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Resolution of the Phosphotransferase Enzymes of *Streptococcus mutans*: Purification and Preliminary Characterization of a Heat-Stable Phosphocarrier Protein

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The sucrose phosphotransferase system of *Streptococcus mutans* catalyzes the phosphorylation of sucrose to sucrose-6-phosphate with concomitant translocation of this disaccharide across the cytoplasmic membrane in reactions requiring intracellular phosphoenolpyruvate. Soluble proteins released by vigorous homogenization of cells with glass beads are shown to be necessary for the phosphoenolpyruvate-dependent phosphorylation of sucrose in combination with one or more proteins that remain tightly associated with the membrane fraction. We have partially purified phosphotransferase enzyme I and have purified a heat-stable phosphocarrier protein (HPr) to apparent homogeneity, by gel filtration and ion-exchange chromatography from the soluble fraction. HPr from *S. mutans* has an apparent molecular weight larger than that of *Escherichia coli* HPr but has properties similar to those of *Staphylococcus aureus* HPr. Furthermore, it appears to be partially complexed with a heat-stable enzyme III-like protein in cell-free fractions from *S. mutans*, and we also report the purification of this complex. Enzyme I from *S. mutans* is a protein (native M_r > 100,000) that cross-complements enzyme I from *S. aureus*. Preliminary characterizations of homogeneous HPr and its complex with the putative enzyme III are also presented.

The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is an active group translocation mechanism that is employed by the majority of anaerobic and facultatively anaerobic bacteria for transport of a variety of sugars (5, 18, 20). Among the bacterial PTSs that have been studied with respect to sucrose translocation, only those of *Streptococcus mutans* (28, 29), *Streptococcus lactis* (31), *Klebsiella pneumoniae* (12), and *Escherichia coli* (12, 24) have been described in any detail. Sucrose PTS activity in the latter organism is specified by a transmissible plasmid, encoding a membrane-bound sucrose enzyme II and a soluble sucrose-6-phosphate hydrolase. It is of interest to identify and characterize the PTS components in *S. mutans* not only to further our understanding of sucrose utilization and its regulation in this organism, but also to help elucidate the process by which *S. mutans*, an oral pathogen, utilizes dietary sucrose to initiate the formation of carious lesions in tooth enamel (7). Ultimately, since sucrose-specific PTS activities serve not only to transport sucrose but also to initiate its catabolism, potential inhibitors designed to preclude colonization by *S. mutans* in the oral cavity might be directed to act at the level of the sucrose PTS. The design of such inhibitors, in part, relies upon elucidation of the mechanism of the PTS in *S. mutans*.

In this report, we characterize the *S. mutans* PTS with regard to the number of proteins required for sucrose phosphorylation and their cellular localization. From the results of these experiments, it is apparent that the following set of reactions is minimally required for this activity:

$$\text{PEP} + \text{HPr} \xleftrightarrow{\text{enzyme I}} \text{phospho-HPr} + \text{pyruvate}$$

$$\text{phospho-HPr} + \text{sucrose} \xrightarrow[(\text{enzyme III}^{\text{Scr}})]{\text{enzyme II}^{\text{Scr}}} \text{sucrose-6-P} + \text{HPr}$$

These reactions are similar to those found for the PTSs of other organisms. In contrast to many other bacteria, however, all of the PTS enzymes were previously found to remain tightly associated with the membrane fraction of *S. mutans* in cells broken by sonication or French pressure cell lysis (G. R. Jacobson, W. L. Ran, and P. J. Scott, Abstr. Annu. Meet. Am. Soc. Microbiol., abstr. no. K122, 1981). Vigorous agitation with glass beads was found in the present report to remove the general, nonspecific PTS components, enzyme I, and a heat-stable phosphocarrier protein (HPr), from the membrane. The sucrose-specific enzyme II (enzyme II^{Scr}), however, remained tightly associated with the membrane under these conditions and is presumed to be the transmembrane permease, as is the case in all other organisms containing a PTS.

HPr, one of the nonspecific general PTS proteins, participates in phosphoexchange between PTS enzyme I (which is phosphorylated by PEP) and sugar-specific enzymes II (or a sugar-specific enzyme III, in some cases) as shown above. HPr proteins from *Streptococcus faecalis* (11), *S. lactis* (11), *Bacillus subtilis* (11), *E. coli* (1), *Salmonella typhimurium* (2), and *Staphylococcus aureus* (27) have been purified to homogeneity and compared with respect to their structures and mechanisms of action (6, 10, 11, 15, 19, 25, 33). In this report, we describe the purification of HPr from *S. mutans*, its complex with an enzyme III-like protein, and the partial purification of *S. mutans* enzyme I. Preliminary characterizations of these proteins are also presented.

(Portions of this work were presented in preliminary form at the annual meeting of the American Society for Microbiology in New Orleans, 1983.)

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MATERIALS AND METHODS

Materials. D-Glucose and sucrose, both uniformly labeled with ^{14}C, were obtained from New England Nuclear Corp. Phosphoenol-1-[^{14}C]pyruvate was purchased from Amersham Corp. DEAE-cellulose and other chemicals used were purchased from Sigma Chemical Co., unless otherwise indicated. Purified *E. coli* HPr was the generous gift of T. Osumi.

Bacterial strains. *S. mutans* V843 (GS-5, serotype *c*) was provided by F. Macrina. The cytoplasmic fraction derived from this strain (see below) contained the soluble PTS components isolated in this study. *S. aureus* S797A, lacking HPr, was obtained from J. Deutscher. *S. aureus* LJ195, defective in enzyme I, was kindly provided by Milton Saier.

Cell growth and preparation of cell-free extracts. *S. mutans* cultures were routinely maintained on mitis salivarius agar (Difco Laboratories) plates containing 15% sucrose and bacitracin (0.2 U/ml), with frequent transfers. For growth in liquid culture, colonies were inoculated into 5 ml of brain heart infusion broth (Difco) containing 1% sucrose and were allowed to grow in stoppered tubes for 24 to 36 h at 37°C. This culture was then used as an inoculum for the final culture medium, which consisted of tryptone (20 g/liter) (Difco) broth and 2% sucrose containing in grams per liter: K_2HPO_4, 4; KH_2PO_4, 1; NaCl, 2; $MgCl_2$, 0.25; and 20 mM DL-threonine to render the cell more susceptible to rupture (4). Cultures were grown in stoppered flasks without shaking at 37°C to early stationary phase and then used to inoculate 2 to 3 liters of the same medium. These were grown under the same conditions to late exponential phase (16 to 24 h), harvested at 15,000 × *g* for 10 min, washed twice with 20 mM Tris-hydrochloride–1 mM dithiothreitol (pH 7.5) (TD buffer), and then resuspended in 1/30 the original culture volume of TD buffer. This cell suspension was then stored at −70°C until used. No loss of total cell PTS activity, or activities of individual PTS components, was detected over a period of at least 3 months under these storage conditions.

S. mutans cells were lysed as follows. Cell suspensions in TD buffer were treated in a Bead-Beater homogenizer (Biospec Products) with 1:2 (vol/vol) cell suspension-glass beads (0.1 mm) and a 400-ml metal chamber. Care was taken to remove as much air as possible from the chamber, and samples were homogenized in a cold room for five 3-min intervals, using the ice jacket provided with the instrument and allowing the suspension to cool for 5 min between each homogenization cycle. The cell extract was then decanted from the settled glass beads and centrifuged at 5,000 × *g* for 10 min to remove any unbroken cells and residual glass beads. The resulting cell-free extracts were subjected to centrifugation at 100,000 × *g* for 90 min at 4°C. $MgCl_2$ was added to the supernatants (cytoplasmic fractions) to a final concentration of 10 mM. These cytoplasmic fractions were then dialyzed at 4°C against 10 volumes of TD buffer containing 10 mM $MgCl_2$ and concentrated 10- to 20-fold at 4°C in an Amicon ultrafiltration apparatus with a YM5 filter. The pellet from the high-speed centrifugation (membrane fraction) was washed with TD buffer, recentrifuged at 100,000 × *g*, and then taken up in 1/10 the volume (relative to the initial extract) of TD buffer containing 10 mM $MgCl_2$. Both washed membrane and dialyzed cytoplasmic fractions were stored at −70°C until used.

S. aureus strains were grown in 4 liters of a complex medium, as described by Beyreuther et al. (3). Cells were harvested and broken in the same manner as for *S. mutans*. Membranes were also prepared as for *S. mutans*, but in contrast to *S. mutans*, neither dialysis nor concentration of the cytoplasmic fraction was found to be necessary for complementation assays (see below).

Assays. For assays of PTS enzymes, membrane or cytoplasmic fractions (or both), prepared as described above, were used. Before the assay, *S. mutans* membranes were treated with 1% toluene (vol/vol) to render any vesicular structures permeable to charged substrates. Unless otherwise indicated, PEP-dependent sugar phosphorylation was estimated in mixtures (0.10 ml) containing 25 mM Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, 0.1 mM [^{14}C]sugar (5 μCi/μmol), 5 mM $MgCl_2$, 10 mM KF, and the sample(s) to be assayed, in the presence and absence of 10 mM PEP. Mixtures were incubated at 37°C for 30 to 60 min, the reaction was stopped by addition of 1 ml of ice-cold dH_2O, and the solutions were then filtered through DEAE-filter disks (Whatman, Inc., DE81) as described by Jacobson et al. (9). After three 10-ml washes with water, the disks were air-dried and counted in standard toluene-based scintillation fluid. PEP-dependent phosphorylation was calculated as the difference between mixtures containing and lacking the phosphodonor in all cases.

Enzyme I of the PTS was determined by the [^{14}C]PEP-pyruvate exchange assay (22). Assay mixtures (0.1 ml) contained 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 0.2 mM [^{14}C]PEP (12 μCi/μmol), 2 mM pyruvate, 10 mM KF, 1 mM dithiothreitol, and the sample to be tested. Mixtures were incubated at 37°C for 30 min and placed on ice to stop the reaction, and 0.4 ml of 0.02% 2,4-dinitrophenylhydrazine in 1 N HCl was added. Tubes were incubated at 37°C for 10 min to allow reaction of pyruvate with the dinitrophenylhydrazine. This solution was extracted by vortexing with 1 ml of ethyl acetate, and 0.6 ml of the upper organic layer was removed and counted in 5 ml of standard Triton X-100-toluene scintillation fluid. Controls were prepared identically, but pyruvate was omitted from the reaction mixture. Alternatively, *S. mutans* enzyme I activity was detected by complementation with *S. aureus* LJ195 membranes containing glucose-specific enzyme II and cytoplasm from LJ195, in the PEP-dependent phosphorylation of glucose. Phosphorylation was quantitated as described previously (9).

S. mutans HPr activity was assayed by complementation with partially purified enzyme I and membranes containing the sucrose-specific enzyme II from *S. mutans*, in the PEP-dependent phosphorylation of sucrose. Alternatively, *S. mutans* HPr activity was detected by complementation with *S. aureus* cytoplasm (derived from a strain defective in HPr) and *S. aureus* membranes to restore PEP-dependent glucose phosphorylation. In both sucrose and glucose phosphorylation assays, the membranes used from *S. mutans* or from *S. aureus* were treated with 1% toluene before assay, as we have found that PTS activities are enhanced by this pretreatment.

Column chromatography. Separation of the soluble PTS enzymes was accomplished with *S. mutans* cytoplasm prepared as described above. A 1-ml portion of this cytoplasmic fraction was applied to a Sephadex G-100 column (23 by 1 cm) (Pharmacia Fine Chemicals) and eluted with 20 mM Tris-hydrochloride–1 mM dithiothreitol–10 mM $MgCl_2$, pH 7.5 (TDM buffer), at 4°C. Eighteen 1-ml fractions were collected, the relative protein content of each fraction was estimated by absorbance at 280 nm, and the enzyme I- and HPr-containing fractions, detected as described above, were pooled and stored at −70°C until used.

Separation of the HPr-enzyme III complex and of free HPr

derived from the Sephadex G-100 step was achieved by DEAE-cellulose column chromatography. A 2-ml portion of pooled, boiled HPr-containing fractions (100°C for 5 min) was applied to a 2-ml DEAE-cellulose column (Sigma, D-8382) and eluted with three 2-ml washes each of 0.05, 0.10, 0.15, and 0.20 M NaCl in dH_2O. The HPr-containing fractions were pooled and stored at −70°C until used.

Other Procedures. Sodium dodecyl sulfate-polyacrylamide slab gels were prepared according to Weber and Osborn (32). The gels were stained either with Coomassie brilliant blue or by the method of Merril et al. (16, 17) using an ultrasensitive silver stain. When necessary, silver-stained gels were destained (to reduce background darkness and to remove surface deposits of silver), as described by Switzer et al. (30).

Total protein determinations were performed by the method of Lowry et al. (13). Amino acid compositions, kindly performed by R. Laursen and Nithya Rajan, were determined on a Beckman 119CL amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 24 h.

RESULTS

PTS activities in cell-free fractions of *S. mutans*. In a preliminary communication (G. R. Jacobson, W. L. Ran, and P. J. Scott, Abstr. Annu. Meet. Am. Soc. Microbiol., 1981 K122, p. 157), we reported that nearly all of the PEP-dependent sucrose-phosphorylating activity in *S. mutans* was associated with the membrane fraction after cell lysis by sonication or French pressure cell treatment. In an effort to dissociate from the membrane PTS proteins that are normally soluble in other organisms (enzyme I, HPr, and enzymes III), we adopted a cell lysis procedure involving vigorous homogenization with glass beads as described above. Membrane and cytoplasmic fractions obtained by this procedure were then tested for total PEP-dependent sucrose-phosphorylating activity and for enzyme I activity. Only a combination of membranes and cytoplasm could catalyze phosphorylation of sucrose, and enzyme I activity resided almost exclusively in the cytoplasmic fraction (Table 1). These results show that this cell-breaking procedure resulted in the release of one or more proteins of the *S. mutans* PTS into the soluble fraction and that both these and a membrane-bound protein (enzyme II) are necessary to reconstitute PTS activity. Interestingly, it was found that only dialyzed *S. mutans* cytoplasm could efficiently complement membranes in sucrose phosphorylation (Table 1, footnote *a*). Thus, there appears to be an as yet unidentified PTS inhibitor of relatively low molecular weight in the cytoplasmic fraction.

TABLE 1. PTS activities in cell-free fractions of *S. mutans* broken by glass bead homogenization

Fractions	Sucrose PTS[a] (pmol of Scr-P/h per mg of protein)	Enzyme I[b] (nmol of [^{14}C]pyruvate/0.5 h per mg of protein)
Membranes	1.5	15.4
Cytoplasm	2.6	484
Membranes + cytoplasm	239	ND[c]

[a] PEP-dependent phosphorylation assays were performed as described in the text. When membranes plus cytoplasm were tested, the assay mixture contained 210 μg of membrane protein and 140 μg of cytoplasmic protein, and the specific activity listed is relative to the amount of the latter in the assay mixture. The cytoplasm used in these experiments was concentrated and dialyzed as described in the text. If the cytoplasm was not dialyzed, no reconstitution of sucrose PTS activity was detected (see text).

[b] Determined by the PEP-pyruvate exchange assay (see the text).

[c] ND, Not determined.

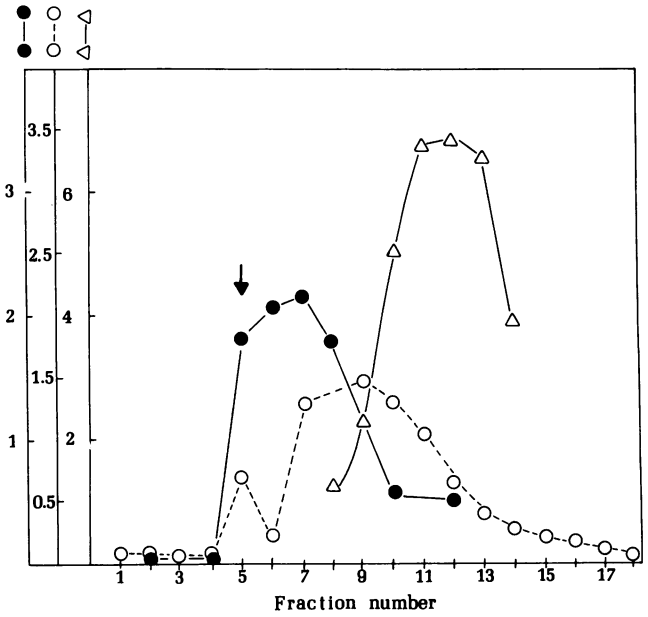

FIG. 1. Sephadex G-100 fractionation of *S. mutans* soluble PTS proteins. Dialyzed and concentrated *S. mutans* cytoplasm was fractionated on Sephadex G-100 and assayed for PTS enzyme activities as detailed in the text. Column fractions were collected and assayed for the relative amounts of total protein by absorbance at 280 nm (○), for [^{14}C]PEP-pyruvate exchange (●, enzyme I activity), and for PEP-dependent phosphorylation of sucrose by complementation of *S. mutans* membranes and pooled enzyme I (△, HPr activity). Ordinate values for the latter two reactions are given in thousands of counts per minute of product ([^{14}C]pyruvate and [^{14}C]sucrose-6-phosphate, respectively). In the enzyme I assay, mixtures (100 μl) contained 20 μl of each column fraction and assays were conducted as described in the text. In the HPr assay, mixtures (100 μl) contained 20 μl of each column fraction, 210 μg of *S. mutans* membranes, 16 μg of enzyme I pool (fractions 4 through 8), and other components as referred to in the text. The arrow denotes the excluded volume of the column.

Resolution of *S. mutans* enzyme I and HPr. For separation of the soluble PTS enzymes from *S. mutans*, cytoplasmic samples from glass bead-ruptured cells were fractionated first by gel permeation chromatography and then by anion-exchange chromatography, as described above. PTS enzyme I activity typically eluted from Sephadex G-100 near the void volume (Fig. 1). Two criteria were used to localize enzyme I activity: the ability to catalyze phosphoexchange between PEP and pyruvate (Fig. 1) and the ability to complement membranes containing enzyme II^{Glc} from *S. aureus* LJ195 (defective in enzyme I) and cytoplasm derived from the same strain in the PEP-dependent phosphorylation of glucose (Table 2). The assays indicated not only that enzyme I was present in column fractions (Fig. 1) but also that enzyme I from sucrose-grown *S. mutans* exhibited cross-complementarity with the glucose-specific *S. aureus* PTS (Table 2). Enzyme I eluted before the bulk of the other cytoplasmic proteins and well before HPr activity, the latter assayed by complementation of PEP-dependent sucrose phosphorylation by using the pooled enzyme I peak and *S. mutans* membranes (Fig. 1). The molecular weight exclusion limit of Sephadex G-100 is ca. 100,000 daltons. Thus, it is not

TABLE 2. *S. mutans* enzyme I complementation of PEP-dependent glucose phsophorylation in *S. aureus* LJ195 defective in enzyme I[a]

Sample	Glucose-phosphate formed (pmol/h per mg of protein)
S. aureus membranes + *S. aureus* cytoplasm ...	0
S. aureus membranes + *S. mutans* concentrated cytoplasm	344
S. aureus membranes + *S. aureus* cytoplasm + enzyme I pool from *S. mutans*.....	159

[a] Enzyme I complementation activities were measured as described in the text. Specific activities are relative to the amount of *S. mutans* protein present. A total of 570 μg of *S. aureus* membrane protein and 124 μg of *S. aureus* cytoplasmic protein were used in each assay containing these proteins.

surprising that HPr and enzyme I activities should elute distinctly one from the other, since in all bacteria possessing a PTS, the relative molecular weights of these proteins differ greatly. (*E. coli* HPr and enzyme I dimer are 9,017 and 134,000 daltons, respectively [2, 18].)

The HPr activity isolated by gel filtration was pooled, boiled for 5 min, and then tested in sucrose phosphorylation assays after lyophilization and resuspension (Table 3). Neither lyophilization nor heat treatment of HPr abolished its ability to complement enzyme I and membranes in PEP-dependent sucrose phosphorylation (Table 3). In fact, the reconstituted phosphorylation activity with boiled HPr was actually enhanced relative to the unheated sample. Presumably, this enhancement is attributable to heat inactivation of proteins or factors (or both) that either mask or inhibit the phosphorylation activity being monitored.

Also shown in Table 3 is evidence that enzyme I activity and HPr had indeed been eluted in distinct fractions in an uncomplexed form, since *S. mutans* membranes containing enzyme II, when reconstituted with enzyme I alone, or with HPr activity alone, had little activity in the PEP-dependent sucrose phosphorylation reaction. Only the combination of enzyme I, enzyme II, and HPr resulted in successful reconstitution of sucrose phosphorylation activity.

TABLE 3. PEP-dependent sucrose phosphorylation in cell-free fractions of *S. mutans*

Assay no.	Fractions	Sucrose-6-phosphate formed (pmol/h per mg of protein)[a]
1	Membranes + cytoplasm[b]	239
2	Membranes + HPr (G-100 pool)[c]	17.7
3	Membranes + enzyme I (G-100 pool)[d]	12.4
4	Membranes + enzyme I + cytoplasm,[b] boiled 10 min	303
5	Membranes + enzyme I + HPr[c]	824
6	Membranes + enzyme I + HPr,[c] boiled 5 min	985
7	Membranes + enzyme I + HPr,[c] boiled 10 min	780
8	Membranes + enzyme I + lyophilized HPr,[e] boiled 5 min	378

[a] Specific activities are relative to the amount of cytoplasmic protein (assays 1 and 4), HPr protein (assays 2 and 5 through 8), or enzyme I pool (assay 3) present. Assays were conducted as described in the text.

[b] A 140-μg amount of cytoplasmic protein was used, and 210 μg of membrane protein was used in this and all subsequent assays.

[c] A 20-μg amount of HPr pool protein was used.

[d] A 16-μg amount of enzyme I pool protein was used in this and all subsequent assays.

[e] Boiled HPr activity from the Sephadex G-100 column was lyophilized and resuspended in 1/10 the volume of dH_2O for assay.

Purification of complexed HPr. HPr activity isolated by gel filtration chromatography and identified as described above was pooled, boiled for 5 min, and subjected to anion-exchange chromatography on DEAE-cellulose. Virtually all of the activity eluted in the 0.10 M NaCl fractions (Table 4). Lane 1 of Fig. 2 is a sodium dodecyl sulfate-polyacrylamide gel showing protein contained in the second 0.10 M NaCl wash. It reveals the presence of only one band, a protein of ca. 17,000 daltons (inferred from the migration of protein standards). This gel was stained with Coomassie brilliant blue. An identical gel containing the same sample as in lane 1, in addition to purified *S. mutans* HPr (see below), purified HPr from *E. coli*, and molecular weight standards, was stained by the method of Merril et al. (16, 17), using an ultrasensitive silver stain capable of protein detection at concentrations of 0.10 ng/mm^2. A second band corresponding to 26,000 daltons is revealed by this staining procedure, in addition to the band at 17,000 daltons (lane 3, Fig. 2). This additional, 26,000-dalton protein apparently copurifies with the 17,000-dalton protein, becoming dissociated only after treatment with sodium dodecyl sulfate. Evidence presented below suggests that the protein with an apparent molecular weight of 17,000 is HPr, whereas the larger protein is an enzyme III-like factor that is also necessary for complementation of the *S. mutans* sucrose PTS. Both proteins migrate considerably more slowly than purified HPr from *E. coli* (lane 4, Fig. 2).

Detection and purification of *S. mutans* HPr based on its ability to complement an *S. aureus* HPr mutant. The *S. mutans* HPr activity purified above was identified by reconstitution of PEP-dependent sucrose phosphorylation, by using *S. mutans* membranes, *S. mutans* enzyme I, and

TABLE 4. DEAE-cellulose anion-exchange chromatography of HPr activity

NaCl concn (M)	Wash no.	Sucrose-6-phosphate formed (pmol/h per mg of protein)[a]
0.05	1	0.0
	2	37.1
	3	25.0
0.10	1	856
	2	2,520
	3	728
0.15	1	26.9
	2	11.4
	3	13.6
0.20	1	15.8
	2	0.0
	3	0.0

[a] HPr activities were measured as described in the text. Specific activities were relative to the amount of protein present in each wash. Each assay contained 210 μg of membrane protein and 16 μg of enzyme I pool protein, in addition to the DEAE column fractions containing various amounts of protein.

Sephadex G-100 column fractions. In additional experiments, HPr activity was more directly localized by complementation of glucose phosphorylation in *S. aureus* S797A, defective in HPr. Sephadex G-100 fractions of *S. mutans* cytoplasm were incubated with *S. aureus* cytoplasm (containing enzyme I, but not HPr) and *S. aureus* membranes in the reconstitution of PEP-dependent glucose activity. As shown in Fig. 3, Sephadex G-100-fractionated *S. mutans* cytoplasm contained HPr activity determined by the sucrose phosphorylation assay that eluted in one peak as before (cf. Fig. 1), whereas two HPr peaks were detected by the *S. aureus* mutant complementation assay. The first of these coeluted with that detected in the *S. mutans* sucrose phosphorylation assay, whereas the second eluted later and thus had a smaller apparent size (Fig. 3).

The HPr activities detected by complementation of *S. mutans* sucrose phosphorylation and by complementation of *S. aureus* glucose phosphorylation were pooled separately and boiled, and each was subjected to DEAE-cellulose anion-exchange chromatography as described earlier. The NaCl step fractions were again assayed for HPr activity, and complementation of sucrose phosphorylation activity eluted at a slightly lower salt concentration (0.10 M) than that for glucose phosphorylation (0.15 M). Sodium dodecyl sulfate-polyacrylamide gels containing these step gradient fractions and stained with Coomassie brilliant blue showed that the 17,000-dalton protein was again present in the HPr activity detected by sucrose phosphorylation and was also present in fractions containing HPr activity detected by the *S. aureus* glucose phosphorylation assay. As before, a silver-stained gel revealed the presence of an additional 26,000-dalton protein in the lane containing HPr activity detected by the *S. mutans* sucrose phosphorylation assay (not shown). However, in the lane of the silver-stained gel containing HPr activity detected by the *S. aureus* glucose phosphorylation assay, only the 17,000-dalton protein was present (Fig. 2, lane 2). Thus, the 17,000-dalton protein is HPr, whereas the 26,000-dalton protein is most likely an enzyme III^{Scr} (see below) with some properties (heat stability and stability to lyophilization) that resemble those of enzyme III^{Glc} in *E. coli* (20) and enzyme III^{Lac} in *S. aureus* (27).

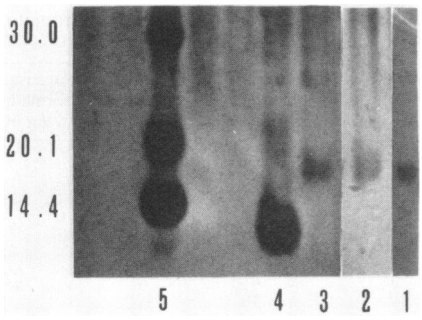

FIG. 2. Coomassie brilliant blue-stained and silver-stained sodium dodecyl sulfate gels of the activities shown in Table 3. Lane 1, Part of a gel stained with Coomassie brilliant blue containing protein eluted in the second wash of the 0.10 M NaCl step fractionation from DEAE-cellulose. Lanes 2 through 5 are silver-stained by the method of Merril et al. (16, 17). Lane 3, Same sample as in lane 1. Lane 2, Protein purified from the peak at fraction no. 14 of Fig. 3. Lane 4, *E. coli* HPr. Lane 5, The following protein standards from top to bottom with their subunit sizes indicated on the left of the gel in kilodaltons: carbonic anhydrase, soybean trypsin inhibitor, and alpha-lactalbumin. Only the lower halves of each lane are shown. No other bands were detectable in the high-molecular-weight region with either staining procedure.

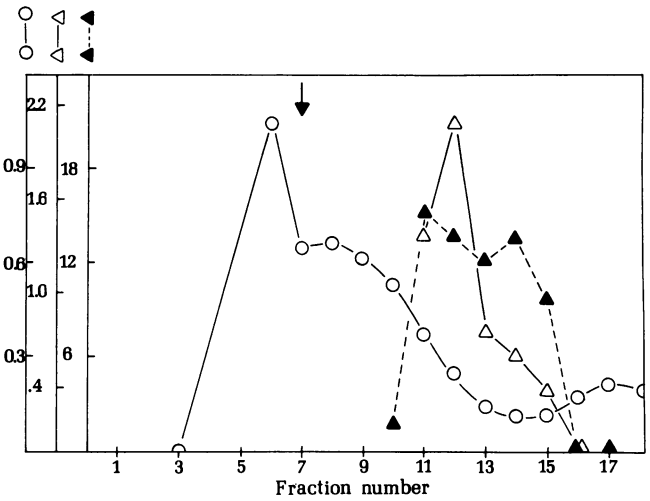

FIG. 3. Sephadex G-100 column fractionation of HPr detected by complementation of glucose phosphorylation in a *S. aureus* mutant lacking HPr. Dialyzed and concentrated *S. mutans* cytoplasm was fractionated on Sephadex G-100 and assayed for PTS enzyme I and HPr activities as detailed in the text and the legend to Fig. 1. Column fractions were assayed for the relative amounts of total protein by absorbance at 280 nm (○); for PEP-dependent phosphorylation of sucrose with *S. mutans* membranes (115 μg), 16 μg of enzyme I pool (see below), and 20 μl of the indicated column fractions (△); and for PEP-dependent phosphorylation of glucose with *S. aureus* membranes (100 μg) and cytoplasm (108 μg) plus 20 μl of the indicated column fractions (▲). Ordinate values for phosphorylation assays have the same significance as in Fig. 1. The enzyme I activity curve has not been included in this graph. Instead, the position where enzyme I peak activity was detected is indicated by the arrow.

PEP-dependent sucrose and glucose phosphorylation activities of the purified HPrs, with and without complexed, putative enzyme III, in homologous and in heterologous reconstitutions are shown in Table 5. It is apparent that both sucrose and glucose phosphorylation were achieved in reconstitutions of *S. mutans* enzyme I with *S. mutans* enzyme II and *S. mutans* HPr-putative enzyme III. However, only glucose phosphorylation was observed in reconstitutions of *S. mutans* enzyme I with *S. mutans* enzyme II and purified *S. mutans* HPr lacking the 26,000-dalton protein. These data indicate that the protein that copurifies with HPr from sucrose-grown *S. mutans* is necessary for PEP-dependent sucrose phosphorylation, but not for glucose PTS activity in the *S. mutans* system. The finding that glucose phosphorylation also was readily achieved in reconstitutions in which *S. aureus* cytoplasm (containing enzyme I but not HPr) and *S. aureus* membranes (containing enzyme II) were used with either *S. mutans* HPr-enzyme III or HPr alone implies that the putative enzyme III^{Scr} purified from *S. mutans* is not involved in glucose phosphorylation in *S. aureus*.

Amino acid composition of *S. mutans* HPr. Table 6 shows the amino acid composition of the uncomplexed, purified *S. mutans* HPr isolated in this study. Amino acid compositions of *S. aureus* HPr (3) and *E. coli* HPr (33) are also shown for comparative purposes. The composition of *S. mutans* HPr more closely resembles that of *S. aureus*, as expected, although there are some differences, especially in several hydrophobic amino acids (see below).

TABLE 5. PEP-dependent sucrose and glucose phosphorylation activities in homologous and heterologous systems[a]

Fractions	Glucose-6-phosphate formed (pmol/h per mg of protein)	Sucrose-6-phosphate formed (pmol/h per mg of protein)
S. mutans membranes + enzyme I pool + HPr-enzyme III[b]	312	788
S. mutans membranes + enzyme I pool + HPr[c]	199	0
S. aureus membranes + *S. aureus* cytoplasm + HPr-enzyme III[b]	5,622	ND[d]
S. aureus membranes + *S. aureus* cytoplasm + HPr[c]	3,199	ND

[a] Specific activities are based on the amount of protein in the HPr-enzyme III sample or in the HPr sample added. *S. aureus* membranes and cytoplasm were derived from strain S797A, lacking HPr. The following amounts of each protein were present in the appropriate 0.1-ml assay mixture: 415 μg of *S. aureus* membrane protein; 130 μg of *S. mutans* membrane protein; 16 μg of enzyme I pool protein; 162 μg of *S. aureus* cytoplasmic protein; 9 μg of HPr-enzyme III.

[b] This fraction contained HPr and a 26,000-dalton protein on silver-stained gels (Fig. 2, lane 3) and was purified from the peak at fraction no. 11 of Fig. 3.

[c] This fraction contained only HPr on silver-stained gels (Fig. 2, lane 2) and was purified from the peak at fraction no. 14 of Fig. 3.

[d] ND, Not determined.

DISCUSSION

We have shown in this report that lysis of *S. mutans* cells by vigorous homogenization with glass beads releases soluble components of the sucrose PTS of this organism, including enzyme I, HPr, and a presumptive HPr-enzyme III^{Scr} complex. These components have been separated by gel filtration, and the latter two have been purified to apparent homogeneity, at least as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Previous reports have suggested the association of complete PTSs for glucose (23; E. S. Liberman and A. S. Bleiweis, Abstr. Annu. Meet. Am. Soc. Microbiol., abstr. no. K163, 1983) and sucrose (G. R. Jacobson et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1981) with the membrane fraction of *S. mutans* cells broken by other procedures. The in vivo significance of these observations, however, remains to be determined.

The HPr of the PTS catalyzes phosphoexchange between PTS enzymes I and II, often via a fourth PTS protein, a sugar-specific enzyme III, in other bacteria that have been examined. Whereas the existence of a heat-stable PTS component in *S. mutans* has been reported by Maryanski and Wittenberger (14), neither HPr nor any enzyme III has previously been purified from *S. mutans*. The other sucrose PTS components from *S. mutans* (including the membrane-bound permease, enzyme II^{Scr}) have yet to be purified, and virtually all of the PTS components from this organism have yet to be characterized.

Our preliminary estimation of the subunit molecular weight of HPr from *S. mutans* based on sodium dodecyl sulfate-gel electrophoresis is 17,000. This is considerably larger than reported molecular weights of HPr from *E. coli* and *S. typhimurium* (9,017 [2]) and from *S. aureus* (7,685 [3]). Whether this represents a true size difference or whether *S. mutans* HPr runs abnormally slowly on sodium dodecyl sulfate gels remains to be determined. Interestingly, although the amino acid composition determined for *S. mutans* HPr closely resembles that for *S. aureus* in charged and polar amino acids (when mole percents are compared), *S. mutans* HPr appears to be somewhat enriched in nonpolar amino acids, especially alanine (Table 6). It is possible, therefore, that *S. mutans* HPr may have an extra domain of hydrophobic amino acids, which could contribute to both its larger apparent molecular weight and its tendency to remain bound to the membrane fraction under cell breakage conditions that usually result in the solubilization of HPrs from other organisms (21).

As also shown from the amino acid composition of the HPr isolated in this study, there is more homology between *S. mutans* and *S. aureus* HPrs than between *S. mutans* and *E. coli* HPrs (Table 6). This finding is supportive of results reported by Kalbitzer et al. (11) that HPr proteins from gram-positive bacteria are structurally similar to one another, whereas HPrs from gram-positive and gram-negative organisms differ substantially.

The protein that copurifies with part of the *S. mutans* HPr fraction has an apparent molecular weight of 26,000 and also exhibits heat stability. These properties and the fact that it is apparently tightly complexed to HPr throughout the purification procedure suggest that it is an enzyme III. Those PTSs that utilize enzymes III employ these proteins as the phosphoacceptor from HPr and as the phosphodonor to the transported sugar via the sugar-specific enzyme II. Further evidence that this protein may be a sucrose-specific enzyme III was obtained. The HPr-26,000-dalton protein complex appears to be at least partially specific for sucrose, since it is not required for glucose phosphorylation by the PTS but is necessary for PEP-dependent sucrose phosphorylation. Thus, in *S. mutans*, it is possible that phospho-HPr acts as the terminal phosphate donor in the glucose PTS and that phosphoenzyme III serves as the terminal phosphate donor to sucrose in the sucrose PTS. Although we have no indication that a cytoplasmic enzyme III^{Glc} exists in *S.*

TABLE 6. Amino acid compositions of HPr proteins from *S. aureus*, *S. mutans*, and *E. coli*

Amino acid	Mol% composition in:		
	S. aureus[a]	*S. mutans*[b]	*E. coli*[c]
Asp + Asn	10.0	12.8	4.0
Thr	7.1	7.1	11.3
Ser	10.0	6.2	7.9
Glu + Gln	12.8	13.7	15.2
Pro	1.4	≤1.0	2.5
Gly	8.6	11.8	7.6
Ala	5.7	21.3	10.6
Cys	0.0	≤1.0	0.0
Val	5.7	3.8	8.0
Met	5.7	≤1.0	2.4
Ile	10.0	5.2	3.8
Leu	5.7	5.7	9.6
Tyr	4.3	≤1.0	0.0
Phe	1.4	2.4	4.5
His	1.4	2.4	2.3
Lys	8.6	7.6	9.2
Arg	1.4	≤1.0	1.1
Trp	0.0	ND[d]	0.0

[a] From reference 3.

[b] Methionine and cysteine most likely were destroyed under the hydrolysis conditions used.

[c] From reference 33.

[d] ND, Not determined.

mutans, it is possible that such an enzyme III might be present tightly bound to the membrane, as appears to be the case with *S. typhimurium* (26).

In view of the fact that in *E. coli* the entire PTS complex appears to be functionally associated under some conditions with the membrane (8, 21), and this may also be the case in *S. mutans* (see above), the copurification of HPr with the putative enzyme III was, perhaps, not surprising. It is possible that these proteins are tenaciously associated with one another (since they catalyze successive steps in the phosphoexchange sequence) and are peripherally associated with the other PTS components of the membrane-bound complex. Homogenization with glass beads might then dissociate these proteins from the membrane-associated complex but not from one another. In any case, there is apparently a considerable excess of HPr over this protein in *S. mutans*, since only part of the *S. mutans* HPr activity appears in this complex in gel filtration experiments (Fig. 3).

Finally, it should be emphasized that as yet we have no direct biochemical evidence that the 26,000-dalton protein is a sucrose-specific enzyme III. Its properties, however, are very suggestive of this, as pointed out above. Purification and characterization of this protein in an uncomplexed form will be necessary to answer this question. Further experiments also will be necessary to determine whether this enzyme III-like protein participates in phosphorylation of any other sugars by the *S. mutans* PTS. In the future, purified *S. mutans* enzymes II for these sugars will not only provide definitive proof of sugar substrate specificities but will also afford kinetic studies, and a clear determination as to the involvement of enzymes III. Studies on the regulation of carbohydrate transport by the sucrose PTS will also be important toward an eventual understanding of the role of sucrose utilization in the pathogenicity of *S. mutans*.

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