INFECTION AND IMMUNITY, Nov. 1979, p. 487-491
0019-9567/79/11-0487/05$02.00/0

Vol. 26, No. 2

Regulation and Function of Sucrose 6-Phosphate Hydrolase in *Streptococcus mutans*

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

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

Received for publication 22 June 1979

Sucrose catabolism by *Streptococcus mutans* is initiated by a phosphoenolpyruvate-dependent sucrose phosphotransferase reaction that produces sucrose 6-phosphate; the latter is then cleaved by a sucrose 6-phosphate hydrolase reaction that yields glucose 6-phosphate and fructose. We have examined the regulation of the sucrose 6-phosphate hydrolase and found that it was synthesized constitutively whereas sucrose phosphotransferase activity was inducible. However, the levels of both sucrose phosphotransferase and sucrose 6-phosphate hydrolase were repressed when fructose was used as a growth substrate. The specific activity of sucrose 6-phosphate hydrolase in permeabilized cells was approximately 30 nmol/min per mg (dry weight of cells), and it had an apparent K_m for sucrose 6-phosphate of 0.3 mM. Analysis of a mutant that was missing sucrose 6-phosphate hydrolase activity revealed that its ability to hydrolyze sucrose was reduced.

We have previously demonstrated that sucrose transport by *Streptococcus mutans* is mediated by a sucrose phosphotransferase (PTS) (7) as seen in reaction 1: sucrose + phosphoenolpyruvate $\xrightarrow{\text{PTS}}$ sucrose 6-phosphate + pyruvate. The product of this reaction is sucrose phosphate, with the phosphate group being located on C-6 of the glucose moiety. In addition, we detected a second enzyme activity that was capable of hydrolyzing sucrose 6-phosphate, as shown in reaction 2: sucrose 6-phosphate $\xrightarrow{\text{HYDROLASE}}$ glucose 6-phosphate + fructose. Because substrate amounts of sucrose 6-phosphate were not available, the assay for sucrose 6-phosphate hydrolase was coupled to the endogenous production of sucrose 6-phosphate by the sucrose PTS activity present in permeabilized cells (7). This assay would not permit us to determine the actual concentration of sucrose 6-phosphate in the reaction nor could we measure sucrose 6-phosphate hydrolase in cells that did not have high levels of sucrose PTS activity. In this report, we describe the biological synthesis of substrate amounts of sucrose 6-phosphate, using permeabilized cells of a mutant strain of *S. mutans* that is constitutive for the synthesis of sucrose PTS activity but missing sucrose 6-phosphate hydrolase. The isolated substrate was then used to examine the regulation and function of sucrose 6-phosphate hydrolase in both the parent and mutant strains of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Stock cultures were maintained in fluid thioglycolate medium by storage at −20°C. *S. mutans* strain 6715-10 was designated DR0001 in our culture collection. The isolation and characterization of a sucrose PTS mutant (DR0001/2) and a sucrose 6-phosphate hydrolase mutant (DR0001/3) have been described previously (7). All cultures were transferred twice in defined medium (8) containing 20 mM mannitol before induction experiments were performed. Substrates were provided at the following concentrations: 20 mM fructose, 20 mM mannitol, 20 mM sorbitol, and 10 mM sucrose. Mutant strains were grown on 20 mM mannitol plus 5 mM sucrose for induction studies.

Synthesis of sucrose 6-phosphate. Sucrose 6-phosphate was prepared by incubating a permeabilized cell suspension of *S. mutans* DR0001/3, which had been grown in 100 ml of defined medium plus 20 mM mannitol, with sucrose and phosphoenolypyruvate. The 100-ml reaction mixture contained: 50 mM sodium-potassium phosphate buffer at pH 7.2, 2.5 mM $MgCl_2$, 10 mM NaF, 10 mM sucrose, 10 mM phosphoenolpyruvate, and 10 ml of concentrated and permeabilized cell suspension (7). The reaction was terminated by removing the cells by centrifugation followed by filtration of the supernatant fluid through a 0.45-μm membrane filter. Synthesis of sucrose 6-phosphate was monitored by assaying for glucose 6-phosphate before and after acid hydrolysis (0.2 N HCl and 100°C for 10 min), using a glucose 6-phosphate dehydrogenase- and nicotinamide adenine dinucleotide phosphate ($NADP^+$)-coupled spectrophotometric assay. Each 1-ml reaction mixture contained: 100 mM sodium-potassium phosphate buffer at pH 7.2, 5 mM $MgCl_2$, 0.5

mM $NADP^+$, 2 U of glucose 6-phosphate dehydrogenase, and 10 to 50 μl of sample.

Isolation of sucrose 6-phosphate. Sucrose 6-phosphate was purified by ion-exchange chromatography on a Dowex 2-X8 (50 to 150 mesh) formate column (1 by 30 cm), using a linear (0 to 4 M) formic acid gradient (4, 6). The column was eluted at a flow rate of 1 ml/min, and 5-ml fractions were collected. Column fractions were monitored for sucrose 6-phosphate using a rapid color assay that detected the fructose released from sucrose 6-phosphate by acid hydrolysis. Samples (0.1 ml) were mixed with 10 μl of 2 N HCl and incubated for 10 min in a boiling-water bath. After the samples were cooled to 37°C, 0.9 ml of reagent (0.1% 2,3,5-triphenyl tetrazolium chloride plus 1% Triton X-100 in 1 N NaOH) was added to each sample (1). The mixture was incubated at 37°C for 20 min, and the amount of fructose in each sample was determined by reading the optical density at a wavelength of 490 nm as compared with hydrolyzed sucrose standards. Column fractions containing sucrose 6-phosphate were pooled (200 ml) and adjusted to pH 6.5 with NH_4OH. Sucrose 6-phosphate was isolated as the barium salt by adding 2 ml of 1 M $BaCl_2$ to the sample followed by 4 volumes of ethanol and storage at 0°C overnight (2).

The resulting precipitate was collected by centrifugation, washed twice with 80% ethanol at 0°C, and dried under vacuum. The barium salt of sucrose 6-phosphate was exchanged with Dowex 50 W hydrogen form and neutralized with NaOH before use in assays. The amount of glucose 6-phosphate and sucrose 6-phosphate in the purified substrate was determined by measuring the amount of glucose 6-phosphate before and after acid hydrolysis, using the glucose 6-phosphate dehydrogenase-coupled assay described above. The amount of fructose 6-phosphate was determined by adding 2 U of phosphoglucose isomerase to the assay. Glucose and fructose were determined by using the same glucose 6-phosphate dehydrogenase assay plus 2 U of hexokinase and 10 mM adenosine 5′-triphosphate with and without 2 U of phosphoglucose isomerase.

Enzyme assays. The preparation of permeabilized cells and the sucrose PTS assay have been described previously (7). Sucrose hydrolysis was determined as the release of both glucose and fructose from sucrose, using a previously described spectrophotometric assay except that 100 mM sucrose was used as substrate (7). Sucrose 6-phosphate hydrolase activity was assayed as the permeabilized cell-dependent release of glucose 6-phosphate from sucrose 6-phosphate, using a glucose 6-phosphate dehydrogenase- and $NADP^+$-coupled spectrophotometric assay. Each 1-ml reaction mixture contained: 100 mM sodium-potassium phosphate buffer at pH 7.2, 5 mM $MgCl_2$, 10 mM NaF, 0.5 mM $NADP^+$, 2 U of glucose 6-phosphate dehydrogenase, 1 mM sucrose 6-phosphate, and 10 to 100 μl of permeabilized cell suspension. Because our sucrose 6-phosphate preparations contained approximately 5% glucose 6-phosphate, the assay reagents were preincubated and monitored to ensure that all the glucose 6-phosphate was consumed by the enzyme couple before initiating the reaction by adding permeabilized cells.

RESULTS

Synthesis of sucrose 6-phosphate. Sucrose 6-phosphate is not commercially available; however, its enzymatic synthesis from glucose 6-phosphate and fructose, using a purified preparation of levan sucrase, has been described previously (3). Because our permeabilized cell preparations had relatively high levels of sucrose PTS activity, 0.1 μmol/min per mg (dry weight of cells), and we had isolated a mutant that was missing sucrose 6-phosphate hydrolase activity, we synthesized sucrose 6-phosphate by using permeabilized cells of our mutant strain. This procedure did not require the purification of an enzyme. The production of sucrose 6-phosphate was monitored enzymatically (Fig. 1). The synthesis of sucrose 6-phosphate was linear for approximately 1 h. We also detected the synthesis of small amounts of glucose 6-phosphate. After removal of the cells, the reaction mixture was subjected to ion-exchange chromatography (Fig. 2). Sucrose 6-phosphate was eluted as a single peak, using a linear (0 to 4 M) formic acid

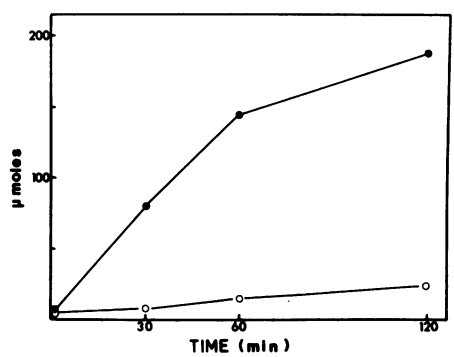

FIG. 1. *Synthesis of sucrose 6-phosphate by permeabilized cells of S. mutans strain DR0001/3. Symbols: sucrose 6-phosphate (●); glucose 6-phosphate (○).*

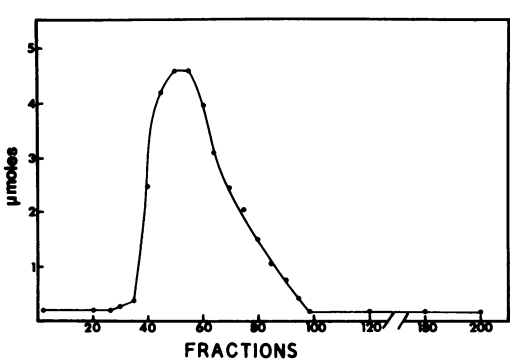

FIG. 2. *Dowex-2 (formate) chromatography of sucrose 6-phosphate, using a linear (0 to 4 M) formic acid gradient and 5-ml fractions.*

gradient. Sucrose 6-phosphate was isolated as the barium salt and characterized enzymatically (Table 1). Before acid hydrolysis, the preparation contained 1.3 μmol of glucose 6-phosphate per ml but no detectable glucose, fructose, or fructose 6-phosphate. After acid hydrolysis, to cleave the glycosidic bond of sucrose 6-phosphate, the preparation yielded 22 μmol of fructose and 25.5 μmol of glucose 6-phosphate per ml. The product was therefore sucrose 6-phosphate with approximately 5% contaminating glucose 6-phosphate.

Regulation of sucrose 6-phosphate hydrolase in the parent strain. *S. mutans* strain DR0001 was grown in defined medium on five different substrates, and permeabilized cell preparations were assayed for three sucrose-metabolizing enzyme activities (Table 2). Growth on sucrose increased sucrose PTS activity approximately 6- to 66-fold. Variations in the increase were due to differences in the uninduced levels found in cells grown on sugars other than sucrose and variations between experiments. However, the level of sucrose 6-phosphate hydrolase activity did not increase significantly in these same sucrose-grown cells. Therefore, the synthesis of sucrose 6-phosphate hydrolase appears to be constitutive and not coordinately regulated with the sucrose PTS. Sucrose hydrolase was also synthesized constitutively, and its levels paralleled the levels of sucrose 6-phosphate hydrolase. All three enzyme activities were repressed when fructose was used as the growth substrate.

TABLE 1. *Enzymatic characterization of the sucrose 6-phosphate preparation*

Compound assayed for:	μmol/ml	
	Before acid hydrolysis	After acid hydrolysis
Glucose	<0.5	<0.5
Fructose	<0.5	22.0
Glucose 6-phosphate	1.3	25.5
Fructose 6-phosphate	<0.5	<0.5

TABLE 2. *Effect of growth substrate on the enzymes of sucrose metabolism in S. mutans DR0001*

Growth substrate	Sp act (nmol/min per mg [dry wt of cells])		
	Sucrose PTS	Sucrose 6-phosphate hydrolysis	Sucrose hydrolysis
Glucose	14 (12)[a]	15[b] (43)	69 (103)
Fructose	2 (2)	4 (3)	20 (23)
Mannitol	5 (18)	12 (29)	69 (95)
Sorbitol	18 (7)	12 (16)	78 (89)
Sucrose	111 (131)	24 (34)	139 (167)

[a] All values in parentheses are the results of a separate experiment.

[b] The values in this column were obtained at a sucrose 6-phosphate concentration of 0.5 mM.

Regulation of sucrose 6-phosphate hydrolase in the mutant strains. Permeabilized cell preparations of the parent and two mutant strains of *S. mutans*, which had been grown on mannitol or mannitol plus sucrose, were assayed for sucrose-metabolizing enzyme activities (Table 3). Growth of the parent strain DR0001 on mannitol plus sucrose led to a fourfold increase in sucrose PTS activity, but no significant increase in sucrose 6-phosphate hydrolase or sucrose hydrolase activity occurred. The first mutant, DR0001/2, had no detectable sucrose PTS activity but constitutive levels of both sucrose 6-phosphate hydrolase and sucrose hydrolase activity. A functional sucrose PTS is, therefore, not required for the synthesis of either hydrolase. The second mutant, DR0001/3, had constitutive levels of sucrose PTS activity but no detectable sucrose 6-phosphate hydrolase activity. Sucrose hydrolase activity was reduced by approximately 80% in this strain. Sucrose 6-phosphate hydrolase may, therefore, also have the ability to hydrolyze sucrose.

Kinetics of sucrose 6-phosphate hydrolysis. We have been unable to detect the accumulation of sucrose 6-phosphate by using radioactively labeled sucrose and permeabilized cell suspensions of the parent strain of *S. mutans* (unpublished data). These results suggested that sucrose 6-phosphate is rapidly hydrolyzed and that sucrose 6-phosphate hydrolase must have a high affinity for sucrose 6-phosphate in order to function effectively even at low concentrations of substrate. Therefore, we measured the sucrose 6-phosphate hydrolase activity in permeabilized cells of strain DR0001/2 at varying concentrations of sucrose 6-phosphate. This strain has no detectable sucrose PTS activity but constitutive levels of sucrose 6-phosphate hydrolase. A velocity-versus-substrate concentration plot of the results produced a sigmoidal curve (Fig. 3). Each point was the average of three determinations. When the data were replotted as $1/v$ versus $1/[s]^2$, a straight line was generated, and the apparent K_m was calculated to be 0.3 mM (data not shown). Sucrose 6-phosphate hydrolase can, therefore, function effectively at low substrate concentrations. Because these assays were performed with permeabilized cells, we are not certain whether the sigmoidal kinetics observed are truly a property of the sucrose 6-phosphate hydrolase or are an artifact of this complex assay system.

DISCUSSION

We have demonstrated that *S. mutans* strain DR0001/3 can be used for the biological synthe-

TABLE 3. *Regulation of sucrose-metabolizing enzymes in mutants of S. mutans*

Strain	Growth substrate	Sp act (nmol/min per mg [dry wt of cells])		
		Sucrose PTS	Sucrose 6-phosphate hydrolysis	Sucrose hydrolysis
DR0001	Mannitol	12 (10)[a]	29[b] (59)	124 (89)
	Mannitol + sucrose	33 (56)	13 (32)	126 (138)
DR0001/2	Mannitol	<1 (<2)	15 (32)	64 (66)
	Mannitol + sucrose	<1 (<2)	20 (37)	88 (76)
DR0001/3	Mannitol	83 (135)	<1 (<1)	16 (21)
	Mannitol + sucrose	45 (31)	<1 (<1)	23 (22)

[a] All values in parentheses are the results of a separate experiment.

[b] The values in this column were obtained at a sucrose 6-phosphate concentration of 0.5 mM.

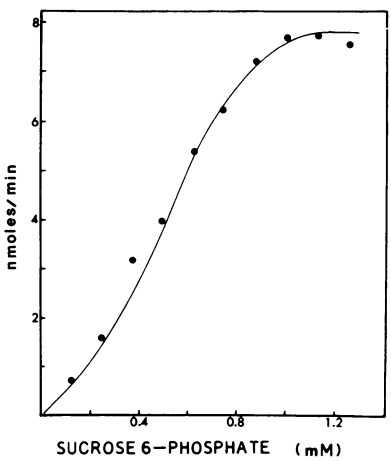

FIG. 3. *Velocity-versus-substrate concentration plot of sucrose 6-phosphate hydrolase activity, using permeabilized cell suspensions of S. mutans strain DR0001/2.*

sis of sucrose 6-phosphate; however, small amounts of glucose 6-phosphate were also produced. The synthesis of substrate amounts of sucrose 6-phosphate has permitted us to examine the regulation and function of sucrose 6-phosphate hydrolase in *S. mutans*. We have found that sucrose 6-phosphate hydrolase was synthesized constitutively, unlike the sucrose PTS, which was only induced during growth on sucrose. A sucrose 6-phosphate hydrolase has also been described in *Bacillus subtilis*; however, the synthesis of this enzyme was inducible (3). When *S. mutans* was grown on fructose, sucrose 6-phosphate hydrolase was repressed. Why fructose but not glucose should cause this repression is not known.

Analysis of sucrose-defective mutants revealed that one mutant had lost sucrose PTS activity while remaining constitutive for sucrose 6-phosphate hydrolase activity. A second mutant was constitutive for sucrose PTS activity but missing sucrose 6-phosphate hydrolase activity. More sucrose 6-phosphate hydrolase-negative mutants will have to be isolated to determine if there is a direct correlation between the loss of sucrose 6-phosphate hydrolase and the constitutive synthesis of sucrose PTS. The loss of sucrose 6-phosphate hydrolase activity was also accompanied by an 80% decrease in sucrose hydrolysis. Sucrose 6-phosphate hydrolase may, therefore, also play a role in sucrose hydrolysis. Sucrose 6-phosphate hydrolase has recently been found to be identical to the previously described intracellular invertase of *S. mutans* (B. M. Chassy and E. V. Porter, Biochem. Biophys. Res. Commun., in press). The identity of the enzyme, or enzymes, responsible for the remaining sucrose hydrolase activity in this strain is not yet known.

The sucrose 6-phosphate hydrolase activity of permeabilized cells was shown to have a high affinity for sucrose 6-phosphate (K_m, 0.3 mM). Low levels of sucrose 6-phosphate can, therefore, be rapidly hydrolyzed. This result supports our earlier observation that permeabilized cells of the wild-type strain are unable to accumulate significant pool levels of sucrose 6-phosphate when incubated with radioactively labeled sucrose and phosphoenolpyruvate (7). The apparent sigmoidal kinetics also suggests that the steady-state pool of sucrose 6-phosphate is not only very small but also tightly regulated. This regulation may be necessary to prevent the intracellular accumulation of inhibitory levels of sucrose 6-phosphate. Growth of the sucrose 6-phosphate hydrolase-negative mutant on mannitol has previously been shown to be rapidly inhibited by the addition of 1 mM sucrose (7). Analogs of sucrose that are substrates for the sucrose PTS but not hydrolyzed by sucrose 6-phosphate hydrolase (5) might also be expected to be very effective growth inhibitors of *S. mutans*.

ACKNOWLEDGMENTS

We thank Jack London and Donald LeBlanc for their comments and criticisms of this manuscript.

LITERATURE CITED

1. **Gabriel, O., and S. F. Wang.** 1969. Determination of enzymatic activities in polyacrylamide gels. I. Enzymes catalyzing the conversion of nonreducing substrates to reducing products. J. Anal. Biochem. **27**:545–554.
2. **Horecker, B. L.** 1962. *L*-Ribulose 5-phosphate-4-epimerase. Methods Enzymol. **5**:253–275.
3. **Kunst, F., M. Pascal, J. A. Lepesant, J. Walle, and R. Dedonder.** 1974. Purification and some properties of an endocellular sucrase from a constitutive mutant of

Bacillus subtilis, Marburg 168. Eur. J. Biochem. **42**: 611–620.

4. **Leloir, L. F., and C. E. Cardini.** 1955. The biosynthesis of sucrose phosphate. J. Biol. Chem. **214**:157–165.
5. **Lepesant, J.-A., F. Kunst, M. Pascal, J. Kejzlarova-Lepesant, M. Steinmetz, and R. Dedonder.** 1976. Specific and pleiotropic regulatory mechanisms in the sucrose system of *Bacillus subtilis* 168, p. 58–69. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
6. **Schmitz, H., and G. Walpurger.** 1959. Trennung von Phosphorsäureestern durch Ionenaustausch-Chromatographie. Angew. Chem. **17**:549–552.
7. **St. Martin, E. J., and C. L. Wittenberger.** 1979. Characterization of a phosphoenolpyruvate-dependent sucrose phosphotransferase system in *Streptococcus mutans*. Infect. Immun. **24**:865–868.
8. **Wittenberger, C. L., A. J. Beaman, and L. N. Lee.** 1978. Tween 80 effect on glucosyltransferase synthesis by *Streptococcus salivarius*. J. Bacteriol. **133**:231–239.