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Regulation of ATP-Dependent P-(Ser)-HPr Formation in *Streptococcus mutans* and *Streptococcus salivarius*

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Sugar transport via the phosphoenolpyruvate (PEP) phosphotransferase system involves PEP-dependent phosphorylation of the general phosphotransferase system protein, HPr, at histidine 15. However, gram-positive bacteria can also carry out ATP-dependent phosphorylation of HPr at serine 46 by means of (Ser)HPr kinase. In this study, we demonstrate that (Ser)HPr kinase in crude preparations of *Streptococcus mutans* Ingbritt and *Streptococcus salivarius* ATCC 25975 is membrane associated, with pH optima of 7.0 and 7.5, respectively. The latter organism possessed 7- to 27-fold-higher activity than *S. mutans* NCTC 10449, GS-5, and Ingbritt strains. The enzyme in *S. salivarius* was activated by fructose-1,6-bisphosphate (FBP) twofold with 0.05 mM ATP, but this intermediate was slightly inhibitory with 1.0 mM ATP at FBP concentrations up to 10 mM. Similar inhibition was observed with the enzyme from *S. mutans* Ingbritt. A variety of other glycolytic intermediates had no effect on kinase activity under these conditions. The activity and regulation of (Ser)HPr kinase were assessed in vivo by monitoring P-(Ser)-HPr formation in steady-state cells of *S. mutans* Ingbritt grown in continuous culture with limiting glucose (10 and 50 mM) and with excess glucose (100 and 200 mM). All four forms of HPr [free HPr, P~(His)-HPr, P-(Ser)-HPr, and P~(His)-P-(Ser)-HPr] could be detected in the cells; however, significant differences in the intracellular levels of the forms were apparent during growth at different glucose concentrations. The total HPr pool increased with increasing concentrations of glucose in the medium, with significant increases in the P-(Ser)-HPr and P~(His)-P-(Ser)-HPr concentrations. For example, while total PEP-dependent phosphorylation [P~(His)-HPr plus P~(His)-P-(Ser)-HPr] varied only from 21.5 to 52.5 μg mg of cell protein^{-1} in cells grown at the four glucose concentrations, the total ATP-dependent phosphorylation [P-(Ser)-HPr plus P~(His)-P-(Ser)-HPr] increased 12-fold from the 10 mM glucose-grown cells (9.1 μg mg of cell protein^{-1}) to 106 and 105 μg mg^{-1} in the 100 and 200 mM glucose-grown cultures, respectively. (Ser)HPr kinase activity in membrane preparations of the cells varied little between the 10, 50, and 100 mM glucose-grown cells but increased threefold in the 200 mM glucose-grown cells. The intracellular levels of ATP, glucose-6-phosphate, and FBP increased with the external glucose concentration, with the level of FBP being 3.8-fold higher for cells grown with 200 mM glucose than for those grown with 10 mM glucose. However, the variation in the intracellular levels of FBP, particularly between cells grown with 100 and 200 mM glucose, did not correlate with the extent of P-(Ser)-HPr formation, suggesting that the activity of (Ser)HPr kinase is not critically dependent on the availability of intracellular FBP.

The principal sugar transport system in oral streptococci is the phosphoenolpyruvate (PEP):sugar phosphotransferase transport system (PTS) (33). The PTS is a group translocation process which utilizes PEP for the phosphorylation of incoming sugars via a phosphoryl transfer process involving the general, non-sugar-specific proteins enzyme I (EI) and HPr and subsequently a sugar-specific, membrane-bound enzyme II (EII) complex, which catalyzes the transport and phosphorylation of the specific carbohydrate (22). During this process, HPr is transiently phosphorylated by P-EI on histidyl residue 15 (His-15) and the phosphate group from phospho-HPr [P~(His)-HPr] is then transferred to the membrane-bound EII complex. The EII complex consists of three functional domains: (i) the IIA domain (also referred to as enzyme III) possessing the first phosphorylation site, (ii) the IIB domain bearing the second phosphorylation site, and (iii) the IIC domain that forms the transmembrane channel and provides the sugar-binding site (30).

In 1983, Deutscher and Saier (5) showed that the HPr of *Streptococcus pyogenes* could also be phosphorylated on a serine residue (Ser-46) at the expense of ATP by a specific HPr kinase. This phosphorylation reaction was subsequently found to be widespread among gram-positive but not gram-negative bacteria (22, 27) and has even been shown to occur in some species lacking a functional PTS (27). Whereas several observations over the past 10 years have suggested that P-(Ser)-HPr possesses regulatory roles, only recently have some of the physiological functions of this phosphoprotein been determined. These functions include the regulation of glucose and lactose permease activity in *Lactobacillus brevis* (38, 39), regulation of inducer expulsion in *Lactococcus lactis* (40), and involvement in catabolite repression in *Bacillus subtilis* (4).

In vitro studies have suggested that the intracellular concentration of P-(Ser)-HPr is controlled by the conjugated action of a metabolite-activated, ATP-dependent protein kinase [(Ser) HPr kinase] and a P_i-dependent P-(Ser)-HPr phosphatase (22–24, 26). Phosphorylation by purified (Ser)HPr kinases from *S. pyogenes*, *Enterococcus faecalis*, *B. subtilis*, and *L. brevis* (23, 26) is stimulated by fructose-1,6-bisphosphate (FBP) and inhibited by P_i. (Ser)HPr kinase activity has been observed in the oral species, *Streptococcus salivarius* (34, 37) and *Streptococcus mu-*

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tans (21, 34), and activity of the enzyme in the latter organism has also been shown to be activated by FBP and inhibited by P_i and some glycolytic intermediates (21).

In vivo studies have confirmed the phosphorylation of HPr on a serine residue and shown that the activity of both the ATP-dependent (Ser)HPr kinase and the PEP-dependent phosphoryl cascade in cells results in the formation of four different forms of HPr in gram-positive bacteria: free HPr, P~(His)-HPr, P-(Ser)-HPr, and a doubly phosphorylated form, P~(His)-P-(Ser)-HPr. All four forms have been detected in *S. salivarius* and *S. mutans* by crossed immunoelectrophoresis following rapid acidification of the cytoplasm followed by grinding of a frozen pellet of the cells to extract cytoplasmic proteins (34). Batch-grown, exponential-phase cells have been shown to contain only P-(Ser)-HPr and P~(His)-P-(Ser)-HPr, whereas stationary-phase cells contained predominately HPr and P~(His)-HPr. These results imply a change in the levels of the HPr forms in response to the availability of the carbon source which was the limiting nutrient, supporting earlier observations describing the dephosphorylation of P-(Ser)-HPr during starvation (17).

The in vitro and in vivo studies have clearly established the phosphorylation of HPr on Ser-46 by a regulated ATP-dependent protein kinase in gram-positive bacteria. Nevertheless, none of these studies have demonstrated a correlation between the cellular levels of the putative effectors of (Ser)HPr kinase and the extent of P-(Ser)-HPr formation. With this observation in mind, we have examined the activity and regulation of (Ser) HPr kinase in *S. salivarius* and various strains of *S. mutans.* Early results with crude membrane preparations indicated that, unlike in other studies with *S. mutans* (21), (Ser)HPr kinase was not activated by FBP. Consequently, we monitored the formation of P-(Ser)-HPr in vivo by assaying the four forms of HPr in steady-state cells of *S. mutans* Ingbritt growing in continuous culture with increasing concentrations of glucose in order to generate increased levels of the key glycolytic intermediates.

MATERIALS AND METHODS

Materials. Pepstatin A, phenylmethylsulfonyl fluoride, polyethylene glycol 3350, gramicidin D, and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Agarose was obtained from Bio-Rad and sodium barbitone was obtained from BDH (Toronto, Canada).

Bacterial strains and growth conditions. The test organisms used were *S. salivarius* ATCC 25975 and *S. mutans* Ingbritt, kindly supplied by J. Sandham, University of Toronto, Toronto, Canada, and *S. mutans* GS-5, kindly supplied by A. S. Bleiweis, University of Florida, Gainesville, Fla., while *S. mutans* NCTC 10449 was obtained from R. Linzer, State University of New York, Buffalo, N.Y. The methods of culture maintenance and purity control for the test organisms have been described previously (6). Batch-grown cells were cultivated in a medium containing (per liter) 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 2.5 g of NaCl, 2.5 g of Na_2HPO_4, and 50 g of glucose. Growth was stopped during the mid-exponential phase (optical density at 660 nm, 0.4) by the addition of chloramphenicol (50 μg/ml). The cells were harvested by centrifugation at 4°C and were kept frozen at −40°C for not more than 3 days.

Anaerobic growth of *S. mutans* Ingbritt in continuous culture was carried out in LH 500 Series III chemostats (L.H. Engineering, Stoke Poges, Buckinghamshire, United Kingdom) with a working volume of 700 ml. Glucose and phosphate salts were autoclaved separately and added to a semidefined medium (1). The glucose concentration in the medium was 10, 50, 100, or 200 mM, with the concentrations at 10 and 50 mM being limiting for glucose, while the cultures at 100 and 200 mM were nitrogen limited or contained an excess of glucose. In all experiments, the pH of the culture was maintained automatically at 7.0 by the addition of 2 M KOH, the dilution rate (D) was held at 0.1 h^{-1} (doubling time of 7 h), and the gas phase was 5% CO_2 in nitrogen. Daily routine maintenance of the chemostats was performed as described previously (8). Cell dry weight determinations were carried out by filtering 5 ml of culture through four preweighed 0.45-μm-pore-size filters. The cultures were grown at each glucose concentration for at least 10 mean generations before a steady state was considered to have been established and the cells were removed from the growth chamber for the appropriate assay.

(Ser)HPr kinase assay. Cells were washed twice with 50 mM Tris-HCl (pH 7.5) buffer containing 50 μg of chloramphenicol per ml to eliminate phosphate, since phosphate interferes with HPr kinase activity (34). The pellet was suspended (20% [wet weight], wt/vol) in 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 M phenylmethylsulfonyl fluoride, 0.1 μM pepstatin A, 0.1 μM leupeptin, and 0.5 g of alumina per ml of the cell suspension. The cells were disrupted in a sonicator (Heat Systems-Ultrasonic, Inc.; model W350) in the pulse mode for three periods of 4 min during which the suspension was maintained in a dry ice-ethanol-water mixture (chunks of dry ice are placed in a mixture of ethanol and water [80:20, vol/vol]). Alumina was removed by centrifugation (2,500 × g for 5 min), and the cells and cell debris were sedimented at 20,000 × g for 20 min at 4°C. Membrane fragments in the supernatant containing the (Ser)HPr kinase activity were collected following centrifugation at 140,000 × g for 24 h at 4°C, resuspended in 50 mM Tris-HCl (pH 7.5) buffer, and stored in 200-μl aliquots at −40°C.

(Ser)HPr kinase activity was assayed in a mixture (50 μl) containing 100 mM Tris-acetate (pH 7.0 for *S. mutans* and pH 7.5 for *S. salivarius*); 2 mM $MgCl_2$; 12.5 mM NaF; 10 μM HPr; 0.05, 0.1, or 1.0 mM [γ-^{32}P]ATP (0.1 μCi/nmol); and 3 to 30 μg of membrane protein, depending on the strain tested. Although the reaction was linear over a 5-min period with up to 40 μg of protein, incubation was carried out at 37°C for 4 min. The reaction was stopped by the addition of 25 μl of denaturing buffer (187.5 mM Tris-HCl [pH 6.8], 6% sodium dodecyl sulfate [SDS], 15% 2-mercaptoethanol, 30% glycerol, 0.003% bromophenol blue) followed by heating at 100°C for 5 min. The product, ^{32}P-HPr, was isolated by SDS-polyacrylamide gel electrophoresis (15) by loading 20-μl samples onto a polyacrylamide gel (1 mm thick; 5 by 8 cm) with a 15% resolving gel and running the gel at 200 V at room temperature until the bromophenol blue reached the bottom of the gel. The ^{32}P-HPr band was located by autoradiography following incubation of the dried gel with Kodak X-ray film (X-Omat AR) for 16 h at room temperature. The labelled gel band was excised and counted in 5 ml of Cytoscint ES (ICN) in a liquid scintillation counter. Preliminary experiments had indicated that no other phosphoprotein migrated in the same position as P-HPr under identical conditions and the level of 3-P~(His)-HPr formation was less than 5% of the total HPr present (37). Assays testing the effects of glycolytic intermediates on kinase activity were carried out with 1.0 mM ATP and included the following intermediates: glucose-6-phosphate (0.2, 0.5, and 2.0 mM), fructose-6-phosphate (0.01, 0.05, and 2.0 mM), dihydroxyacetone phosphate (0.5, 1.0, and 2.0 mM), glyceraldehyde-3-phosphate (0.05, 0.2, and 0.5 mM), 2-phosphoglycerate (0.1, 0.25, and 0.5 mM), 3-phosphoglycerate (0.5, 2.0, and 5.0 mM), 2,3-diphosphoglycerate (0.2, 2.0, and 10.0 mM), and pyruvate (0.5, 1.0, and 2.0 mM). The effects of FBP were assayed with the *S. salivarius* enzyme at various concentrations of up to 10 mM with 0.05, 0.1, and 1.0 mM ATP, while the *S. mutans* enzyme was assayed with 1.0 mM ATP at FBP concentrations of up to 15 mM. Units of (Ser)HPr kinase activity are expressed as picomoles of P-(Ser)-HPr formed per microgram of membrane protein per minute.

HPr determinations. Cells for the quantitative analysis of the various forms of HPr were rapidly collected directly from the chemostat under a vacuum into a stirred solution containing 100 mM Tris-citrate buffer (pH 4.0) with chloramphenicol (50 μg/ml) and gramicidin D (1 μM), and the pH of the sample was immediately lowered to 4.5 with 5 N HCl. The cell suspension was centrifuged at 16,000 × g for 10 min, resuspended in a minimal amount of the supernatant to consolidate the cells, recentrifuged at 27,000 × g for 20 min, and frozen at −70°C. The frozen cell suspension was used within 2 to 3 h to prepare a membrane-free cellular extract by a slight modification of the method of Vadeboncoeur and coworkers (34). Our method involved a reduction in the volume of the buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer [pH 7.0] containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10^{-7} M pepstatin A) to 1.5 ml from 3.0 ml. In addition, the amount of cells ground with alumina was held relatively constant, at approximately 0.60 g per sample. The HPr determinations were repeated at least four times and as many as nine times for each condition.

The various forms of HPr were separated by crossed immunoelectrophoresis as described previously (34), with minor alterations to the protocol. Agarose (1%) was dissolved in a Tris-barbitone buffer containing (in grams per liter) sodium barbitone (5.01), Tris base (8.86), calcium lactate (0.11), and sodium azide (0.13). The samples were diluted to 2 mg of protein ml^{-1} with 10 mM HEPES buffer (pH 7.0), and 5 μl of the solution was deposited at the cathodic end of the gel. Electrophoresis in the first dimension was conducted for 75 min at 10 V/cm at 10°C on an LKB Bromma 2117 Multiphor (Pharmacia, Baie d' Urfe, Quebec, Canada). The second dimension, conducted for 19 h at 2 V/cm at 4°C, involved electrophoresis against a polyclonal rabbit antibody (8 mg per plate) directed against HPr of *S. mutans* DR0001. Following electrophoresis, the gels were dried at room temperature overnight before being stained with Coomassie blue.

Quantitation of HPr in the various peaks was obtained by scanning of each gel in a 300 DPI Apple Scanner (Apple Computers Inc., Cupertino, Calif.) followed by transfer of the image to the Image 1.37 program (National Institute of Mental Health, Bethesda, Md.) and determination of the number of pixels under each peak. These values were then compared with a standard curve (100 to 2,000 ng of protein) to determine the number of nanograms of each HPr form present in each cell sample. The final values were expressed as micrograms of HPr per milligram of cellular protein.

Intermediate assays. The intracellular concentrations of glucose-6-phosphate and FBP in steady-state cells were determined by a modification of the sampling method originally described by Iwami et al. (12). A total of approximately 40 to 60 mg of chemostat-grown cells was collected directly from the chemostat, with the amount never exceeding 10% of the chamber volume to ensure maintenance of steady-state conditions. To obtain sufficient cells, six sequential samples (6 to 8 ml) were rapidly collected directly from the growth chamber and were filtered through 0.4-μm-pore-size Acropor filters. The filters were rapidly washed with 5 ml of cold 10 mM Tris-HCl buffer (pH 7.0) before immediately being placed into ice-cold 0.5 M perchloric acid. The cellular ATP concentration, on the other hand, was determined with an equivalent amount of cells removed directly from the chemostat into perchloric acid (1 M). This method was faster (~6 s) than the modified Iwami method and was made possible since the extracellular level of ATP was less than 1.3 pmol ml^{-1} and the intra- to extracellular ATP ratios were always greater than 1,000-fold. In both assays, cell extraction proceeded for 30 min at room temperature before the samples were neutralized to pH 7.6 with 10 mM K_2CO_3 and cooled in ice. The potassium perchlorate was removed by centrifugation, and any further precipitate formed during the incubation on ice (30 min) was removed by filtration through a 0.2-μm filter. On the day of sampling, the cell biomass in the chemostat, as milligrams (dry weight) per milliliter, was determined and cell-free medium samples were collected by filtration.

All intermediate assays were repeated a minimum of 6 times and at most 15 times. Glucose-6-phosphate was assayed with glucose-6-phosphate dehydrogenase by the method of Lang and Michal (16), while FBP was assayed by the method of Michal and Beutler (20). ATP was measured by a luciferin-luciferase assay (31) with a model 2000 Integrating Photometer (Science Application Inc., La Jolla, Calif.). Prior to the ATP determinations, all glassware was acid washed (1 N HCl) and autoclaved. The total volume was 1.0 ml and contained the following components (final concentrations): 8 mM Tris-acetate buffer, 5 mM $MgCl_2$, 0.06 mM dithiothreitol, 0.1 mM EDTA, 0.28 mM luciferin, and 0.14 μg of luciferase per ml. All unknowns were assayed in triplicate with and without an internal standard (2.5 pmol) to correct for sample quenching. In addition, to correct for the ATP concentration in the extracellular medium, ATP was assayed in the cell-free medium filtrate obtained on the day of sampling by filtering (0.2-μm filter) an aliquot of the culture. The micromolar concentrations of all three intermediates are reported on the basis of the intracellular water volumes, with the cell dry weight and actual internal water volume determined for each condition as described below.

Estimation of internal water volumes. The internal water volume of the cells was estimated by subtracting the extracellular water volume determined with ^3H-polyethylene glycol from the total aqueous space measured by using ^3H_2O (13). Four milliliters of culture suspension received either 40 μl of ^3H_2O (20 μCi/ml) or ^3H-polyethylene glycol (15 μCi/ml), and following a 5-min incubation period, 10 samples (0.4 ml) were removed and centrifuged through silicone oil as previously described (7). The internal water volume values were determined for each growth condition along with cell dry weight determinations and represented the averages (± standard errors) of six determinations. The values for the chemostat cultures were 2.01 ± 0.10 and 1.98 ± 0.05 μl/mg (dry weight) of cells for the 10 and 50 mM glucose-limited cells and 2.00 ± 0.03 and 1.99 ± 0.09 μl/mg of cells for the 100 and 200 mM glucose-excess cells, respectively.

Protein and glucose determinations. The initial (medium) and residual (culture filtrate) glucose concentrations were determined as described by Kingsley and Getchell (14), while protein concentrations were estimated by the method of Lowry et al. (18).

RESULTS

In vitro (Ser)HPr kinase activity. In order to compare the intracellular levels of (Ser)HPr kinase activity in oral streptococci, we assayed the enzyme in membrane and cytoplasmic fractions of glucose-grown, exponential-phase cells of *S. salivarius* ATCC 25975 and *S. mutans* Ingbritt, GS-5, and 10449 strains. In all strains, (Ser)HPr kinase was associated with the cell membrane, with less than 1% of the activity appearing in the cytoplasmic fraction. The pH optimum for the enzyme in *S. salivarius* was 7.5, while that for *S. mutans* Ingbritt was 7.0 (Fig. 1) and the latter enzyme was more acid tolerant, with half-maximum activities at pH 5.5 and 8.0 compared with those at pH 6.3 and 8.8 for the *S. salivarius* enzyme. Comparative (Ser) HPr kinase assays with the four strains indicated that *S. salivarius* possessed 7- to 27-fold-higher activity than the three *S. mutans* strains. The (Ser)HPr kinase activities determined for *S. salivarius* ATCC 25975 and *S. mutans* NCTC 10449, GS-5, and Ingbritt were 32.0, 4.8, 1.2, and 3.9 pmol/μg of membrane protein min^{-1}, respectively. This result could simply be due to inherent differences in the specific activities of the enzymes from these different bacteria, or it may indicate that the enzyme in the various strains was dependent on different effectors.

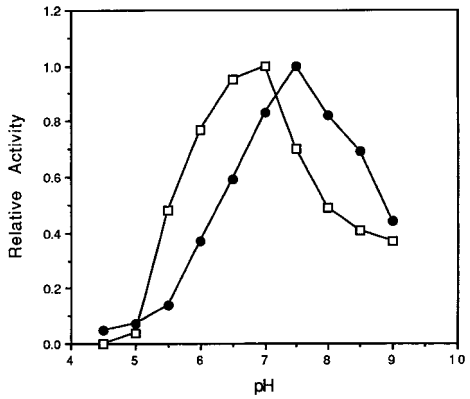

FIG. 1. Effect of pH on (Ser)-HPr kinase activity in membrane preparations of *S. mutans* Ingbritt (□) and *S. salivarius* ATCC 25975 (●).

As previous research has demonstrated the importance of glycolytic intermediates in the regulation of (Ser)HPr kinase activity, we employed a ^{32}P-labelled HPr gel isolation assay to examine this regulation in *S. salivarius* ATCC 25975 and *S. mutans* Ingbritt. For this assay, enzyme activity in membrane preparations from each organism was measured at the pH optimum in the presence of various intermediates at three concentrations typical of that in the cytoplasmic compartment of oral streptococci at pH 7.0 (12). With 0.05 mM ATP, the activity of the *S. salivarius* enzyme was stimulated twofold by FBP, with maximum activation at a 5 mM concentration (Fig. 2). However, if the ATP concentration was increased to 0.1 mM in the assay, FBP activation was less than 20% at 4 mM and a further increase to a 1.0 mM ATP concentration resulted in slight inhibition which increased with the FBP concentration. With 1.0 mM ATP, the activity of the *S. mutans* enzyme was also progressively inhibited, and it was inhibited to a slightly greater extent by FBP, with a reduction in activity of 45% at 15 mM (Fig. 2). The intermediates glucose-6-phosphate, fructose-6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, 2,3-phosphoglycerate, and pyruvate had no effect on (Ser)HPr kinase activity (data not shown). These results sug-

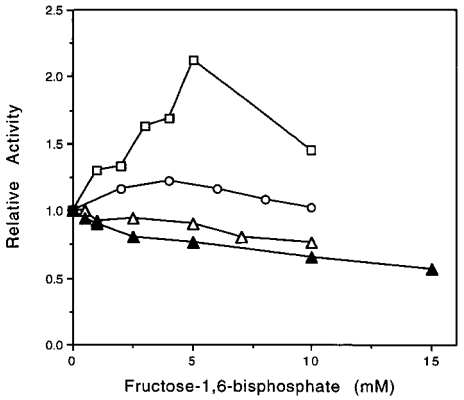

FIG. 2. Effect of FBP on (Ser)HPr kinase activity by membrane preparations of *S. mutans* Ingbritt (solid symbols) and *S. salivarius* ATCC 25975 (open symbols). The ATP millimolar concentrations used were 0.05 (□), 0.1 (○), and 1.0 (△ and ▲).

TABLE 1. Effects of glucose concentration on yield and glucose transport by cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 and a dilution rate of 0.1 h^{-1}

Nominal glucose concn (mM)	Glucose concn (mM)			Cell dry wt (mg/ml)	Cell yield[a]	Glucose transport in chemostat[b]
	Actual initial	Residual	Utilized			
10	8.2	0	8.2	0.39	53.7	32.8
50	46.4	0	46.4	1.34	25.7	57.7
100	82.3	22.7	59.6	1.44	23.6	69.0
200	227	106	121	1.30	16.1	155.1

[a] Grams (dry weight) of cells per mole of glucose.
[b] Nanomoles of glucose per milligram (dry weight) of cells per minute.

gest that the level of P-(Ser)-HPr in these streptococci is not regulated by the intracellular concentrations of the major glycolytic intermediates, including FBP, contrary to findings of studies with *S. mutans* GS-5 (21), *S. pyogenes* (26), and *E. faecalis* (2).

Glucose concentration and intracellular HPr. The in vitro assay results have raised questions about the extent of (Ser) HPr kinase activity in cells of *S. mutans* and the nature of the intracellular regulation of the enzyme. To determine whether the activity of (Ser)HPr kinase was influenced by environmental factors and governed by the energetic status of the cell, we examined these questions by measuring the intracellular levels of the various forms of HPr in cells of *S. mutans* Ingbritt grown at various glucose concentrations in continuous culture. The cells were grown at pH 7.0, (D, 0.1 h^{-1}) with four glucose concentrations (10, 50, 100, and 200 mM). As seen in Table 1, growth with 10 and 50 mM glucose resulted in the complete utilization of the energy source in the growth chamber and, therefore, the cultures were glucose limited. On the other hand, significant residual glucose was present with the 100 and 200 mM glucose chemostats and these cultures were nitrogen limited or contained an excess of glucose.

Inspection of the yields and the rates of glucose utilization by the cells in each culture (Table 1) clearly indicated differences in the physiological status of the cells at each glucose concentration. As expected, the cell yields decreased with an increase in the medium glucose concentration and although both the 10 and 50 mM cultures were glucose limited, there were significant physiological differences in these cultures, as would be expected on the basis of the different concentrations of glucose utilized by each culture (i.e., 8.2 versus 46.4 mM [Table 1]). This difference resulted in a twofold-higher cell yield for the 10 mM culture (53.7 g [dry weight] of cells per mol of glucose) than for the 50 mM culture (25.7 g mol^{-1}). Similarly, the 100 and 200 mM cultures contained a glucose excess; however, the 200 mM culture utilized twice as much glucose (121 mM) as the 100 mM culture (59.6 mM) and this difference was reflected in a significantly lower cell yield for the 200 mM culture (16.1 g mol^{-1}) than for the 100 mM culture (23.6 g mol^{-1}). Calculation of the actual rate of glucose uptake in the chemostat (11) revealed increases from 32 nmol mg of $cells^{-1}$ min^{-1} at 10 mM glucose to a maximum (155 nmol mg of $cells^{-1}$ min^{-1}) at 200 mM (Table 1). The progressive increase in the rate of glucose transport observed with respect to the initial glucose concentration was consistent with the gradual change in the amount of glucose utilized observed during cellular growth, being the minimum for cells having the lowest rate of glucose transport and the maximum for cells that exhibited the highest rate.

The different forms of HPr were quantitatively estimated for steady-state cells growing in continuous cultures under the four conditions described above. As shown previously (34), four forms of HPr are possible in steady-state cells of oral streptococci: free HPr, P~(His)-HPr, P-(Ser)-HPr, and the doubly phosphorylated product, P~(His)-P-(Ser)-HPr. Although P~(His)-HPr and P-(Ser)-HPr appear in the same location on crossed immunoelectrophoresis gels, they can be separated by boiling a portion of the cell extract to degrade the phosphoamidate bond of P~(His)-HPr since the phosphomonoester bond of P-(Ser)-HPr is heat stable (19). Thus, boiling the sample quantitatively converted P~(His)-HPr to free HPr and the doubly phosphorylated form to heat-stable P-(Ser)-HPr (Fig. 3A and B). Subsequent quantitative comparison of the peaks resulting from the boiled sample with those from an unboiled sample of the same cell extract permitted the estimation of the cellular concentration of each of the four forms of HPr (Table 2). In addition, the actual total PEP-dependent phosphorylating activity is represented by the sum of P~(His)-HPr and P~(His)-P-(Ser)-HPr, while total ATP-dependent phosphorylation is represented by the sum of P-(Ser)-HPr and the doubly phosphorylated form.

All four forms of HPr could be detected in the four chemostat-grown cell cultures of *S. mutans* Ingbritt; however, contin-

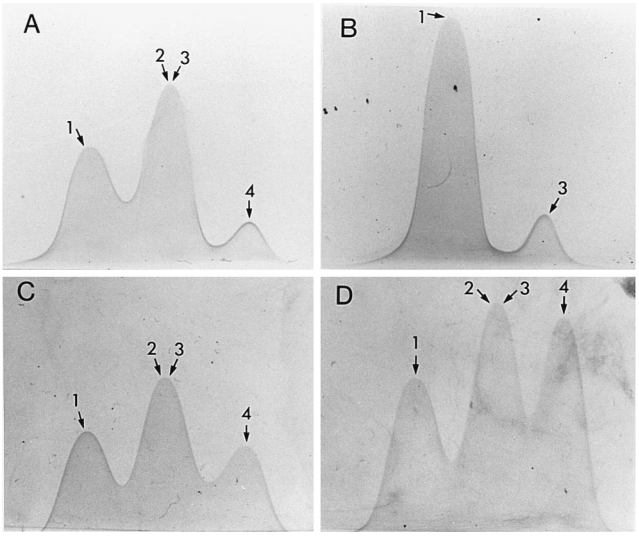

FIG. 3. Crossed immunoelectrophoresis of membrane-free cellular extracts of cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (D, 0.1 h^{-1}) with 10, 50, and 200 mM glucose. Each sample (5 μl) contained 10 μg of cytoplasmic proteins and was probed with polyclonal anti-HPr rabbit antibodies directed against *S. mutans* DR0001. (A) Cells grown with 10 mM glucose; (B) cells grown as for panel A incubated at 100°C for 3 min prior to electrophoresis; (C) cells grown with 50 mM glucose; (D) cells grown with 200 mM glucose. The numbered arrows indicate immunoprecipitate peaks which correspond to nonphosphorylated HPr (1), P~(His)-HPr (2), P-(Ser)-HPr (3), and P~(His)-P-(Ser)-HPr (4).

TABLE 2. Effects of glucose concentration on concentrations of the four forms of HPr in cells of *S. mutans* Ingbritt grown in continuous culture at a dilution rate of 0.1 h^{-1} and pH 7.0[a]

Glucose concn (mM)	Cellular concn[b] of:						
	HPr	P~(His)-HPr	P-(Ser)-HPr	P~(His)-P-(Ser)-HPr	Total HPr	Total PEP-dependent phosphorylation	Total ATP-dependent phosphorylation
10	63.9 ± 5.8	18.2 ± 6.8	5.8 ± 1.4	3.3 ± 1.4	91.2 ± 5.7	21.5 ± 8.0	9.1 ± 1.7
50	59.2 ± 11.2	31.8 ± 12.4	28.6 ± 11.5	14.3 ± 3.1	134 ± 11.3	46.1 ± 12.7	42.9 ± 10.6
100	52.1 ± 8.0	11.2 ± 3.7	84.7 ± 13.8	21.5 ± 8.1	170 ± 19.0	32.7 ± 11.2	106 ± 9.9
200	52.5 ± 2.9	13.5 ± 6.2	66.4 ± 4.9	39.0 ± 11.9	171 ± 13.5	52.5 ± 14.6	105 ± 8.9

[a] Determinations were carried out by crossed immunoelectrophoresis, and the values represent the means of at least four determinations.
[b] Micrograms per milligram of cell protein ± standard error.

uous growth at the various glucose concentrations resulted in differences in both the concentration of the total pool of HPr in the cells and in the intracellular levels of the various forms of HPr (Table 2 and Fig. 3A, C, and D). The total pool of HPr, expressed as micrograms per milligram of cell protein, almost doubled for the 200 mM glucose-starved cells (171 μg mg^{-1}) compared with the glucose-starved cells grown at 10 glucose mM (91.2 μg mg^{-1}).

The cellular concentrations of P~(His)-HPr and P-(Ser)-HPr were of particular interest in these experiments. As seen in Table 2, the P~(His)-HPr levels alone varied only from 11.2 to 31.8 μg mg of cell $protein^{-1}$ in cells grown at between 10 and 200 mM, with the highest concentration in the 50 mM glucose-grown cells. The cellular levels of the doubly phosphorylated form, P~(His)-P-(Ser)-HPr, increased 12-fold over the complete range of glucose concentrations. Thus, the total PEP-dependent phosphorylation of HPr [i.e., P~(His)-HPr plus P~(His)-P-(Ser)-HPr] changed from 21.5 to 52.5 μg mg of cell $protein^{-1}$, with concentrations from 10 to 200 mM glucose. The level of P-(Ser)-HPr, on the other hand, increased 15-fold from that for the 10 mM glucose-grown cells (5.8 μg mg of cell $protein^{-1}$) to a high for 100 mM glucose of 84.7 μg and declined with 200 mM glucose (66.4 μg). As a consequence, total ATP-dependent phosphorylation of HPr [i.e., P-(Ser)-HPr plus P~(His)-P-(Ser)-HPr], increased progressively with respect to the medium glucose level from 9.1 μg at 10 mM to a maximum of 106 μg mg of $protein^{-1}$ in cells grown with 100 mM glucose. Increasing the glucose concentration to 200 mM did not increase the level of total P-(Ser)-HPr or ATP-dependent phosphorylation.

Since the total cellular HPr concentration changes with increasing glucose in the medium, a more informative method of analyzing the differences in the levels of the four forms of HPr is calculation of the relative proportions of the various forms within each growth condition. As seen from the data in Fig. 4, the relative amount of free, nonphosphorylated HPr in *S. mutans* Ingbritt cells grown in continuous culture was the highest for cells grown with 10 mM glucose (70%) and this declined to 31% for the cells grown with 100 and 200 mM glucose. As for P~(His)-HPr, the 10 and 50 mM glucose-limited cultures contained the highest levels of this form (20 and 24%, respectively) with the 100 and 200 mM glucose-excess-growth cells exhibiting the lowest relative amounts (6.6 and 7.9%, respectively). On the other hand, P-(Ser)-HPr increased from 6% for the 10 mM glucose-grown cells to a maximum of 50% with the 100 mM glucose-grown cells. The level of the doubly phosphorylated form, P~(His)-P-(Ser)-HPr, increased progressively from 3.6 to 23% as the medium glucose concentration was increased from 10 to 200 mM.

Further analysis of the data in Table 2 reveals the extent of the PEP- and ATP-dependent phosphorylating activity as a fraction of total HPr phosphorylation. This fraction is obtained by combining the relative values for P~(His)-HPr and P-(Ser)-HPr peaks with those of the doubly phosphorylated form, P~(His)-P-(Ser)-HPr. From these calculations, it can be determined that phosphorylation dependent on PEP ranged from 70% at 10 mM glucose to 24% at 100 mM glucose, while that dependent on ATP was 30% with 10 mM glucose and increased to 76% at 100 mM glucose, reflecting the increased uptake of glucose into the cell (Table 1).

(Ser)HPr kinase activity and medium glucose. In association with the estimation of the levels of various forms of HPr in vivo, (Ser)HPr kinase activity was measured in membrane preparations from the same cells grown in chemostats at the four concentrations of glucose. As seen from the data in Fig. 5, enzyme activities were very similar in cells grown with 10, 50, and 100 mM glucose, varying from 2.7 to 3.5 U μg of membrane $protein^{-1}$ min^{-1}. Growth with 200 mM glucose, however, resulted in a threefold increase in (Ser)HPr kinase activity to 10.6 U, suggesting a significant change in enzyme synthesis. Nevertheless, this increase in the cellular amount of the enzyme did not result in an increase in the intracellular concentration of P-(Ser)-HPr (Table 2 and Fig. 4).

Cellular concentrations of key glycolytic intermediates. Results from our earlier in vitro (Ser)HPr kinase assays have suggested that, contrary to findings of earlier studies (21), the enzyme in *S. mutans* and *S. salivarius* was not stimulated by FBP (Fig. 2) and other glycolytic intermediates. In view of this

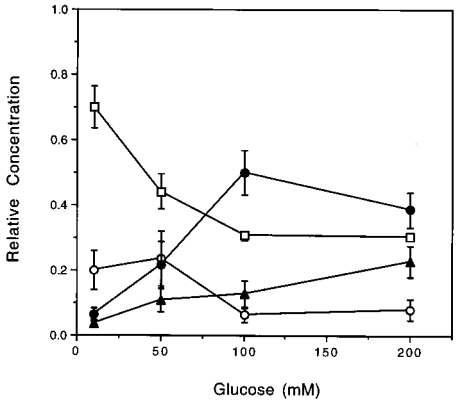

FIG. 4. Comparison of relative amounts of the four forms of HPr in cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (*D*, 0.1 h^{-1}) with concentrations of glucose ranging from 10 to 200 mM. The symbols correspond to nonphosphorylated HPr (□), P~(His)-HPr (○), P-(Ser)-HPr (●), and P~(His)-P-(Ser)-HPr (▲). The results (in micrograms per milligram of protein) represent the means of at least four determinations, and the bars represent the standard errors.

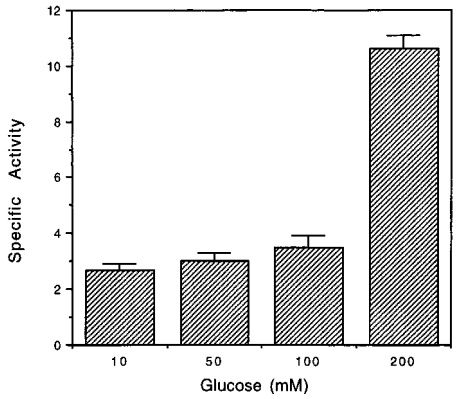

FIG. 5. (Ser)-HPr kinase activity in membrane preparations derived from cells of *S. mutans* Ingbritt grown anaerobically in continuous culture at pH 7.0 (*D*, 0.1 h^{-1}) with 10, 50, 100, and 200 mM glucose. Activity units are expressed in picomoles of P-(Ser)-HPr formed per microgram of membrane protein per minute. The standard errors are indicated by T bars.

observation and the data for enzyme activity in the chemostat-grown cells (Fig. 5), we undertook to determine whether there was an in vivo relationship between the intracellular concentrations of FBP and glucose-6-phosphate, as well as the substrate, ATP, and enzyme activity, with cells grown in continuous culture under the same conditions as those for the HPr determinations.

As seen from the data in Table 3, with the exception of the glucose-6-phosphate concentration in cells from the 10 mM glucose chemostat culture, the glycolytic intermediate levels increased with the medium glucose concentration in a predictable manner. In particular, the intracellular levels of FBP increased in the chemostat-grown cells such that the 200 mM glucose-grown cells contained a 3.8-fold-higher level than the 10 mM glucose-grown cells. The cellular concentration of ATP increased from 415 μM with 10 mM glucose and reached a relatively stable level in the 100 and 200 mM glucose-grown cells (1,416 to 1,494 μM).

DISCUSSION

Steady-state chemostat-grown cells of *S. mutans* Ingbritt possessed all four forms of HPr during growth at all four glucose concentrations. A notable effect of increasing the glucose concentration from 10 to 200 mM was the increase in the total cellular content of all forms of HPr from 91.2 to 171 μg mg of cell protein^{-1}, an observation contrary to our previous results (10). In that previous study, the cellular HPr concentration decreased fourfold from medium glucose concentrations of 6.8 to 304 mM, which confirmed an earlier observation (36) that changes from glucose limitation to nitrogen limitation (glucose excess) resulted in a small decrease in the synthesis of HPr. The reason for this difference is not immediately apparent, since the current experiments were carried out with the same organism in the same medium and under similar conditions (pH 7.0; *D*, 0.1 h^{-1}). A notable difference between these studies, however, was the fact that the HPr concentrations in the earlier studies were estimated by rocket immunoelectrophoresis, a technique that does not allow separation of the chemically different forms of HPr. Even though the antibodies against free HPr reacted with all four forms of HPr, some or all of them may be regarded as distinct molecules. Therefore, the peak observed in rocket immunoelectrophoresis would result from the reaction of the antibody with the major form of HPr present in the tested cells, representing only a portion of the total HPr. Furthermore, some forms would not be detected because their concentrations would be too low or because their presence would be masked by the major peak. In the present study, this problem was avoided by calculating the amount of HPr from the total amount of each form of HPr separated by crossed immunoelectrophoresis.

TABLE 3. Effects of glucose concentration on the concentrations of FBP, glucose-6-phosphate, and ATP in cells of *S. mutans* Ingbritt grown in continuous culture at a dilution rate of 0.1 h^{-1} and pH 7.0

Glucose concn (mM)	Intracellular concn (μM) ± SE[a] of:		
	FBP	Glucose-6-phosphate	ATP
10	290 ± 10	801 ± 56	415 ± 46
50	637 ± 22	497 ± 47	1,004 ± 101
100	976 ± 109	662 ± 56	1,494 ± 80
200	1,114 ± 67	1,227 ± 39	1,416 ± 86

[a] Determinations were carried out enzymatically (see Materials and Methods), and the values represent the means of at least five determinations.

The most notable effect of glucose on the intracellular forms of HPr in *S. mutans* Ingbritt was a progressive increase in the concentration of the doubly phosphorylated form with the medium glucose concentration, accompanied by a small decrease in the amount of P~(His)-HPr (Table 2). The concentration of P~(His)-P-(Ser)-HPr was minimal in cells grown under conditions of glucose limitation (10 mM), a status dominated by P~(His)-HPr, and reached a maximum in cells grown with excess glucose (200 mM), being threefold higher than that for P~(His)-HPr. Consequently, total ATP-dependent phosphorylation of HPr, calculated from P-(Ser)-HPr and P~(His)-P-(Ser)-HPr concentrations, increased 12-fold from 12.4 to 144 μg mg of cell protein^{-1} with increases in the medium glucose from 10 to 200 mM, while the PEP-dependent phosphorylation varied only 2-fold from a low of 21.5 μg with 10 mM glucose to a high of 52.5 μg mg of cell protein^{-1} with 200 mM glucose.

These results are consistent with those obtained with batch-grown cells of *S. mutans* DR0001 and *S. salivarius* 25975 (34). In that prior study, using the same methods, exponential-phase cells possessed no free HPr and no or very small amounts of P~(His)-HPr, with most of the HPr in the form of P-(Ser)-HPr and with lesser amounts in the doubly phosphorylated form, P~(His)-P-(Ser)-HPr. On the other hand, the dominant forms in stationary-phase cells were free HPr and P~(His)-HPr, with the cells devoid of P-(Ser)-HPr and the doubly phosphorylated form. Globally, these results suggest that in energy-deprived cells, phosphate is transferred to the EII complexes of the PTS mainly by P~(His)-HPr, whereas under conditions of an abundant energy source, this function is taken over by the doubly phosphorylated product. Although our results are consistent with this hypothesis, further research is needed to determine whether the doubly phosphorylated form of streptococcal HPr is efficient at transfer of phosphate groups to the EII complexes.

Physiologically, replacement of the P~(His)-HPr by the doubly phosphorylated protein as the phosphocarrier in the PTS phosphoryl cascade is obviously linked to the function of P-(Ser)-HPr. The results reported here, as well as those obtained with batch-grown cells (34), indicate that the assigning of the regulatory functions to P-(Ser)-HPr must take into account the existence of the doubly phosphorylated product. Recent studies have suggested that P-(Ser)-HPr is involved in regulating non-PTS transport systems of *L. brevis* (38, 39), a bacterium that apparently lacks EI and EII complexes of the

PTS. Such a role for P-(Ser)-HPr is compatible with the presence of the doubly phosphorylated protein in cells possessing a PTS. Hence, when cells were growing in the presence of a glucose excess, P-(Ser)-HPr would be produced to prevent utilization of other less rapidly degraded non-PTS sugars, whereas the doubly phosphorylated protein would be synthesized to maintain the transfer of phosphate from PEP to the EII complex of the glucose PTS at an optimal rate. Under conditions of energy deprivation, the cells would produce very low levels of P-(Ser)-HPr, thus permitting the concomitant utilization of several sugars.

Several years ago, it was suggested that phosphorylation of HPr on Ser-46 might serve to regulate the activity of the PTS (3). This hypothesis was supported by the observation that P-(Ser)-HPr was phosphorylated in vitro at a very much slower rate by PEP than nonphosphorylated HPr. However, the data reported in this study, as well as those obtained with batch-grown cells (34), are not fully consistent with this proposal since the amount of the doubly phosphorylated protein found in growing cells suggested that the phosphorylation of P-(Ser)-HPr by EI of the PTS and, conversely, the phosphorylation of P~(His)-HPr by HPr kinase, are not limiting steps in vivo. Other in vivo studies also failed to provide evidence supporting the hypothesis that P-(Ser)-HPr controls PTS activity (25, 28, 29, 32). Nevertheless, this last hypothesis has recently reappeared as a suggestion that P-(Ser)-HPr is involved in the control of lactose expulsion in *L. lactis* by controlling the activity of both a sugar-P phosphatase and lactose EII (40). Although control of phosphatase by P-(Ser)-HPr is compatible with the presence of a doubly phosphorylated HPr in the cell, control of lactose permease raises some questions since it would imply that this PTS permease could not be phosphorylated by P~(His)-P-(Ser)-HPr and would be inhibited by P-(Ser)-HPr, whereas the glucose PTS would be immune to the inhibitory effect of P-(Ser)-HPr and be able to react with the doubly phosphorylated protein. This is unlikely, as it has already been reported that the IIA^{Lac} and IIA^{Mtl} enzymes from *B. subtilis* could catalyze in vitro the phosphorylation of lactose or mannitol, respectively, with a phosphocarrier that consisted of a mutated HPr in which the serine at position 46 was replaced by an aspartate, resulting in a protein having properties similar to those of P-(Ser)-HPr (28). Therefore, the control of PTS activity by P-(Ser)-HPr is not consistent with the presence of large amounts of doubly phosphorylated HPr in rapidly growing cells or in cells growing in the presence of excess glucose. It should be pointed out that even though the presence of doubly phosphorylated HPr in streptococci has been confirmed (in this study and in previous work [34]), it remains to be established for other species, including lactococci.

Notwithstanding the *L. lactis* results, our data, together with previously published kinetic data (29), provide further evidence that phosphorylation of HPr on Ser-46 has no physiological consequences with respect to activity of the PTS. Assuming that 0.5 mg of protein is the equivalent of 1 mg of cells (dry weight), simple calculations reveal that the cellular concentrations of free HPr and P~(His)-HPr under all growth conditions were approximately 1.5 mM and 0.3 to 0.75 mM, respectively. These concentrations are substantially higher than the apparent K_m values of the relevant enzymes for these substrates; e.g., the K_m of streptococcal EI for HPr is 25 μM (35) and that of *Bacillus* IIA^{Glu} for P~(His)-HPr is 0.5 μM (29). Similar calculations indicate that the cellular concentrations of P-(Ser)-HPr are also higher than that required for saturation of EI and IIA^{Glu} of *B. subtilis* (28, 29). We are aware that determination of these kinetic parameters with protein of streptococcal origin is required to substantiate this conclusion. However, the data reported in this study clearly indicate that phosphorylation of HPr on Ser-46 is not dedicated to reducing the entry of glucose into these cells since the rate of glucose transport by growing cells increases with respect to the cellular level of total P-(Ser)-HPr (Tables 1 and 2).

Recent observations also suggest that (Ser)-HPr phosphorylation plays a role in repression of several catabolite genes in *B. subtilis* (4). A form of catabolite repression has been observed with chemostat-grown cells of *S. mutans* Ingbritt growing on glucose. In this case, the glucose PTS is subjected to concentration-dependent repression by glucose itself (6), a process known to inhibit the synthesis of EII^{Glc} and EII^{Man} in membrane preparations of the organism (10). In these latter experiments, the syntheses of EII^{Glc} and EII^{Man} were repressed during growth at all glucose concentrations above 2.6 mM, with a 33-fold level of inhibition observed with 271 mM glucose. EII for fructose (EII^{Fru}) was also repressed fourfold, although its activity level in glucose-grown cells was low. In view of the data for *B. subtilis* (4) and the concomitant effects seen with the formation of P-(Ser)-HPr and EII^{Glc} synthesis in the presence of high concentrations of glucose, it is tempting to speculate that P-(Ser)-HPr is involved in catabolite repression of the glucose PTS in what would, in fact, be a form of autorepression by the substrate itself. Obviously, much more work is required in order to ascertain whether this mechanism is functional in oral streptococci.

Comparison of the observed cellular P-(Ser)-HPr levels (Table 2 and Fig. 4) and the (Ser)HPr kinase assays (Fig. 5) from chemostat-grown cells at the four glucose concentrations indicated two separate effects on the kinase in *S. mutans* Ingbritt. With between 10 and 100 mM glucose, there was no increase in enzyme specific activity (Fig. 5), whereas the intracellular concentration of total P-(Ser)-HPr increased progressively from 9.1 to 106 μg mg of cell protein^{-1}, indicating control of the enzyme at the protein level. On the other hand, specific enzyme activity increased threefold with glucose between 100 and 200 mM, suggesting a stimulation of kinase synthesis, presumably at the transcriptional level; however, this increase in enzyme synthesis did not result in higher levels of P-(Ser)-HPr (Table 2). A plausible explanation for this result would be that with 200 mM glucose, cells accumulate one or more metabolites that down-regulate (Ser)HPr kinase. To keep the cellular level of total P-(Ser)-HPr at the appropriate level, the cells compensate by increasing enzyme synthesis. This hypothesis is consistent with the facts that (i) the rate of glucose transport increased significantly with glucose concentrations between 100 and 200 mM (Table 1), which increases the cellular concentration of metabolites (12) (Table 3), and (ii) the kinase was inhibited in vitro by high concentrations of FBP (Fig. 2). These effects are undoubtedly influenced by the nature of the enzyme in the particular strain, the effects of FBP (Fig. 2) and other metabolites, and subtle differences in the pH profile of kinase activity. For example, the difference between the (Ser)HPr kinase pH profile of *S. mutans* Ingbritt and that of *S. salivarius* (Fig. 1) is in keeping with the acid-tolerant characteristics of the *S. mutans* strain (9) and is a factor at pH values below 6.0.

The effect of glycolytic intermediates on the activity of (Ser) HPr kinase in membrane preparations of *S. salivarius* and *S. mutans* suggests differences from the results reported by other workers. FBP activation of (Ser)HPr kinase has been a consistent feature of the studies with *S. pyogenes* (26), *E. faecalis* (2), *L. brevis* (27), and *B. subtilis* (23), as well as *S. mutans* GS-5 (21). Our in vitro and in vivo results suggest that the intracellular concentrations of glucose-6-phosphate and FBP are not critical factors in the stimulation of (Ser)HPr kinase activity. In

the in vitro assay, only with 0.05 mM ATP was FBP slightly stimulatory with membrane preparations of the enzyme from *S. salivarius*. When the concentration of ATP was increased to 1.0 mM, the *S. salivarius* and *S. mutans* enzymes either were not affected or were inhibited by FBP, as well as being unaffected by other intermediates.

Correlation of the in vivo concentration of FBP seen in Table 3 and the level of the total P-(Ser)-HPr reported in Table 2 showed that both concentrations increased with respect to the medium glucose concentration. This relationship is apparently consistent with the suggestion that FBP activates (Ser)HPr kinase. However, the concentration of FBP observed in vivo is far below the concentration that activated the kinase of other organisms in vitro (~5 mM) (24) as well as the kinase in *S. salivarius*, even in the presence of 0.05 mM ATP (Fig. 2). As we have seen from the data in Table 3, other than for the cells at concentrations of 10 mM, cellular ATP levels were at or above 1.0 mM, suggesting that cellular ATP levels would not be low enough to promote FBP activation similar to that for *S. salivarius* in Fig. 2. Moreover, in spite of the fact that the 200 mM glucose-grown cells contained the highest cellular level of FBP (1.1 mM), as well as a 10-fold-higher level of the enzyme, the total intracellular concentration of P-(Ser)-HPr did not change from that at 100 mM glucose. Considering the in vitro and in vivo results, we suggest that the intracellular concentrations of FBP are not related to those of P-(Ser)-HPr in *S. salivarius* and *S. mutans*. Obviously, increased levels of other metabolites, alone or in combination, may contribute to an increase in (Ser)HPr kinase activity in vivo and may also be involved in increased synthesis of the enzyme in cells at 200 mM concentrations.

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