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Streptococcal Phosphoenolpyruvate:Sugar Phosphotransferase System: Purification and Characterization of a Phosphoprotein Phosphatase Which Hydrolyzes the Phosphoryl Bond in Seryl-Phosphorylated Histidine-Containing Protein

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Histidine-containing protein (HPr) of gram-positive bacteria was found to be phosphorylated at a seryl residue (P-ser-HPr) in an ATP-dependent reaction catalyzed by a protein kinase (J. Deutscher and M. H. Saier, Jr., Proc. Natl. Acad. Sci. U.S.A. 80:6790–6794, 1983). Here we describe the purification and characterization of a soluble enzyme of *Streptococcus faecalis* which splits the phosphoryl bond in P-ser-HPr. The enzyme has a molecular weight of ca. 7.5×10^4, as determined by its migration behavior on a Sephacryl S-200 column. On native polyacrylamide gels the purified enzyme produced only one protein band. On sodium dodecyl sulfate-polyacrylamide gels we found one major protein band of molecular weight 2.9×10^4 and two minor protein bands of molecular weights 2.3×10^4 and 7×10^4. Fructose 1,6-diphosphate, which stimulated the ATP-dependent, protein kinase-catalyzed phosphorylation of HPr, had no effect on the phosphatase activity. Other glycolytic intermediates also had no effect. However, inorganic phosphate, which inhibited the ATP-dependent HPr kinase, stimulated the P-ser-HPr phosphatase. EDTA at a concentration of 0.1 mM completely inhibited the phosphatase. Divalent cations like Mg^{2+}, Mn^{2+}, and Co^{2+} overcame the inhibition by EDTA. Fe^{2+}, Zn^{2+}, and Cu^{2+} had no effect, whereas Ca^{2+} slightly inhibited the phosphatase. ATP was also found to inhibit the phosphatase. Under conditions in which ATP severely inhibited the phosphatase, ADP was found to have no effect on the enzyme activity. Besides P-ser-HPr of *S. faecalis*, the phosphatase was also able to hydrolyze the phosphoryl bond in P-ser-HPr of *Streptococcus lactis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, and *Lactobacillus casei*. Phosphoenolpyruvate-dependent *o*-nitrophenyl-β-D-galactopyranoside phosphorylation, catalyzed by the *S. aureus* phosphoenolpyruvate:lactose phosphotransferase system, was about 150-fold decreased in the presence of P-ser-HPr of *S. aureus*, as compared with HPr. However, when P-ser-HPr was first incubated with P-ser-HPr phosphatase to allow complete hydrolysis of the phosphoryl bond, it had the same activity as HPr. Besides this cytoplasmic phosphoprotein phosphatase, we detected a membrane-bound phosphatase which also hydrolyzed the phosphoryl bond in P-ser-HPr.

Most anaerobic and facultatively anaerobic bacteria possess a phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) as the main carbohydrate transport system (13, 34). The PTS is usually composed of three soluble proteins, enzyme I, HPr, and factor III (III), and membrane-bound enzyme II (33). Enzyme I and histidine-containing protein (HPr) are the two nonspecific proteins of the PTS, whereas III and enzyme II are specific for a certain sugar. The four proteins of the PTS form a phosphorylation chain. Enzyme I is phosphorylated by PEP at the N-3 position of a single histidyl residue (1a, 42). Phosphorylated enzyme I transfers the phosphoryl group to the N-1 position of His-15 in HPr (3, 12), which then phosphorylates III proteins at the N-3 position of a single histidyl residue (5, 9, 19). Recently, a phosphorylated enzyme II intermediate was documented (29, 41). In the last reaction step, phosphorylated enzyme II catalyzes the transport and concomitant phosphorylation of the carbohydrate. Besides being phosphorylated at His-15 by PEP and enzyme I in the PTS phosphorylation chain, HPr was also found to be phosphorylated at a seryl residue (P-ser-HPr) in an ATP-dependent, protein kinase-catalyzed reaction (8). The ATP-dependent HPr phosphorylation is stimulated by glycolytic intermediates, mainly fructose 1,6-diphosphate, and is severely inhibited by P_i (6, 30). In contrast to the histidyl-bound phosphoryl group, the seryl-bound phosphoryl group cannot be used for sugar phosphorylation (8). The formation of P-ser-HPr was therefore believed to be a regulatory response of carbohydrate uptake. It was found that P-ser-HPr of *Streptococcus lactis* was about 5,000 times more slowly phosphorylated by PEP and enzyme I than HPr was (7). III proteins stimulated the PEP-dependent phosphorylation of P-ser-HPr. In the presence of *Streptococcus faecalis* III specific for gluconate (III^{gct}), phosphorylation of P-ser-HPr was as fast as phosphorylation of HPr, whereas in the presence of *Staphylococcus aureus* III specific for lactose (III^{lac}), phosphorylation of P-ser-HPr was still about 80 times slower than that of HPr (7). Based on these data an inducer exclusion mechanism was proposed. The formation of P-ser-HPr was believed to narrow the broad specificity of HPr to a restricted specificity for only a few III proteins. In this paper we describe the purification and preliminary characterization of P-ser-HPr phosphatase,

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an enzyme which is important for controlling the intracellular amount of P-ser-HPr.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. faecalis* 26487 (Streptococcen Zentrale, Kiel, Federal Republic of Germany) *S. lactis* 11454 (Streptococcen Zentrale), and *Bacillus subtilis* W1360 (a gift from U. Winkler, Bochum, Federal Republic of Germany) were grown in 100-liter batches at 37°C in a Chemap fermentor. The medium (100 liters) was composed of 1,000 g of yeast extract (Ohly, Hamburg, Federal Republic of Germany), 200 g of tryptone (Difco Laboratories, Detroit, Mich.), 250 g of Na_2HPO_4, and 1,000 g of glucose. During growth, the pH was kept at 7 by the addition of 10% KOH. After 5 to 6 h, the absorbance at 580 nm reached 10 to 11, and the culture was harvested with a Westfalia continuous-flow centrifuge. The yield was about 1 kg of wet cell paste.

S. aureus S305A and S797A were grown under the same conditions but without the addition of glucose to the growth medium. In both strains the proteins III^{lac}, enzyme II specific for lactose (enzyme II^{lac}), and 6-phospho-β-galactosidase are expressed constitutively. S797A carries, in addition, a mutation in the *ptsH* gene (14).

Lactobacillus casei 64H (National Institute of Dental Research, Bethesda, Md.) was grown in 100-liter batches at 37°C in a Chemap fermentor. The medium (100 liters) contained 1,000 g of yeast extract (Ohly), 500 g of casein hydrolysate (E. Merck AG, Darmstadt, Federal Republic of Germany), 500 g of tryptone (Difco), 200 g of sodium citrate, 100 g of ammonium acetate, 100 g of sodium acetate, 20 g of cysteine, 300 g of K_2HPO_4, 300 g of KH_2PO_4, 57.5 g of $MgSO_4$, 3.5 g of $FeSO_4$, 12 g of $MnSO_4$, and 200 g of lactose. A 5-liter overnight culture was used to seed a 100-liter batch, and after 6 to 7 h of growth, we obtained ca. 1 kg of wet cell paste.

Streptococcus pyogenes type 12 was grown as previously described (32).

Protein purification. P-ser-HPr phosphatase was purified from *S. faecalis* 26487. Wet cell paste (300 g) was suspended in 500 ml of standard buffer (50 mM Tris-chloride [pH 7.5], 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium azide). Cells were broken in a Dynomill (Bachofen, Basel, Switzerland) with glass beads (diameter, 0.5 mm). After centrifugation at 25,000 × *g* for 30 min, the crude extract was loaded on a DEAE-cellulose column (Whatman DE-23, 9 by 20 cm) equilibrated with standard buffer. Membrane fragments were washed from the column with 1 liter of standard buffer before a 7-liter linear gradient of 0 to 1 M NaCl in standard buffer was applied. P-ser-HPr phosphatase eluted from the column at about 0.45 M NaCl between HPr and enzyme I, which eluted at 0.3 and 0.6 M NaCl, respectively. The pool containing P-ser-HPr phosphatase was subjected to ammonium sulfate precipitation (50% saturation). After centrifugation at 25,000 × *g* for 30 min, the pellet, which contained P-ser-HPr phosphatase, was dissolved in 30 ml of standard buffer. It was applied to a Sephacryl S-200 column (5 by 100 cm; Pharmacia, Uppsala, Sweden) equilibrated with standard buffer (pH 6.5). P-ser-HPr phosphatase eluted from the column at a molecular weight of ca. 7.5×10^4. Fractions containing P-ser-HPr phosphatase were pooled and loaded on a second DEAE-cellulose column (Whatman DE-52, 2.5 by 12 cm) equilibrated with standard buffer (pH 6.5). Proteins were eluted with a 400-ml linear gradient of 0 to 0.35 M NaCl. P-ser-HPr phosphatase eluted at about 0.17 M NaCl and was dialyzed against 5 mM potassium phosphate buffer (pH 6.5). It was then loaded on a hydroxyapatite column (HA ultrogel, 2.5 by 17 cm; LKB Instruments, Inc., Rockville, Md.) equilibrated with 5 mM potassium phosphate buffer (pH 6.5). Proteins were eluted with a 500-ml linear gradient of 5 to 200 mM potassium phosphate (pH 6.5). P-ser-HPr phosphatase eluted from the column at about 70 mM potassium phosphate in a broad peak. The pool containing P-ser-HPr phosphatase was concentrated to 5 ml by pressure dialysis with an Amicon YM-2 membrane and was further purified by high-pressure liquid chromatography. Aliquots (1 ml) were loaded on a DEAE column (TSK IEX545, 150 by 6 mm; Bio-Rad Laboratories, Richmond, Calif.). Proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM potassium phosphate buffer (pH 6.5) in 45 min at a flow rate of 0.8 ml/min at 30°C. P-ser-HPr phosphatase eluted at 0.35 M NaCl.

Enzyme I was purified from *S. faecalis* and *S. aureus* (1a).

HPr proteins of the different microorganisms were purified as previously described (20). P-ser-HPr was obtained with partially purified ATP-dependent HPr kinase. Different HPr proteins (10 mg) were incubated at 37°C with partially purified HPr kinase (150 μl of the concentrate after the DE-52 run [6]), 20 mM $MgCl_2$, 5 mM fructose 1,6-diphosphate, and 20 mM ATP in the presence of 25 mM Tris-chloride buffer (pH 7.5). After 1 h of incubation, the assay mixture (total volume, 2 ml) was loaded on a DEAE-cellulose column (Whatman DE-52, 2.5 by 10 cm). HPr and P-ser-HPr were separated by the application of a 500-ml linear gradient of 0 to 0.2 M NaCl in 50 mM Tris-chloride. Both HPr- and P-ser-HPr-containing fractions were pooled, desalted on a Sephadex G-25 column (3 by 25 cm), and lyophilized. This procedure yielded 6 to 7 mg of P-ser-HPr and 2 to 3 mg of HPr.

For the preparation of [^{32}P]P-ser-HPr of *S. faecalis*, 1 mg of HPr was incubated at 37°C with partially purified HPr kinase (15 μl of the concentrate after the DE-52 run [6]), 20 mM $MgCl_2$, 5 mM fructose 1,6-diphosphate, 25 mM Tris-chloride, and [γ-^{32}P]ATP (10 μCi, 3,000 Ci/mmol). After 30 min of incubation, unlabeled ATP was added to a final concentration of 0.5 mM. After a further 30 min of incubation at 37°C, the assay mixture (total volume, 200 μl) was separated by high-pressure liquid chromatography on a DEAE column (TSK IEX545, 150 by 6 mm; Bio-Rad) by the application of a linear gradient of 0 to 0.4 M NaCl in 10 mM Tris-chloride (pH 6.8) for 50 min. The temperature was 30°C, and the flow rate was 0.8 ml/min. [^{32}P]P-ser-HPr overlapped with ATP and was desalted on a Sephadex G-25 column (0.8 by 10 cm) and lyophilized in a vacuum centrifuge (Savant Instruments, Inc., Hicksville, N.Y.).

III^{lac} of *S. aureus* and 6-phospho-β-galactosidase were purified from strain S305A (37). Enzyme II^{lac} of *S. aureus* was obtained from membranes of strain S305A. The enzyme was purified by NaOH extraction as previously described (36). Treatment of the membrane fragments with NaOH at pH 12.4 destroyed the membrane-associated phosphoprotein phosphatase activity.

Assay for P-ser-HPr phosphatase. P-ser-HPr (20 μg) was incubated at 37°C with 20 mM $MgCl_2$, 50 mM P_i, 100 mM Tris-chloride buffer (pH 7.5), and the enzyme sample. The incubation time was 10 min to 1 h, depending on the amount of P-ser-HPr phosphatase added. P-ser-HPr and HPr were separated by electrophoresis on 7.5% polyacrylamide gels. The amount of P-ser-HPr converted to HPr could be detected after staining and destaining the gels. For a more quantitative assay we incubated [^{32}P]P-ser-HPr with the

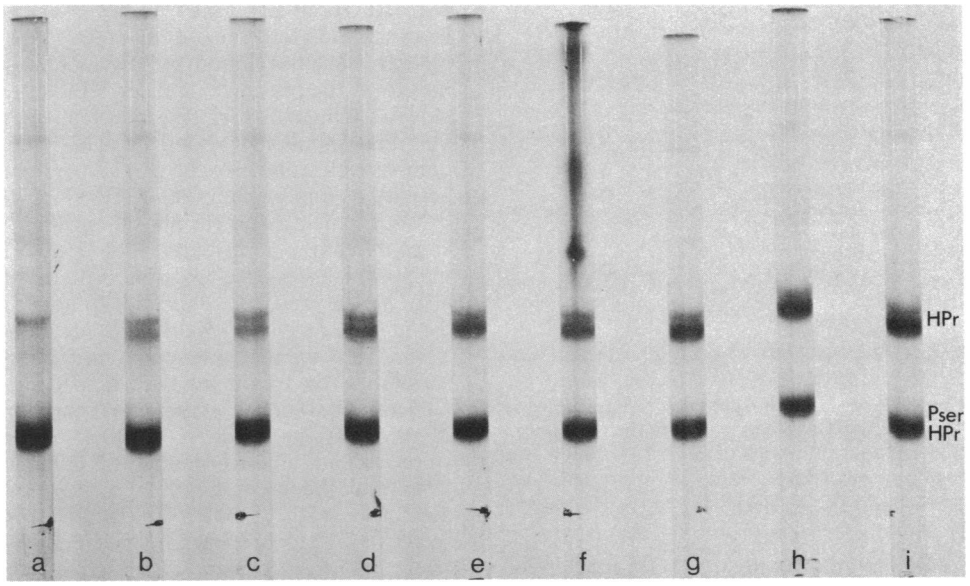

FIG. 1. Requirement of Mg^{2+} for P-ser-HPr phosphatase activity. The assay of P-ser-HPr phosphatase activity was carried out in the presence of 0.1 mM EDTA and different concentrations of Mg^{2+} (0, 0.2, 0.4, 0.8, 1.5, 3, 5, 10, and 20 mM; lanes a through i, respectively). The assay mixture did not contain P_i. P-ser-HPr phosphatase from the DE-52 run was used for the assay.

phosphatase as described above. After incubation, P_i and [^{32}P]P-ser-HPr were separated by gel filtration on a Sephadex G-25 column (0.8 by 10 cm). Radioactivity was determined by liquid scintillation counting.

PEP-dependent ONPG phosphorylation. PEP-dependent *o*-nitrophenyl-β-D-galactopyranoside (ONPG) phosphorylation was carried out in the presence of either HPr or P-ser-HPr of *S. aureus* with purified or partially purified components of the lactose-specific PTS of *S. aureus*. The ONPG 6-phosphate formed was hydrolyzed by 6-phospho-β-galactosidase, and *o*-nitrophenol was measured at 405 nm. The assay mixture contained 50 μg of enzyme I, 100 μg of III^{lac}, 20 μg of 6-phospho-β-galactosidase, 5 mg of enzyme II^{lac} (wet membrane fragments after extraction with NaOH), 10 mM $MgCl_2$, 5 mM ONPG, 5 mM PEP, and 20 mM Tris-chloride in a total volume of 300 μl. To this mixture HPr (1.25 to 100 μg), P-ser-HPr (5 to 400 μg), or P-ser-HPr previously incubated for 15 min at 37°C with P-ser-HPr phosphatase (1.25 to 100 μg) was added. The reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of 1 ml of 0.5 M Na_2CO_3. The *o*-nitrophenol formed was measured at 405 nm.

Polyacrylamide gels. Native polyacrylamide gels contained 0.4 M Tris-glycine (pH 9.3) and 7.5% acrylamide containing 2.5% methylenebisacrylamide. Sodium dodecyl sulfate-polyacrylamide gels contained 15% acrylamide and were prepared as previously described (22).

RESULTS

Physical properties of P-ser-HPr phosphatase. P-ser-HPr phosphatase was purified from *S. faecalis* 26487 as described above. After the last purification step, P-ser-HPr phosphatase produced only one band on native polyacrylamide gels. However, on sodium dodecyl sulfate-polyacrylamide gels we obtained one major protein band corresponding to a molecular weight of 2.9×10^4 and two minor protein bands of molecular weights 2.3×10^4 and 7×10^4.

After the last purification step, the enzyme was stored in a refrigerator at 4°C. No loss of activity was observed over 3 months. It seemed that the enzyme was more stable at a slightly acidic pH than at an alkaline pH. However, when the enzyme was precipitated at pH 5.1 to 4.9, it lost its activity irreversibly. We therefore did not include acid precipitation in the purification procedure previously described (7). The pH optimum of the phosphatase reaction was found to be in the range of 7.2 to 7.5.

Regulation of P-ser-HPr phosphatase activity in vitro. The activity of P-ser-HPr phosphatase was dependent on the presence of divalent cations. EDTA (0.1 mM) completely inhibited the phosphatase. An excess of Mg^{2+}, Mn^{2+}, or Co^{2+} restored the phosphatase activity. At a concentration of 5 mM Mg^{2+} the enzyme was fully active (Fig. 1). Cu^{2+}, Zn^{2+}, and Fe^{2+} (all at 1 mM) were found to have no effect on the phosphatase activity. Ca^{2+} (1 mM) slightly inhibited P-ser-HPr phosphatase in the absence of EDTA and P_i. Glycolytic intermediates such as glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, 2-phosphoglycerate, 3-phosphoglycerate, and PEP were found to have no effect on P-ser-HPr phosphatase activity at concentrations of 2.5 and 5 mM. As fructose 1,6-diphosphate was found to stimulate ATP-dependent HPr kinase at concentrations of up to 20 mM (6), the influence of fructose 1,6-diphosphate on P-ser-HPr phosphatase was tested at concentrations of up to 40 mM. However, no effect was observed. On the other hand, P_i, which was found to inhibit ATP-dependent HPr kinase, stimulated P-ser-HPr phosphatase at concentrations higher than 5 mM in the absence as well as in the presence of 5 mM $MgCl_2$ (Fig. 2). Maximal stimulation was reached at about 50 mM P_i. ATP was found to inhibit P-ser-HPr phosphatase. However, inhibition by ATP was dependent on the amount of Mg^{2+} present in the assay mixture. At 3 mM Mg^{2+} inhibition of the phosphatase was observed at an ATP concentration of 3.5 mM. When the concentration of Mg^{2+} was raised to 10 mM, 14 mM ATP was required to inhibit the phosphatase (Fig. 3). Under conditions in which ATP severely inhibited the enzyme, ADP was found to have no effect on the activity of P-ser-HPr phosphatase. Only at a

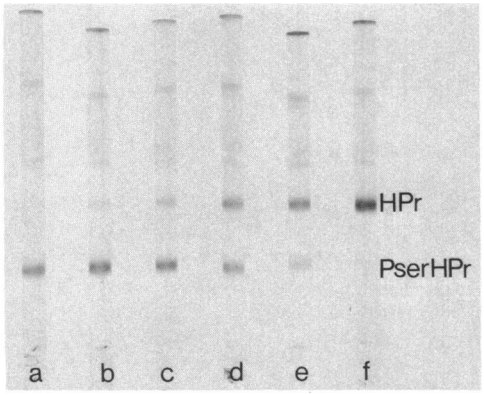

FIG. 2. Stimulation of P-ser-HPr phosphatase by P_i. The assay of P-ser-HPr phosphatase activity was carried out in the presence of 4 mM EDTA and different concentrations of P_i (1, 2, 5, 10, 20, and 50 mM; lanes a through f, respectively). No Mg^{2+} was present in the reaction mixture.

20-fold excess of ADP (30 mM) over Mg^{2+} (1.5 mM) could inhibition of P-ser-HPr phosphatase be observed.

Substrate specificity of P-ser-HPr phosphatase. P-ser-HPr was prepared from HPr of *S. faecalis*, *S. lactis*, *S. pyogenes*, *S. aureus*, *B. subtilis*, and *L. casei* with ATP-dependent HPr kinase isolated from *S. faecalis* (see above). All the different kinds of P-ser-HPr were found to be substrates of P-ser-HPr phosphatase isolated from *S. faecalis*. The rate of hydrolysis of the phosphoryl bond was found to be similar for P-ser-HPr from all the different organisms, with the exception of P-ser-HPr from *S. faecalis*. In mixtures of 10 μg of P-ser-HPr from *S. faecalis* and 10 μg of P-ser-HPr from other microorganisms, P-ser-HPr from *S. faecalis* was always dephosphorylated about two times faster. Other kinds of P-ser-proteins or seryl-phosphorylated proteins have so far not been tested. P-ser-HPr from gram-negative bacteria is not available, as the formation of P-ser-HPr in these organisms has not yet been proven. ONPG 6-phosphate was not hydrolyzed by P-ser-HPr phosphatase. The doubly phosphorylated HPr (P-ser,P-his)-HPr was also a substrate of P-ser-HPr phosphatase. [^{32}P]P-ser-HPr (10 μg) was first phosphorylated by PEP (5 mM) and enzme I (2 μg) for 10 min at 37°C before it was incubated with P-ser-HPr phosphatase. [^{32}P]P-ser-HPr and radioactive P_i were separated on a Sephadex G-25 column. In (P-ser,P-his)-HPr the phosphoryl group bound to the seryl residue was hydrolyzed about twice as slowly as that in P-ser-HPr. In a single experiment we found the following distribution of radioactivity: (i) for hydrolysis of [^{32}P]P-ser-HPr, 642 cpm in the P-ser-HPr peak and 853 cpm in the P_i peak; (ii) for hydrolysis of ([^{32}P]P-ser,P-his)-HPr, 1,244 cpm in the protein peak and 319 cpm in the P_i peak.

PEP-dependent ONPG phosphorylation in the presence of HPr or P-ser-HPr. ONPG is a substrate of the lactose-specific PTS of *S. aureus*, which catalyzes the PEP-dependent phosphorylation of ONPG (14). ONPG 6-phosphate is hydrolyzed by 6-phospho-β-galactosidase, and the amount of *o*-nitrophenol formed can be measured at 405 nm (14). Besides PEP, ONPG, and Mg^{2+}, the assay mixture contained the following purified or partially purified proteins: enzyme I; different amounts of HPr, P-ser-HPr, or P-ser-HPr after treatment with P-ser-HPr phosphatase; III^{lac}; enzyme II^{lac} (membrane fragments after NaOH extraction); and 6-phospho-β-galactosidase. All proteins were purifed from *S. aureus* S305A. Extraction of the membrane with NaOH at pH 12.4 was necessary to destroy a membrane-associated phosphoprotein phosphatase activity. ONPG phosphorylation by the lactose-specific PTS of *S. aureus* in the presence of P-ser-HPr was about 150 times as slow as that with HPr (Fig. 4). However, when P-ser-HPr was first incubated with P-ser-HPr phosphatase to allow complete hydrolysis of the phosphoryl bond, it had the same activity as unphosphorylated HPr (Fig. 4).

Membrane-associated phosphoprotein phosphatase. Besides the above-described soluble P-ser-HPr phosphatase, a membrane-associated phosphoprotein phosphatase which also hydrolyzed the phosphoryl bond in P-ser-HPr was found in *S. aureus*, *S. faecalis*, *S. lactis*, and *S. pyogenes*. In *S. faecalis* the enzyme could not be removed from the membrane by treatment with high concentrations of salts (200 mM NaCl or potassium phosphate) or by treatment with 20 mM EDTA. Triton X-100 (0.05%) and Brij 58 (0.05%) were also not able to remove the phosphoprotein phosphatase from the membrane. In *S. aureus* extraction of the membrane with NaOH (pH 12.4) destroyed the enzymatic activity of the phosphatase. No activity could be detected after NaOH extraction and high-speed centrifugation in either the supernatant or the pellet. No further attempts have been made to characterize the membrane-associated phosphatase.

DISCUSSION

ATP-dependent protein phosphorylation has been shown to be involved in the regulation of almost all kinds of cellular processes in higher organisms (21, 35). The importance of the phosphoprotein phosphatases in the regulation of different cellular processes by counteracting ATP-dependent protein phosphorylation has been recognized, and these enzymes have been studied intensively in higher organisms (15, 16, 18, 28). Two classes of phosphoprotein phosphatases, including four different enzymes which seem to be responsible for most of the cellular phosphoprotein phosphatase activity in animal cells, have been established (1, 17, 40). However, ATP-dependent protein phosphorylation was established in bacteria only recently (4, 10, 11, 26). The first ATP-dependent protein kinase isolated from bacteria phosphorylated a single seryl residue in isocitrate dehydrogenase. The protein kinase was composed of two subunits and was found to be bifunctional; it also carried the opposing phosphatase activity (23, 25). Besides divalent metal ions,

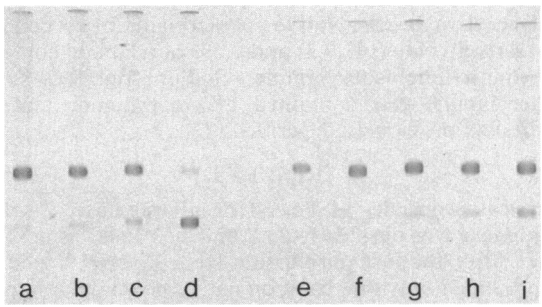

FIG. 3. Inhibition of P-ser-HPr phosphatase by ATP. The assay mixture contained 30 mM P_i and 3 mM $MgCl_2$ (lanes a through d) or 10 mM $MgCl_2$ (lanes e through i). ATP was present at different concentrations (1, 2, 3.5, and 7 mM [lanes a through d, respectively] and 1, 2, 3.5, 7, and 14 mM [lanes e through i, respectively]). Upper band, HPr; lower band, P-ser-HPr.

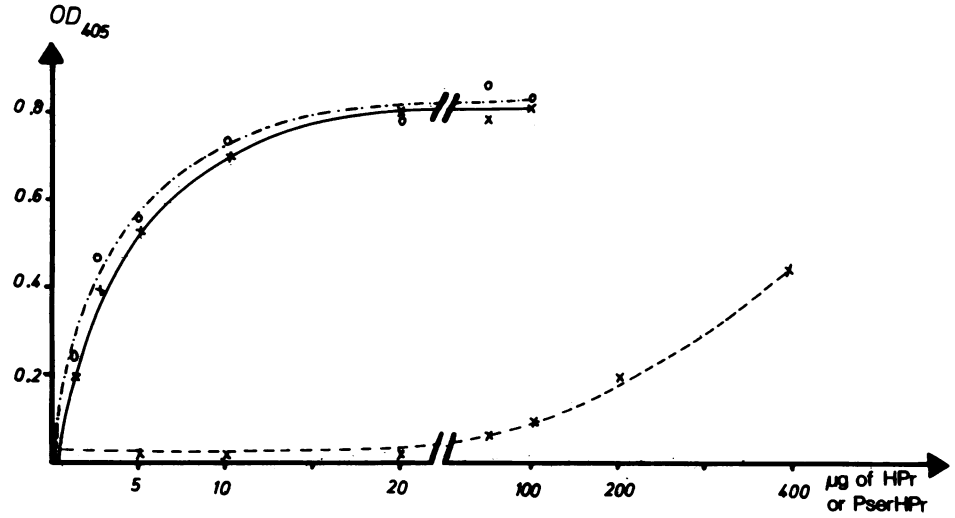

FIG. 4. PEP-dependent ONPG phosphorylation catalyzed by the lactose-specific PTS of *S. aureus* in the presence of HPr (——), P-ser-HPr (-----), or P-ser-HPr after incubation with P-ser-HPr phosphatase (-·-·-·). The incubation of P-ser-HPr with P-ser-HPr phosphatase was carried out at 37°C for 15 min. The assay mixtures contained different amounts of HPr and P-ser-HPr after treatment with P-ser-HPr phosphatase (1.25, 2.5, 5, 10, 20, 50, and 100 μg) and of P-ser-HPr (5, 10, 20, 50, 100, 200, and 400 μg). OD_{405}, Optical density at 405 nm.

isocitrate dehydrogenase phosphatase requires either ADP or ATP for full activity. The P-ser-HPr phosphatase described here is the second phosphoprotein phosphatase isolated from bacteria. It hydrolyzes the phosphoryl bond in P-ser-HPr. HPr is a phosphate carrier protein of the bacterial PTS. During the PTS-mediated sugar uptake reaction, HPr becomes phosphorylated at His-15 in a PEP-dependent reaction catalyzed by enzyme I (3, 12). A second ATP-dependent, protein kinase-catalyzed phosphorylation of a seryl residue in HPr has recently been documented (8). HPr kinase, an enzyme of molecular weight 6.5×10^4, was stimulated by fructose 1,6-diphosphate and strongly inhibited by P_i (6). P-ser-HPr phosphatase was stimulated by P_i and inhibited by ATP. The in vivo formation of P-ser-HPr was found to be dependent on the uptake of a metabolizable carbohydrate and on functioning glycolyis (31). Starved streptococcal cells were shown to contain low concentrations of fructose 1,6-diphosphate (2 mM) and ATP (0.2 mM) and high concentrations of P_i (50 mM) (24, 39). Under these conditions, HPr kinase is inactive and P-ser-HPr phosphatase is fully active. If glucose is added to starved streptococcal cells, the P_i concentration drops to 4 mM within seconds, and the concentrations of fructose 1,6-diphosphate and ATP are elevated to 20 and 2 mM, respectively (24, 39). Under these conditions, HPr kinase is fully stimulated and P-ser-HPr phosphatase is inactive. ATP may regulate the phosphatase activity by lowering the concentration of free Mg^2, which is essential for phosphatase activity. ADP, which forms an Mg^2 complex with a much lower stability constant than that of the Mg^2-ATP complex (27), was found to inhibit the phosphatase only at a concentration 20 times higher than that of ATP. However, the direct interaction of ATP with the phosphatase cannot be excluded.

The discovery of P-ser-HPr was connected to a regulatory phenomenon in bacteria termed inducer expulsion (32, 38). However, so far no involvement of P-ser-HPr in inducer expulsion has been demonstrated. Instead, P-ser-HPr was found to be a poor substrate for PEP-dependent phosphorylation catalyzed by enzyme I of the PTS. Surprisingly, *S. faecalis* III specific for gluconate was able to completely relieve the slower phosphorylation of P-ser-HPr than of HPr, whereas with III^{lac} of *S. aureus*, P-ser-HPr phosphorylation was still about 100 times slower than that of HPr (7). An inducer exclusion mechanism was proposed based on these results, suggesting that after P-ser-HPr formation, some PTS sugars are taken up in preference to others. However, as HPr and P-ser-HPr of *S. lactis* were used in these experiments, the results may simply reflect the fact that PTS proteins from different bacteria interact with each other at different rates. In this paper we report that PEP-dependent phosphorylation of ONPG catalyzed by the lactose-specific PTS is about 150 times slower in the presence of P-ser-HPr than in the presence of HPr. In these experiments we only used proteins isolated from *S. aureus* S305A. When P-ser-HPr was first incubated with P-ser-HPr phosphatase, it had the same activity as HPr. Bacterial cells may therefore use the formation of P-ser-HPr to control the PTS-mediated uptake of carbohydrates. If glycolytic intermediates and ATP are accumulated, P-ser-HPr will be formed, lowering the rate of uptake of PTS substrates. In preliminary experiments aimed at determining the amount of P-ser-HPr formed during growth on different PTS substrates, we found that during growth of *S. faecalis* cells on mannitol, most of the HPr was converted to P-ser-HPr, whereas during growth on glucose or gluconate, only a small amount of P-ser-HPr was formed (unpublished results). Mannitol is metabolized via the Embden-Meyerhoff pathway, whereas gluconate and possibly also glucose are metabolized mainly via the pentose phosphate pathway (2). Gluconate 6-phosphate, formed during the PTS-mediated uptake of gluconate, was reported to stimulate the ATP-dependent HPr kinase (30). However, its effect was much less pronounced than the effect of fructose 1,6-diphosphate. The formation of P-ser-HPr may therefore be mediated by carbohydrates, the metabolism of which leads to high concentrations of fructose 1,6-diphosphate. If glycolytic intermediates are not used up via energy-consuming reactions and biosynthetic pathways, the buildup of mainly fructose 1,6-diphosphate may lower the PTS sugar uptake rate by stimulating P-ser-HPr formation. Thus, bacterial cells could control their different needs for energy and for biosynthetic building blocks during cell growth and cell division by this mechanism.

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LITERATURE CITED

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