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Characterization of a Phosphoenolpyruvate-Dependent Sucrose Phosphotransferase System in *Streptococcus mutans*

EDWARD J. ST. MARTIN* AND CHARLES L. WITTENBERGER

Microbiology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20205

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A phosphoenolpyruvate-dependent sucrose phosphotransferase system has been identified in *Streptococcus mutans*. Sucrose phosphotransferase activity was inducible by sucrose and had an apparent K_m for sucrose of 70 μM. The product of the sucrose phosphotransferase reaction was isolated and identified as sucrose phosphate. Additional analysis revealed that the phosphate group was on the glucose moiety. Mutants unable to grow in media containing low concentrations of sucrose were isolated and found to be missing either sucrose phosphotransferase activity or the ability to hydrolyze sucrose phosphate.

Streptococcus mutans and dietary sucrose have consistently been implicated in the etiology of dental caries. The metabolism of sucrose by this microorganism has therefore received a great deal of attention. It has been found that *S. mutans* can hydrolyze a small portion of available sucrose by the action of extracellular glucosyl- and fructosyltransferases (11, 12, 14). The remainder of the sucrose has been thought to be hydrolyzed by invertases (2, 13).

However, the relatively high K_m values for sucrose that have been reported for the extracellular (7 to 20 mM) and intracellular (30 to 140 mM) invertases suggested that these enzymes might function only at high sucrose concentrations (3, 6, 8, 13; E. V. Palumbo and B. M. Chassy, Fed. Proc. **33**:1315, 1974). In this report, we describe a different pathway for sucrose catabolism, which can function at low substrate concentrations. We have found that sucrose utilization by *S. mutans* can be initiated by a highly efficient phosphoenolpyruvate (PEP)-dependent sucrose phosphotransferase (PTS) reaction.

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MATERIALS AND METHODS

Growth conditions. Stock cultures of *S. mutans* strain 6715-10 (designated DR0001/1 in our collection) were grown in fluid thioglycolate medium and stored at 4°C. All growth experiments were conducted with a chemically defined medium (DM) (15). Solid media were prepared at one-half strength by mixing equal volumes of DM with a 3% agar solution immediately after autoclaving. Agar plates were incubated in an anaerobic atmosphere (H_2-CO_2, GasPak). Growth substrates were provided at the following final concentrations (millimolar): sucrose, 10; maltose, 10; lactose, 10; glucose, 20; fructose, 20; mannitol, 20; and sorbitol, 20.

Preparation of permeabilized cells. Overnight cultures grown at 37°C in DM with the appropriate substrate were harvested by centrifugation and washed twice with 0.1 M sodium-potassium phosphate buffer, pH 7.2, which contained 5 mM $MgCl_2$ (7). The cells were then resuspended in 0.1 volume of the same buffer and treated with 0.05 volume of toluene-acetone (1:9, vol/vol) during 1 min of vigorous agitation on a Vortex-type mixer. The cells were then subjected to three more 1-min periods of agitation with intermittent cooling in an ice bath.

Total sucrose hydrolysis assay. The hydrolysis of sucrose to yield free glucose or fructose or both was determined by using a three-enzyme-coupled continuous spectrophotometric assay. The use of this assay permitted the detection of total glucosyl- and fructosyltransferase activity, as well as invertase activity. Each 1-ml reaction mixture contained 100 mM sodium-potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 10 mM NaF, 10 mM adenosine 5'-triphosphate, 0.2 mM nicotinamide adenine dinucleotide phosphate, 1 U of hexokinase (Sigma type C-302), 1 U of phosphoglucose isomerase, 1 U of glucose-6-phosphate dehydrogenase, 1 mM sucrose, and 10 to 100 μl of cell-free culture fluid or permeabilized cell suspension.

PTS assay. PTS activity was determined by a lactate dehydrogenase-coupled continuous spectrophotometric assay (7). Each 1-ml reaction mixture contained 100 mM sodium-potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 5 mM PEP, 0.2 mM reduced nicotinamide adenine dinucleotide, 10 mM NaF, 1.0 mM substrate, 10 U of lactate dehydrogenase, and 10 to 100 μl of permeabilized cell suspension. Reaction rates were linear with time and proportional to cell concentration.

Isolation of sucrose-negative mutants. *S. mutans* was grown on DM plus 20 mM glucose to an optical density of 0.9 U at 550 nm. The culture was then treated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 30 min. After treatment, the cells were washed with fresh medium and grown for

10 generations on DM plus glucose. These cells were then diluted and plated out on DM that contained 0.5 mM glucose and 5 mM sucrose. After incubation for 72 h, small colonies were picked with sterile toothpicks and replicated to DM plus glucose and DM plus sucrose plates. By using this technique, mutants that could grow on glucose but not on sucrose were identified.

PEP-dependent sucrose phosphate hydrolysis. Because substrate amounts of purified sucrose phosphate were not available, we devised an assay for sucrose phosphate hydrolysis that was based upon the endogenous production of sucrose phosphate by the sucrose PTS activity present in permeabilized cells. Each 1-ml reaction mixture contained 100 mM sodium-potassium phosphate (pH 7.2), 10 mM NaF, 0.2 mM nicotinamide adenine dinucleotide phosphate, 1 mM sucrose, 5 mM PEP, 1 U of glucose-6-phosphate dehydrogenase, and 10 to 100 μl of permeabilized cell suspension. The reaction mixture minus PEP served as a control. No reaction was observed when adenosine 5'-triphosphate was substituted for PEP in the above reaction.

Synthesis of sucrose phosphate. To identify the product of the sucrose PTS reaction, radioactively labeled sucrose was incubated with PEP and permeabilized cells of the sucrose phosphate hydrolase-negative mutant (DR0001/3). A 1-ml reaction mixture contained 50 mM sodium-potassium phosphate (pH 7.2), 2.5 mM $MgCl_2$, 1 mM PEP, 0.1 mM [U-^{14}C]sucrose (specific activity, 1.0 μCi/μmol), and 20 μl of permeabilized cell suspension. Zero-time and 10-min samples were obtained by terminating the reaction with a 0.45-μm pore size filter to remove the cells, followed by quick freezing of the samples in an ethanol–dry ice bath. Reaction samples were applied to a thin-layer cellulose chromatogram and developed in a 1-butanol–acetic acid–water (5:3:2) solvent system. Radioactive sucrose, glucose, and fructose and unlabeled glucose 6-phosphate and fructose 6-phosphate served as standards. Radioactive compounds were located by applying cellophane tape to the thin-layer chromatogram and removing 5-mm fractions for liquid scintillation counting. The unlabeled sugar phosphate controls were visualized with a perchloric acid–molybdate spray (9).

RESULTS

Sucrose hydrolysis. It was previously demonstrated that when *S. mutans* 6715-10 is grown in DM only low levels of extracellular glucosyl- and fructosyltransferase activities can be detected (15). Under these growth conditions, sucrose hydrolysis would be expected to proceed primarily by the action of invertases. Cell-free culture fluid, as well as permeabilized cells of *S. mutans* that had been grown on various carbohydrates in DM, were therefore examined for the ability to hydrolyze sucrose at a 1 mM concentration (Table 1). No activity was detected in the cell-free culture fluids regardless of the growth substrate. Sucrose hydrolysis could only be detected when permeabilized cells that had been grown on sucrose were used. The specific activity at 1 mM sucrose was relatively low (5 nmol/min per mg [dry weight] of cells). The apparent K_m for sucrose hydrolysis with permeabilized cells was found to be approximately 40 mM (data not shown). Thus, if splitting of sucrose by an intracellular invertase were the first step in sucrose utilization at low substrate concentrations, only a limited amount of sucrose hydrolysis could occur in the absence of a highly concentrative sucrose permease system.

Identification of a sucrose PTS reaction. Sucrose transport by *S. mutans* has not been described previously. However, a mechanism for sucrose transport has been described for other gram-positive bacteria. *Bacillus subtilis* (10), *Staphylococcus aureus* (5), and *Streptococcus sanguis* (J. A. Mayo, T. W. Feary, and P. L. Doerr, Abstr. Int. Assoc. Dent. Res. 1978, p. 235) were found to possess a PEP-dependent sucrose PTS system. We have detected a similar sucrose PTS activity in *S. mutans* (Table 2). The sucrose PTS activity is present in sucrose-grown cells but not in cells grown on glucose, fructose, or sorbitol. One or more components of the system, therefore, appear to be inducible. Although the exact levels of PTS activity detected in different cell preparations were somewhat variable, uninduced cells consistently had specific activities below 0.005 μmol/min per mg (dry weight), whereas induced levels varied between 0.05 and 0.2 μmol/min per mg (dry weight). The reaction requires PEP, and 2-phosphoglycerate can only replace PEP in the absence of NaF. The apparent K_m for sucrose in this reaction was deter-

TABLE 1. *Total sucrose hydrolysis activity*[a]

Growth substrate	Cell-free culture fluid sp act (μmol/min per ml)	Sp act of permeabilized cells (μmol/min per mg)[b]
Glucose	<0.001	<0.001
Fructose	<0.001	<0.001
Mannitol	<0.001	<0.001
Sucrose	<0.001	0.005

[a] Sucrose hydrolysis was determined at a 1 mM sucrose concentration.

[b] Specific activity is expressed as micromoles of nicotinamide adenine dinucleotide phosphate reduced per minute per milligram (dry weight) of cells.

TABLE 2. *Induction of sucrose PTS activity*

Growth substrate	Sucrose PTS sp act (μmol/min per mg [dry wt] of cells)
Glucose	0.003
Fructose	0.002
Sorbitol	0.001
Sucrose	0.050

mined to be 70 μM. A similar sucrose PTS activity was detected in other strains of *S. mutans* that include representatives of the four major serotypes (Table 3).

Analysis of sucrose-negative mutants. To assess the possible physiological role of the sucrose PTS system, we attempted to isolate mutants of *S. mutans* that could not grow at low sucrose concentrations. Two representative mutants are described in Table 4. The parent strain was capable of inducing both sucrose PTS and PEP-dependent sucrose phosphate-hydrolyzing activities when grown on DM containing 20 mM mannitol plus 5 mM sucrose for induction. The first mutant (DR0001/2) was unable to induce both sucrose PTS and sucrose phosphate-hydrolyzing activities. Addition of sucrose to a culture of this mutant that was actively growing on mannitol did not inhibit growth. The second mutant (DR0001/3) induced sucrose PTS activity but not PEP-dependent sucrose phosphate-hydrolyzing activity. Growth of this mutant on mannitol was inhibited by sucrose. This inhibition is most likely due to the intracellular accumulation of sucrose phosphate (4). The PTS-negative mutant (DR0001/2) was also examined for the ability to induce other PTS activities (Table 5). The mutant retained PTS activity for glucose, fructose, mannose, mannitol, sorbitol, lactose, and maltose. This sucrose-negative mutant is, therefore, not a pleiotrophic PTS-negative mutant. In addition, the sucrose PTS activity that we observed in the parent strain could not have been due to the action of one of these other PTS activities on sucrose.

TABLE 3. *Sucrose PTS activity in S. mutans*

Strain	Serotype	Sucrose PTS sp act (μmol/min per mg [dry wt] of cells)
AHT	a	0.113
BHT	b	0.109
FA-1	b	0.069
10449	c	0.098
JC-2	c	0.053
SL-1	d	0.089
6715-10	d	0.146

TABLE 4. *Characterization of sucrose-negative mutants*

Strain	Genotype	Sucrose PTS sp act (μmol/min per mg [dry wt] of cells)	Sucrose phosphate hydrolysis sp act (μmol/min per mg [dry wt] of cells)	Sucrose inhibition[a]
DR0001/1	*sac*$^+$	0.111	0.055	No
DR0001/2	*sac-2*	<0.004	<0.002	No
DR0001/3	*sac-5*	0.178	<0.002	Yes

[a] Inhibition of growth on mannitol by sucrose.

Identification of the sucrose PTS reaction product. To isolate the product of the sucrose PTS reaction, radioactively labeled sucrose was incubated with PEP and permeabilized cells of strain DR0001/3. This mutant had sucrose PTS activity but lacked sucrose phosphate-hydrolyzing activity (Table 4) and, unlike the parent strain, permitted the accumulation of the suspected intermediate sucrose phosphate. Analysis of the reaction products by thin-layer chromatography revealed that the radioactively labeled sucrose was converted to a compound that migrated more slowly than either the glucose 6-phosphate or fructose 6-phosphate standards, as would be expected if sucrose had been phosphorylated. The unknown compound was eluted from the chromatogram and characterized further (Fig. 1). A sample of the unknown was rechromatographed and detected in fraction 6 of the chromatogram. After treatment of a portion of the unknown sample with alkaline phosphatase (5 U; pH 10 at 37°C for 10 min), the product cochromatographed with the sucrose standard.

TABLE 5. *Other PTS activities present in strain DR0001/2*

Substrate	PTS activity (μmol/min per mg [dry wt] of cells)
Glucose	0.059
Fructose	0.065
Mannose	0.037
Mannitol	0.095
Sorbitol	0.054
Lactose	0.063
Maltose	0.084

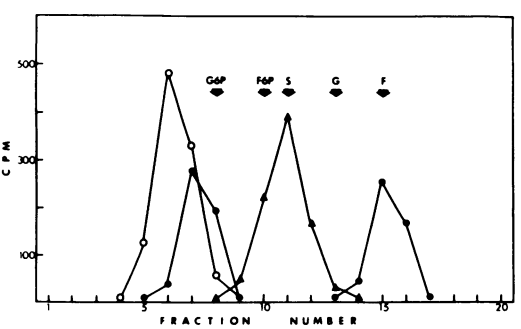

FIG. 1. *Characterization of sucrose phosphate by thin-layer chromatography. Symbols: ○, unknown; ▲, unknown treated with alkaline phosphatase; ●, unknown subjected to mild acid hydrolysis. Standards: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; S, sucrose; G, glucose; F, fructose.*

This result confirmed that the unknown was sucrose phosphate. A sample of the unknown was also subjected to mild acid hydrolysis (0.1 N HCl for 10 min at 100°C) in order to cleave the glycosidic bond. Analysis of the product revealed two equivalent peaks of activity that comigrated with the glucose 6-phosphate and fructose standards. The product of the PTS reaction is therefore sucrose phosphate, and the phosphate group is situated on the glucose moiety.

DISCUSSION

S. mutans has been shown to possess a PEP-dependent sucrose PTS activity that can initiate the catabolism of sucrose. This reaction is specific for sucrose because sucrose PTS activity was only induced in cells that were grown on sucrose. The product of this reaction was isolated and identified as sucrose phosphate, with the phosphate group being situated on the glucose moiety. A sucrose phosphate-hydrolyzing activity was also detected. Hydrolysis of sucrose phosphate would be expected to yield glucose 6-phosphate and fructose. The intracellular fructose that is generated can be metabolized further by a specific fructokinase that has been identified in this strain (E. V. Porter, personal communication). Mutant analysis has confirmed the physiological role of this pathway, because mutants that are unable to metabolize low concentrations of sucrose were found to be missing either sucrose PTS or sucrose phosphate-hydrolyzing activity. The most significant difference between this PTS-initiated pathway of sucrose dissimilation and the other pathways that are present in *S. mutans* lies in its efficiency. The sucrose PTS reaction has an apparent K_m for sucrose of 70 μM, which would permit it to function as an effective scavenger of low levels of sucrose from its environment. Transport of sucrose via a PTS reaction versus a permease is also more economical because the energy of only one high-energy phosphate bond is required to both transport and phosphorylate sucrose (1). This ability would thus be expected to play a major role in the ecology of *S. mutans* by permitting it to compete effectively for the utilization of dietary sucrose within the oral cavity.

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