-bound fraction.

The final and perhaps most significant observation comes from the study of mutant membranes. The amount of EI associated with mutant membranes was greatly reduced as compared with wild-type membranes (Table 7). If entrapment of enzyme within membrane vesicles is to be considered as a prime cause of EI association with membrane preparations, certainly mutant membranes should afford the same opportunities as wild-type membranes. It would seem, therefore, that a specific receptor site exists in membranes for this factor and that this site is altered in mutants. The finding that $glcPTS^-$ mutants lack EII^{glc} activity (Table 6) provides an attractive basis for the supposition that this permease is a major membrane receptor for EI (or HPr), to which it is functionally related. The loss of EII^{glc} would result in the inability of EI to maintain a high degree of membrane association (Table 7).

Data previously reported by Jacobson et al. (G. R. Jacobson, W. L. Ran, and P. J. Scott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K122, p. 157) for the sucrose PTS of *S. mutans* 6715 (serotype *g*) are quite consistent with those reported here for the glcPTS of strain GS5. These authors found EI and HPr to be strongly bound to cell-free membranes and able to resist dissociation by passage through a French pressure cell or by sonication. Only agitation with glass beads caused these PTS factors to become solubilized. Jacobson et al. also concluded that EI and HPr are bound to the cell membrane under physiological conditions conducive to sugar transport. Again, the possibility of EI entrapment within vesicles seems unlikely since such bodies are easily disrupted by the physical means employed by these investigators.

Still not explained is the unexpected loss of fructose phosphorylating activity by $glcPTS^-$ mutant membranes (Table 5), whereas whole decryptified cells of these same mutants possess normal abilities to transport fructose. Assuming there are no secondary lesions in the mutant membranes that alter the structure of EII^{frc} upon mutanolysin treatment of mutant cells, it would seem plausible to assume that a limiting concentration of a PTS component is preventing the phosphorylation of fructose. The loss of membrane association by EI in these mutants (Table 7) could be the basis of such a limitation. Thus, EII^{glc} in wild-type membranes would retain sufficient EI to allow the phosphorylation of glucose, mannose, or fructose in vitro as shown in Table 2. With an altered EII^{glc}, however, such reactions could be prevented by the nonretention of EI by membranes.

It is tempting to extrapolate these findings to in vivo situations. If the permease for glucose transport by the PTS is, in fact, a major receptor of EI, it should be predicted that glucose would be transported before other hexoses; thus, the EII^{glc} would be a prime regulator of sugar transport. This would conform nicely with the catabolite inhibition theory of Thompson et al. (20), who observed a preferential utilization of glucose over galactose in *Streptococcus lactis*. It also would explain, in part, our own earlier findings (8) that *S. mutans* GS5 takes up glucose before lactose when grown in equimolar mixtures of the two substrates. The preferred binding of EI to EII^{glc} while glucose is plentiful in the medium would lead to the exclusion of the lactose inducer. Only upon glucose exhaustion would EI be free to react with the minimal amounts of EII^{lac} present in membranes of noninduced cells. Lactose then could enter and induce the synthesis of a full complement of lacPTS components. This explanation fully agrees with our previous data and predicts the diauxic growth curves we have generated when growing strain GS5 in these sugars (8).

This report has described one of the first attempts to characterize the *S. mutans* PTS at the molecular level. It should be clear that further studies employing a variety of substrates are required to draw a more complete picture of this vital transport system in this cariogenic species.

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