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Properties of ATP-Dependent Protein Kinase from *Streptococcus pyogenes* That Phosphorylates a Seryl Residue in HPr, a Phosphocarrier Protein of the Phosphotransferase System

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Transport of sugars across the cytoplasmic membranes of gram-positive bacteria appears to be regulated by the action of a metabolite-activated, ATP-dependent protein kinase that phosphorylates a seryl residue in the phosphocarrier protein of the phosphotransferase system, HPr. We have developed a quantitative assay for measuring the activity of this enzyme from *Streptococcus pyogenes*. The product of the in vitro protein kinase-catalyzed reaction was shown to be phosphoseryl-HPr by several independent criteria (rates of hydrolysis in the presence of various agents, detection of serine-phosphate in acid hydrolysates, immunological assay, and electrophoretic migration rates). HPrs isolated from four different gram-positive bacteria (*S. pyogenes*, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Bacillus subtilis*) were shown to be phosphorylated by the kinase from *S. pyogenes*. In contrast, *Escherichia coli* HPr was not a substrate of this enzyme. The soluble kinase released from the particulate fraction of the cells with high salt in the presence of a protease inhibitor was shown to have an approximate molecular weight of 60,000 as estimated by gel filtration. Its activity was dependent on divalent cations, with Mg^{2+} and Mn^{2+} being most active. EDTA, P_i, and high concentrations of salt were strongly inhibitory. The enzyme was optimally active at pH 7.0, exhibited high affinity for its substrates, and was dependent on the presence of one of several metabolites. Of these compounds, fructose 1-6-diphosphate was most active, with gluconate 6-phosphate, 2-phosphoglycerate, 2,3-diphosphoglycerate, phosphoenolpyruvate, and pyruvate exhibiting moderate to low stimulatory activities. Other compounds tested, including a variety of sugar phosphates, pyridine nucleotides, and other metabolites, were without effect. The ATP-dependent phosphorylation of HPr on the seryl residue was strongly inhibited by phosphoenolpyruvate-dependent phosphorylation of the active histidyl residue of this protein. Treatment of the kinase with diethyl pyrocarbonate strongly inhibited the ATP-dependent phosphorylation activity, although the sulfhydryl reagents *N*-ethylmaleimide, *p*-chloromercuribenzoate, and iodoacetate were without effect. These results serve to characterize the HPr (serine) kinase, which apparently regulates the rates of carbohydrate transport in streptococcal cells via the phosphotransferase system. A primary role of this kinase in the control of cellular inducer levels and carbohydrate metabolic rates is proposed.

Carbohydrate transport in bacteria is regulated by a multiplicity of mechanisms. At least five distinct mechanisms regulating the uptake of sugars have been demonstrated (12, 20a, 25, 26), whereas the regulatory expulsion of sugars from the cell occurs in some bacterial species (5, 20, 21, 23, 33, 35). The term for the latter phenomenon, inducer expulsion, was coined (21) to emphasize the potential significance of this phenomenon to the transcriptional regulation of carbohydrate catabolic enzyme systems. Subsequent studies led to the important conclusion that ATP and glycolytic intermediates are required for the expulsion process (20, 21, 35). This suggestion led first to the identification of a low-molecular-weight phosphoprotein in intact streptococcal cells (20) and subsequently to the detection of a protein kinase and protein-phosphate phosphatase that apparently catalyze the reversible phosphorylation of this small protein (11). The protein substrate of the kinase was identified as HPr, the phosphocarrier protein of the bacterial phosphotransferase system (PTS), and the amino acid residue phosphorylated in vivo was shown to be a seryl residue (11). Thus, phosphorylation of HPr in streptococci is catalyzed by two distinct protein kinases, a phosphoenolpyruvate (PEP)-dependent kinase (enzyme I) and an ATP-dependent kinase that phosphorylates histidyl and seryl residues in the protein, respectively.

The primary function of histidyl phosphorylation in HPr is to drive the concomitant uptake and phosphorylation of exogenous sugars into the cells (19). The ATP-dependent phosphorylation of HPr on a seryl residue is presumed to be of physiological significance with respect to regulation of sugar transport and metabolism. To characterize this ATP-dependent protein kinase we have developed a quantitative assay for HPr phosphorylation. With the aid of this assay we show that the HPr proteins isolated from several gram-positive bacteria (but not that isolated from *Escherichia coli*) are kinase substrates. These phosphorylated products are rigorously characterized. The quantitative dependencies of the kinase activity on divalent cations, pH, ATP, HPr, metabolic intermediates of glycolysis, and the pentose phosphate shunt are documented. Inhibitory effects of phosphorylation of the active histidyl residue in HPr, of P_i, and of high monovalent salt concentrations are demonstrated. These results serve to preliminarily characterize the protein kinase that presumably regulates the rates of carbohydrate transport mediated by the PTS in streptococcal cells.

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MATERIALS AND METHODS

Organisms, growth conditions, and preparation of crude extracts. The bacterial strains used, including *Streptococcus pyogenes*, *Streptococcus faecalis*, *Bacillus subtilis*, and *Staphylococcus aureus* (strains S305A, S797A, and S710A), were described previously (14, 15, 20a, 22). *S. pyogenes*, *B. subtilis*, and *S. aureus* S797A and S710A were grown to the late logarithmic phase in complex medium containing the following (grams per liter): proteose peptone, 5; tryptone, 15; yeast extract, 2; and NaCl, 5 (21). The media and growth of *S. faecalis* and *S. aureus* strain S305A were as described previously (2, 15). Cells were harvested by centrifugation, washed three times with 50 mM Tris-hydrochloride buffer (pH 7.2) containing 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (TTP buffer), and suspended in a minimal volume of the same buffer. *B. subtilis* cells were ruptured by treatment with 1 mg of lysozyme per ml in the presence of 5 μg of DNase per ml for 30 min at 37°C with gentle agitation followed by passage through a French pressure cell at 10,000 lb/in^2. Whole cells and cell debris were removed by centrifugation for 20 min at 7,000 rpm. *S. aureus* S710A and *S. pyogenes* were ruptured by repeated sonication as follows: the tube containing the cell suspension and glass powder (5 μm; 30% [vol/vol]) was immersed in an acetone-dry ice bath and subjected to four cycles of sonication, 4 min each, with a sonifier cell disrupter (Branson Sonic Power Co., model W140D) employed at maximal output. The extracts were centrifuged for 30 min at 10,000 rpm to remove whole cells and cell debris.

Protein purification. HPr from *S. faecalis* and *S. aureus* was purified by the procedure of Beyreuther et al. (2). HPrs from *B. subtilis* and *S. pyogenes* were similarly purified, but the heat precipitation step was omitted, and rechromatography on DEAE-cellulose was included as the last step. Enzyme I was purified from *S. faecalis* as detailed previously (15). For assay of HPr, crude extracts of a *ptsH* mutant of *S. aureus* (strain S797A) were employed, whereas enzyme I was assayed by employing a crude extract derived from a *ptsI* mutant of *S. aureus* (strain S710A). Assays were as previously described (14). HPr(Ser) kinase was prepared from *S. pyogenes* as follows. The crude extract, prepared as described above, was subjected to high-speed centrifugation (100,000 × *g*) for 10 h, although most of the enzyme pelleted within 1 h at this speed. The collected membranes were washed with TTP buffer and suspended in a minimal volume of the same buffer containing 1.5 M $MgCl_2$. After a 2-h incubation period at 4°C with stirring, the membranes were removed by centrifugation as described above. The supernatant fraction, containing approximately 80% of the kinase activity, was dialyzed overnight against TTP buffer to remove $MgCl_2$. The dialysate was again centrifuged for 10 h at 100,000 × *g*. The pellet contained less than 10% of the kinase activity.

The extracted kinase was purified by ion-exchange and gel filtration as follows. Soluble kinase (5 ml) was applied to DEAE-Sephacel in a 25-ml syringe (10-ml bed volume) equilibrated with TTP buffer. The column was washed with 50 ml of TTP buffer, and proteins were eluted with 15-ml volumes of 0.15, 0.20, and 0.25 M NaCl in TTP buffer. The kinase, recovered in the 0.25 M fraction, was dialyzed against TTP buffer, concentrated by ultrafiltration (Amicon Corp.; YM-10) and then applied to a calibrated column (2.2 by 34 cm) of Sephadex G-75. The kinase activity eluted as a single peak near the void volume with an estimated molecular weight of 60,000. Size exclusion chromatography of the kinase preparation that was eluted from the Sephadex column was also performed by high-pressure liquid chromatography with a Bio-Sil TSK-125 column (Bio-Rad Laboratories) and a Beckman 342 gradient liquid chromatograph. The sample applied contained 10 to 15 μg of protein, and elution was performed with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.1 M NaCl at a flow rate of 0.7 ml/min. The major protein and activity peaks coincided and corresponded to a molecular weight of 60,000. Sodium dodecyl sulfate-gel analyses confirmed that the major protein had a subunit molecular weight of 60,000; however, five or six minor contaminating bands were also detected.

Quantitative assay for ATP-dependent, protein kinase-catalyzed phosphorylation of HPr. The standard assay mixture for quantitation of HPr(Ser) phosphorylation (50-μl final volume) contained 50 mM Tris-acetate buffer (pH 7.2), 2 mM dithiothreitol, 2 mM $MgCl_2$, 4 mM fructose 1,6-diphosphate, 0.1 mM γ-[^{32}P]ATP (approximate specific activity, 500 to 2,000 cpm/pmol), 10 to 20 μg (25 to 50 μM) of HPr from *B. subtilis*, *S. aureus*, *S. pyogenes*, *S. faecalis*, or *E. coli* and 3 to 12 μg of the kinase preparation. The assay mixture was incubated at 37°C for 10 to 30 min before termination of the reaction by the addition of 50 μl of 50% trichloroacetic acid (TCA). The assay solution was placed in a boiling water bath for 4 min, chilled to 0°C for 15 min, and centrifuged in an Eppendorf table-top centrifuge at top speed for 5 min. The pellet was washed three times by centrifugation with 15% TCA containing 0.5% (wt/vol) sodium pyrophosphate and 0.5% sodium tungstate. The bottom segments of the Eppendorf tubes containing the protein pellets were sliced from the remainder of the tubes and transferred to scintillation vials containing Triton X-100–toluene scintillation fluid (22). Radioactivity was determined with a Beckman liquid scintillation spectrometer model LS-230. Controls lacking HPr were simultaneously run to determine the amount of nonspecific radioactivity due to inadequate washing, simple adherence to test tubes, or phosphorylation of other proteins in partially purified kinase preparations. In no case was control radioactivity greater than 10% of the experimental sample. Results are expressed in picomoles of HPr(Ser)-phosphate [HPr(Ser)-P] formed per minute per milligram of protein.

Identification of HPr, HPr(Ser)-P, HPr(His)-P, and HPr(Ser)-P(His)-P by polyacrylamide gel electrophoresis and immunoblot techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of HPr and phosphorylated HPr derivatives or of HPr kinase were performed as described previously (20). Native polyacrylamide gels (12.5%) were prepared in 0.375 M Tris-hydrochloride buffer (pH 8.8). The stacking gel (3.3% polyacrylamide) was polymerized in 0.125 M Tris-hydrochloride buffer (pH 6.8). Electrophoresis was conducted at 25 mA for 1.5 to 3 h with 0.025 M Tris-glycine (pH 8.3) as the running buffer. After electrophoresis, the gels were washed with 50% aqueous methanol containing 0.1 N NaOH for 1 to 2 h with gentle shaking at room temperature to remove [^{32}P]ATP, [^{32}P]PEP, and P_i. Both HPr(His)-P and HPr(Ser)-P are stable under these conditions. This treatment completely eliminated background radioactivity from the gels and thus eliminated the need for laborious procedures used previously to overcome this problem (B. Erni, personal communication). To distinguish between HPr(Ser)-P and HPr(His)-P, the gels were incubated for 30 to 60 min in boiling TCA (15%). Whereas HPr(Ser)-P was stable to this treatment, HPr(His)-P was quantitatively hydrolyzed. By contrast, HPr(Ser)-P was hydrolyzed during a 30- to 60-min exposure of the gels to 1 N NaOH at 60°C, whereas HPr(His)-P was stable under these

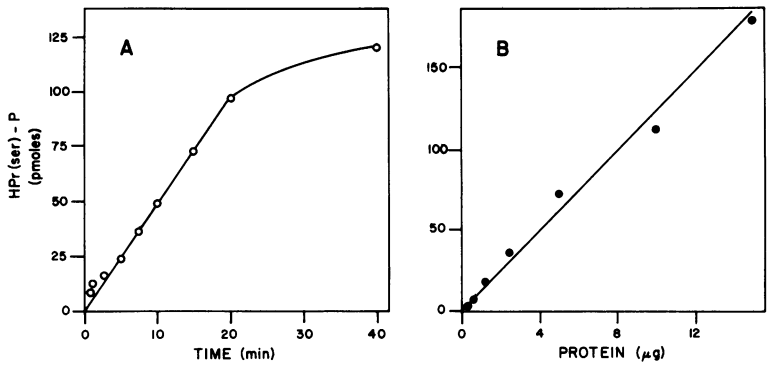

FIG. 1. ATP-dependent protein kinase-catalyzed phosphorylation of HPr from *B. subtilis* (A) and *S. faecalis* (B). The assay procedure was as described in the text. HPr(Ser) kinase was extracted from the membranes of *S. pyogenes* as described in the text and was used in A at a concentration of 4.5 μg per assay tube. The time interval used in B was 10 min. Time courses for kinase-catalyzed phosphorylation and enzyme concentration curves obtained with HPr preparations from *B. subtilis*, *S. faecalis*, and *S. pyogenes* were all shown to be linear.

conditions. Incubation of the gels in boiling TCA or hot NaOH resulted in a slight expansion of the gels, but the relative positions of the protein bands were not affected as compared with untreated gels. Autoradiography of either frozen or dried gels were performed with Kodak X-ray film (X-Omat, XAR-5) with exposure times ranging between 2 and 24 h at −70°C. Gels were stained for protein with Coomassie blue R in methanol-acetic acid-water (5:1:5; 2 h at 55°C) and destained with the same solvent at room temperature.

Immunodetection of HPr and ^{32}P-labeled phosphorylated HPr derivatives on nitrocellulose membranes was accomplished by immunoblotting analysis of native gels by a modified procedure (37) as follows. Protein samples were electrophoresed on 12.5% native polyacrylamide gels (1 mm thick). The developed gels were soaked in running buffer containing 20% methanol, and the protein bands were electrophoretically transferred to a nitrocellulose sheet (Bio-Rad) in the same buffer. Transfer was carried out at 20 V (0.5 A) for 2 h. The membranes were washed three times (5 min each) in 20 mM Tris-hydrochloride buffer (pH 7.4) containing 146 mM NaCl (TBS) and then incubated for 2 h with 3% bovine serum albumin in TBS. After washing in TBS the blots were overlaid and incubated (2 h, room temperature) with 30 ml of TBS containing 3% bovine serum albumin and rabbit antiserum (0.6 ml) directed against staphylococcal HPr. After washing with TBS (six changes, 5 min each) the membranes were incubated for 30 min with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad; 1:2,500 dilution in TBS containing 3% bovine serum albumin). Staining of the immunoperoxidase complex was performed by soaking the blots in a solution of 4-chloro-1-naphthol according to the manufacturer's instructions (Bio-Rad). Autoradiography of the developed blots was as described above.

Synthesis of ^{32}P-labeled-PEP. The original procedure (16) for synthesis of [^{32}P]PEP was modified as follows: Carrier-free $H_3{}^{32}PO_4$ (0.4 ml, 0.8 mCi) was evaporated (2 h) to dryness in a vacuum centrifuge, and 0.1 ml of 0.62% β-chlorolactic acid (Sigma Chemical Co.) in acetonitrile was added. The solvent was evaporated as above, and drying was repeated twice after two additions of 0.1-ml volumes of acetonitrile. The reaction mixture was further dried (30 min) over P_2O_5 under vacuum, and then 50 μl of dimethyl sulfoxide, 5 μl of triethylamine, and 1 μl of trichloroacetonitrile were added. After a 2-h incubation period at 37°C the reaction was stopped by the addition of 50 μl of water. Care was taken to use dry redistilled solvents. Production of PEP was verified by thin-layer chromatography on polyethyleneimine cellulose (Macherey-Nagel) with either 0.5 M KH_2PO_4 containing 5 mM EDTA or 0.25 M LiCl containing 1 M acetic acid as the eluent. An approximate yield of 70% was usually obtained.

Reagents. [γ-^{32}P]ATP (13 Ci/mmol) and carrier-free $^{32}P_i$ were purchased from New England Nuclear Corp., Boston, Mass., or ICN Pharmaceuticals, Irvine, Calif. Materials for polyacrylamide gel electrophoresis were from Bio-Rad. DEAE-Sephacel and Sephadex resins were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Diaflo ultrafiltration membranes were from Amicon Corp., Lexington, Mass. HPr from *E. coli* was kindly provided by H. Kornberg. ATP, alkaline phosphatase, and PEP were products of Sigma. All other chemicals used were of reagent grade from standard sources.

RESULTS

The development of a quantitative assay for HPr kinase has allowed us to characterize the catalytic properties of the enzyme. Phosphorylation of HPr was linear with respect to time and enzyme concentration under the conditions employed (Fig. 1). In all subsequent experiments enzyme concentrations and time intervals used were within the linear range.

Product characterization was achieved by several techniques. Phosphorylated HPr, generated *in vitro* by ATP-dependent kinase-catalyzed phosphorylation, was stable in 15% TCA at 100°C, but was unstable in 1 N NaOH at 60°C (Table 1). It was essentially insensitive to hydroxylamine at pH 5.5, but became soluble in TCA after treatment with pronase or alkaline phosphatase. These results suggest that, as for the in vivo product (11, 20), HPr phosphorylated in vitro is derivatized on a seryl, threonyl, or tyrosyl residue. Analysis of the ^{32}P-labeled product after total acid hydrolysis of phosphorylated HPr as described previously for the in vivo product (11) revealed only a single ^{32}P-phosphoamino acid, which was identified as seryl-P. No tyrosyl-P or threonyl-P was detected.

The products of PEP and ATP-dependent phosphorylation of HPr by enzyme I and the kinase, respectively, could be

TABLE 1. Properties of HPr(Ser)-P[a]

Treatment	% Acid-insoluble ^{32}P
Untreated	100
15% TCA (15 min, 100°C)	83
1 N NaOH (30 min, 60°C)	21
Tris-acetate, pH 5.5 (30 min, 37°C)	91
Tris-acetate, pH 5.5, + hydroxylamine (30 min, 37°C)	79
Pronase (30 min, 37°C)	48
Alkaline phosphatase (30 min, 37°C)	49

[a] HPr from *B. subtilis* was phosphorylated with [^{32}P] ATP in the presence of HPr kinase at 37°C for 30 min and then subjected to treatment with the reagents indicated above. Residual HPr(Ser)-^{32}P was determined by TCA precipitation as described in the text. The final concentrations of hydroxylamine, pronase, and alkaline phosphatase were 1 M, 4 mg/ml, and 20 U/ml respectively.

identified and distinguished according to their migration rates in native polyacrylamide gel electrophoresis and by subjecting the developed gels to selective hydrolytic procedures. Thus, of the two phosphorylated derivatives, HPr(Ser)-P migrated more rapidly as compared with HPr(His)-P (Fig. 2). In addition, alkaline treatment (30 min at 60°C in 1 N NaOH) of the developed gels selectively hydrolyzed the kinase-generated phosphoryl protein, whereas 15% TCA (60 min at 95°C) selectively hydrolyzed the enzyme I-generated phosphoryl-HPr (Fig. 2). These methods allow identification of and simple distinction between the two phosphorylated forms of HPr.

In addition to HPr(His)-P and HPr(Ser)-P, a doubly phosphorylated protein, HPr(His)-P(Ser)-P, was identified when HPr was sequentially phosphorylated by the ATP-dependent kinase followed by incubation with enzyme I and PEP. The reciprocal experiment, i.e., phosphorylation of HPr by PEP and enzyme I and subsequent phosphorylation of HPr(His)-P by the ATP-dependent kinase, was also attempted. In both experiments (Fig. 3), [^{32}P]ATP and unlabeled PEP or unlabeled ATP and [^{32}P]PEP were used alternately for radiochemical labeling of HPr-P. Two phosphorylated bands were obtained when phosphorylation of HPr by the ATP-dependent kinase was followed by that of enzyme I (Fig. 3, lanes 1 and 2). Thus, when unlabeled ATP and [^{32}P]PEP were used to phosphorylate HPr, the labeled

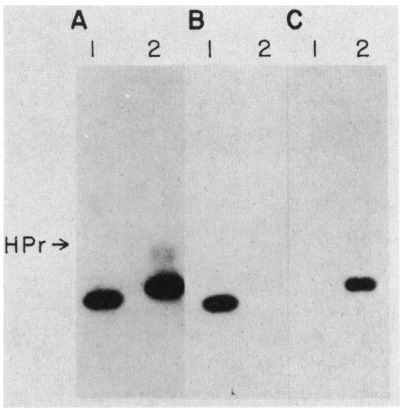

FIG. 2. Identification of HPr(Ser)-P and HPr(His)-P by native polyacrylamide gel electrophoresis and by selective hydrolytic procedures. The autoradiograms show the methanolic NaOH washed gel (A), the same gel treated with 15% TCA for 30 min at 100°C (B), and a duplicate gel treated with 1 N NaOH for 30 min at 60°C (C). In all three gels, lane 1 is HPr from *S. pyogenes* exposed to [^{32}P]ATP plus kinase, and lane 2 is HPr exposed to [^{32}P]PEP plus enzyme I. Phosphorylation of HPr by [^{32}P]PEP (250 to 1,000 cpm/pmol; 0.1 mM) was performed as described for that by ATP, except that enzyme I (2 μg) replaced the ATP-dependent kinase, and fructose 1,6-diphosphate and ATP were omitted. The arrow denotes the position of unphosphorylated HPr as observed on a Coomassie blue-stained gel.

derivatives formed were HPr(His)-^{32}P and a doubly phosphorylated protein, HPr(His)-^{32}P(Ser)-P (Fig. 3, lanes 1). A similar doubly phosphorylated protein labeled on the seryl residue, HPr(His)-P(Ser)-^{32}P, was obtained when [^{32}P]ATP and unlabeled PEP were used (Fig. 3, lanes 2). In contrast, only a single phosphorylated derivative was observed when phosphorylation of HPr by enzyme I and PEP preceded that by the kinase and ATP (Fig. 3, lanes 3 and 4). These findings suggest that HPr(His)-P does not serve as a substrate for the kinase, and this result was confirmed employing the quantitative assay (see below).

The kinase from *S. pyogenes* was capable of phosphorylating HPr isolated from four different gram-positive species: *S. aureus*, *B. subtilis*, *S. faecalis*, and *S. pyogenes* (Fig. 4). For

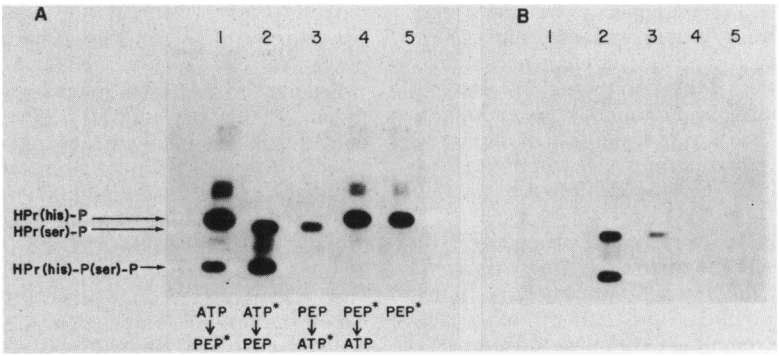

FIG. 3. Sequential phosphorylation of HPr by ATP-dependent kinase and enzyme I. Shown are autoradiograms of (A) the native gel washed in methanolic NaOH and (B) the same gel treated with TCA. The sequence of *S. faecalis* HPr phosphorylation by the ATP-dependent kinase or by *S. faecalis* enzyme I was as indicated at the bottom of the autoradiogram for the following lanes: 1, kinase plus unlabeled ATP followed by enzyme I plus [^{32}P]PEP; 2, kinase plus [^{32}P]ATP followed by enzyme I plus unlabeled PEP; 3, enzyme I plus unlabeled PEP followed by the kinase plus [^{32}P]ATP; 4, enzyme I plus [^{32}P]PEP followed by the kinase and unlabeled ATP; 5, enzyme I plus [^{32}P]PEP. The slightly labeled bands above HPr(His)-^{32}P in lanes 1, 4, and 5 correspond to the position of HPr. Note that only HPr(Ser)-^{32}P or HPr(Ser)-^{32}P(His)-P survives the treatment with TCA (Fig. 3B). Each of the two successive incubation periods was 15 min.

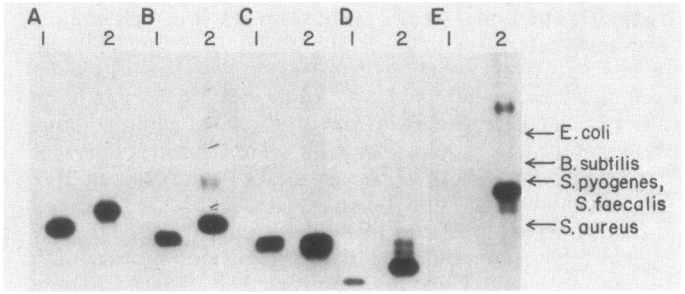

FIG. 4. Phosphorylation of HPr from different bacterial species by the ATP-dependent kinase from *S. pyogenes* or by enzyme I from *S. faecalis*. The autoradiograms of native gels show phosphorylated HPr species from *S. pyogenes* (A), *S. faecalis* (B), *B. subtilis* (C), *S. aureus* (D), and *E. coli* (E). Odd-numbered lanes show HPr preparations exposed to [^{32}P]ATP and the HPr kinase, and even-numbered lanes show HPr preparations exposed to [^{32}P]PEP and enzyme I. The arrows denote the positions of the corresponding nonphosphorylated HPrs as obtained from stained gels.

comparative purposes the locations of HPr(His)-Ps from the various bacteria were also demonstrated. HPr from *E. coli* was not phosphorylated by the ATP-dependent kinase (Fig. 4), although it was phosphorylated by PEP and enzyme I from *S. faecalis* (J. Reizer and M. H. Saier, unpublished data) (see also reference 28). All HPrs from gram-positive bacteria can be phosphorylated to a similar extent by enzyme I from *S. faecalis*. In contrast, the ATP-dependent phosphorylation of HPr from *S. aureus* was only 10 to 20% of that obtained with HPr from *S. faecalis* or *B. subtilis*. It is not known yet whether the reduced rate of ATP-dependent phosphorylation of *S. aureus* HPr is due to the specificity of the ATP-dependent kinase from *S. pyogenes* or to the presence of HPr proteolytic fragments in the former preparation (Fig. 4, lane D2).

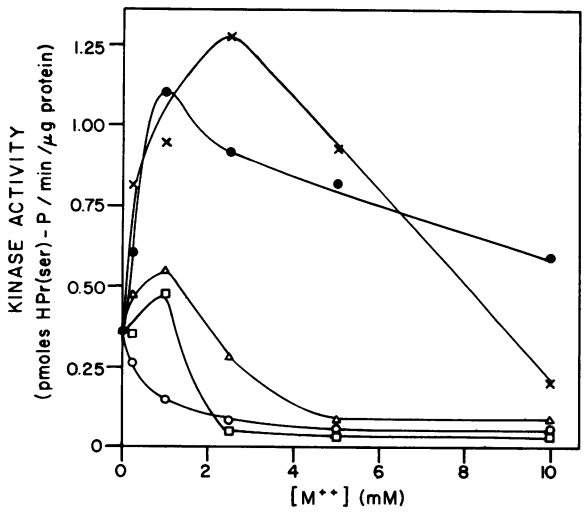

FIG. 5. Dependency of kinase activity on divalent cation concentration. Assays were performed as described in the text with HPr from *S. faecalis*. Activity was measured as a function of the concentration of Mg^{2+} (●), Mn^{2+} (×), Zn^{2+} (△), Co^{2+} (□), or Ca^{2+} (○). EDTA (2.5 mM) quantitatively inhibited kinase activity, and this activity was restored by the addition of 5 mM Mg^{2+} (100%), Mn^{2+} (80%), Zn^{2+} (14%), or Ca^{2+} (15%).

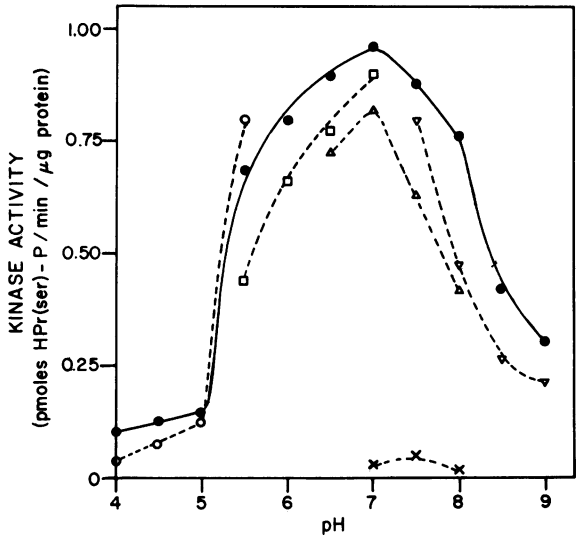

FIG. 6. pH dependency of HPr kinase activity in various buffers. Buffers (used at a final concentration of 50 mM) were Tris-acetate (●); sodium acetate (○); sodium 2-(*N*-morpholino)ethanesulfonic acid (□), potassium 3-(N-morpholino)propanesulfonic acid (△), sodium *N,N*-bis(2-hydroxyethyl)glycine (▽), and potassium phosphate (×). Assays were performed as described in the text with *B. subtilis* HPr.

To verify the identification of the phosphoproteins generated in vitro, immunoblot analysis of HPr from *S. aureus* was performed with antibody directed against *S. aureus* HPr. Immunological activity was observed with free HPr as well as with HPr(His)-P and HPr(Ser)-P (data not shown). This result contrasts with those obtained previously in immunoprecipitation experiments conducted with antibodies directed against other proteins of the PTS (Enzyme III^{lac} and enzyme III^{xtl} [10, 17]). It should be noted that *B. subtilis* HPr or HPr(His)-P(Ser)-P could not be detected by this technique when antiserum against *S. aureus* HPr was employed (unpublished results).

Quantitative evaluation of the divalant cation dependency of the kinase (Fig. 5) revealed that the enzyme was maximally activated by Mg^{2+} and Mn^{2+}. Other divalent cations were less active. Sn^{2+}, Ni^{2+}, and Cu^{2+} did not activate. The essential requirement of divalent cations for enzyme activity was demonstrated by the addition of EDTA (2.5 mM), which completely abolished the activity of the enzyme unless an excess of an appropriate divalent cation was added (Fig. 5). Thus, the low kinase activity observed without the addition of divalent cations (Fig. 5) is most probably due to residual or tightly bound Mg^{2+} in the kinase preparation.

The dependence of kinase activity on pH is illustrated in Fig. 6. Regardless of the buffer employed, the pH optimum was 7.0, with half-maximal activities at about pH 5.3 and 8.4. Interestingly, phosphate completely inhibited the enzyme regardless of pH (Fig. 6). A variety of monovalent salts (the chloride salts of K^+, Na^+, NH_4^+, and Li^+) were inhibitory, with 50% inhibition occurring at about 120 mM, regardless of the salt (data not shown). Qualitatively similar results have been published for protein kinases from viruses and animal cells (24).

The quantitative kinase assay was used to examine the activating effects of a variety of metabolites. In contrast to results reported previously in which no quantitation was

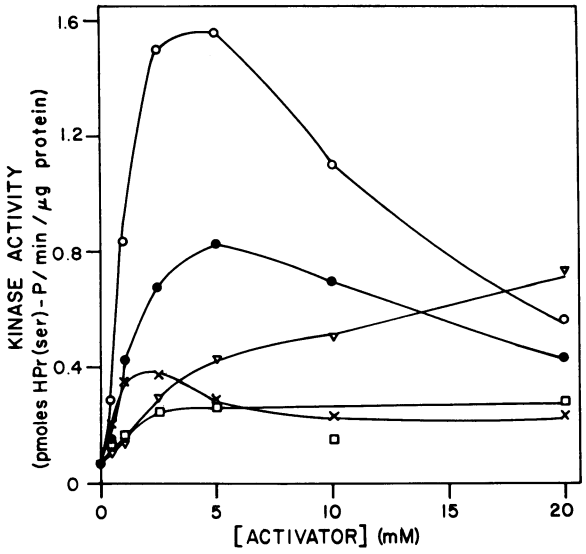

FIG. 7. Activation of HPr kinase by various intermediates of the glycolytic and pentose phosphate pathways. Activators were fructose 1,6-diphosphate (○), 2-phosphoglycerate (●), phosphoenolpyruvate (×), pyruvate (□), and gluconate 6-P (△). Assay conditions were as described in the text with *S. faecalis* HPr and kinase extracted from *S. pyogenes* membranes.

possible (11), the most potent allosteric activator of the kinase was found to be fructose 1,6-diphosphate (Fig. 7). 2-Phosphoglycerate was much less effective, and only slight activation was observed with pyruvate or PEP. Gluconate 6-P was effective at a concentration of 5 mM or higher; 2,3-diphosphoglycerate was similarly effective (data not shown). Metabolites that did not significantly activate at 2.5 or 25 mM included glucose 6-P, glucose 1-P, glucose, fructose 1-P, 3-P-glycerate, mannose 6-P, mannitol 1-P, glucitol 6-P, glucosamine 6-P, galactose 6-P, galactose 1-P, acetyl-P, NADH, NAD^+, lactate, and L-α-glycerophosphate. Nonmetabolizable carbohydrate analogs that did not significantly activate the kinase included 5-thioglucose, glucose 6-sulfate, 2-deoxyglucose, and 2-deoxyglucose 6-P.

The kinetic properties of the kinase-catalyzed reaction were estimated (Fig. 8). The enzyme followed Michaelis-Menten (hyperbolic) kinetics with respect to both substrates. The apparent K_m values were 66 and 61 μM for ATP and HPr, respectively, when assayed under the conditions described in the legend to Fig. 8. An absolute K_m value of 30 μM was calculated for HPr from a secondary plot depicting the apparent $1/V_{max}$ values for ATP, as measured at different fixed concentrations of HPr, versus the reciprocals of HPr concentrations (data not shown).

Phosphorylation of the histidyl residue in HPr strongly inhibited phosphorylation of the seryl residue by the ATP-dependent kinase. Thus, in the presence of 2.5 mM PEP and an excess of enzyme I, the fructose 1,6-diphosphate-activated kinase was inhibited approximately 90% (Fig. 3). PEP in the absence of enzyme I did not inhibit (Fig. 7). A reciprocal effect of HPr(Ser) phosphorylation by ATP on phosphorylation of the histidyl residue by PEP and enzyme I has recently been suggested (J. Deutscher et al., unpublished results). Similar inhibition (95%) of HPr(Ser) phosphorylation was observed after preincubation (10 min, 25°C) of the protein kinase with diethylpyrocarbonate (2 mM). In contrast to the strong inhibition exerted by diethylpyrocarbonate, no significant inhibition was observed after similar preincubation of the protein kinase with any one of the sulfhydryl reagents, *N*-ethylmaleimide (4 mM), *p*-chloromercuribenzoate (1 mM), or iodoacetate (5 mM).

DISCUSSION

Previous reports from this laboratory have served to implicate an ATP-dependent phosphorylation of HPr in the regulation of sugar phosphate accumulation (11, 20). Thus, the phosphorylation of this pivotal PTS protein might be used to regulate the extent of sugar phosphate accumulation by an exclusion mechanism or by activating expulsion of preaccumulated sugar phosphates, or both. The properties of this ATP-dependent protein kinase, a regulatory protein of the PTS which may also regulate cytoplasmic sugar phosphate hydrolysis (34), is the subject of this communication.

We have rigorously characterized the product of the protein kinase-catalyzed reaction synthesized in vitro, both with respect to the protein target and with respect to the residue phosphorylated. Examination of the electrophoretic mobilities of phosphorylated HPrs derivatized on serine or histidine revealed that the former proteins migrated substan-

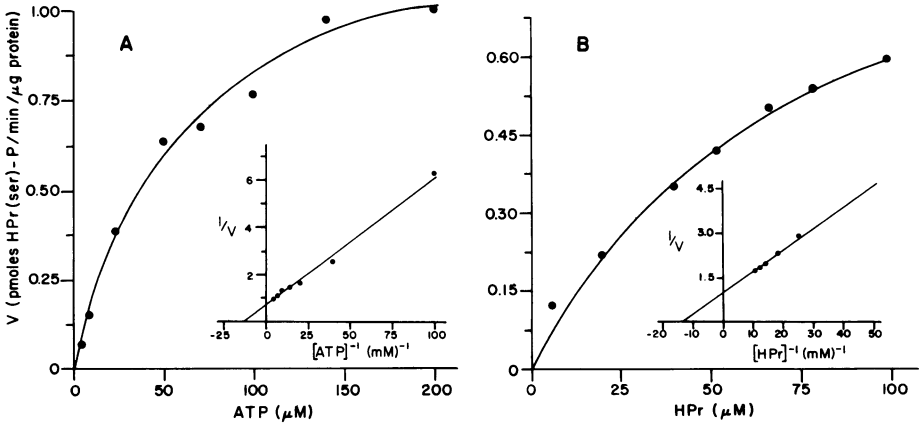

FIG. 8. Estimation of the apparent affinities of the kinase for ATP and HPr. (A) Kinase activity as a function of ATP concentration with *B. subtilis* HPr (0.2 mg/ml). (B) Kinase activity as a function of HPr concentration with 1 mM [^{32}P]ATP. The insets show double-reciprocal plots of the data. The apparent K_m values for ATP and HPr were 66 and 61 μM, respectively.

tially faster than the latter when streptococcal or staphylococcal HPrs were used as substrates. By contrast, similar electrophoretic mobilities were observed for HPr(His)-P and HPr(Ser)-P from *B. subtilis*.

Of further interest is the observation that an antiserum prepared against HPr from *S. aureus* reacted with *S. aureus* HPr(Ser)-P and HPr(His)-P as well as with HPr itself. This result suggests that phosphorylation of the protein on either residue does not greatly alter its antigenic characteristics. The opposite conclusion was reached for two other PTS proteins, the lactose-specific enzyme III or *S. aureus* (10) and the xylitol-specific enzyme III of *Lactobacillus casei* (17; M. H. Saier, Jr., F. C. Grenier, C. A. Lee, and E. B. Waygood, J. Cell. Biochem, in press).

In a previous publication, the ATP-dependent HPr kinase was found to exhibit decreased sensitivity to activation by glycolytic intermediates and a rather low molecular weight after its extraction from the membrane (11). In this report, a much higher molecular weight and full dependency on metabolites were demonstrated with the soluble kinase. Proteolytic cleavage of the protein during extraction in the previous studies may provide an explanation for these differences. Thus, in the original procedure (11), extraction was conducted for 24 h without a protease inhibitor, whereas in the present procedure, extraction was for 2 h in the presence of a serine protease inhibitor. We believe that the protein described in the present report resembles the native enzyme.

We have shown that the streptococcal kinase phosphorylates HPrs from *S. faecalis*, *S. pyogenes*, *B. subtilis*, and *S. aureus*. A 5- to 10-fold lower rate of phosphorylation was observed with the staphylococcal HPr as compared with the other HPr proteins. *E. coli* HPr was not a substrate of this protein kinase. Although preliminary results suggested that *E. coli* HPr might be phosphorylated by an *E. coli* ATP-dependent kinase (11), subsequent work has failed to confirm this suggestion (M. J. Novotny, J. Reizer, and M. H. Saier, Jr., unpublished results). The involvement of an HPr kinase in transport regulation may therefore be restricted to gram-positive bacteria.

In this regard it is interesting to note that the four gram-positive HPr proteins studied have a seryl residue at position 27 which is lacking in the *E. coli* HPr. In addition all five proteins possess a seryl residue at position 31 (K. Beyreuther and W. Hengstenberg, unpublished data). The former residue is therefore a prime candidate for the target of the streptococcal kinase, although residue 31 can by no means be excluded at this time.

The present study serves to characterize many of the catalytic characteristics of the ATP-dependent HPr kinase from *S. pyogenes*. The enzyme exhibits a neutral pH optimum, a strict dependency on a divalent cation, a requirement for one of several metabolic intermediates of glycolysis or the pentose-phosphate pathway, and high affinity for its two natural substrates, ATP and HPr. Simple calculations revealed that the intracellular concentration of HPr in *S. pyogenes* is approximately 0.16 mM. This concentration is considerably higher than the K_m determined for the ATP-dependent kinase and allows saturation of the enzyme under physiological conditions. Moreover, the inhibitory effects of phosphate, high salt concentrations, and phosphorylation of the active histidyl residue have been documented. All of these phenomena may be of physiological significance with regard to sugar uptake and expulsion. For example, starved streptococci have been shown to possess high cytoplasmic concentrations of P_i, whereas glycolyzing cells do not (18, 36). Only the latter cells catalyze HPr(Ser) phosphorylation and rapid hydrolysis of cytoplasmic sugar phosphate followed by expulsion of the free sugar to the external medium (20, 21). Furthermore, expulsion of the free sugar and phosphorylation of HPr(Ser) were also observed in fluoride-poisoned cells supplemented with both glucose and arginine (20). The limited metabolism of glucose and the metabolism of arginine via the deiminase pathway (9, 27) provide these poisoned cells with fructose 1,6-diphosphate and nonglycolytic ATP, respectively, as well as a sink for the highly inhibitory cytoplasmic P_i. Strong inhibition of streptococcal sugar phosphate phosphatase by P_i has also been noted (34).

Of the metabolites studied, fructose 1,6-diphosphate was by far the most potent positive effector of the kinase. This same intermediate had been previously shown to allosterically activate streptoccocal pyruvate kinase (1, 8, 32) and lactate dehydrogenase (31, 38) and to inhibit 6-phosphogluconate dehydrogenase (4). The central role of fructose 1,6-diphosphate in the regulation of various key irreversible steps in carbohydrate amphibolic pathways presumably reflects the facts that fructose 1,6-diphosphate, the product of the allosterically regulated phosphofructokinase (13), is the first common intermediate in the glycolytic metabolism of all sugars except galactose, which is metabolized via tagatose 1,6-diphosphate (3, 6, 7, 29). The latter phosphorylated epimer of fructose is not available commercially and therefore was not tested for activation of the kinase in the present studies. Fructose 1,6-diphosphate and tagatose 1,6-diphosphate can be considered to be the common end products of the first sequences of carbohydrate metabolic interconversions that are initiated by the PTS. They are therefore reasonable feedback effectors of PTS function.

The discovery that gluconate 6-P also activates the HPr kinase may prove to be of physiological significance. Gluconate 6-P is the product of the allosterically regulated glucose 6-P dehydrogenase reaction and the only metabolite common to the pentose phosphate pathway and the Entner-Doudoroff pathway (30). These two pathways provide reducing power and carbon compounds for biosynthetic purposes. Thus, sensitivity to gluconate 6-P may allow the uptake process to sense the biosynthetic needs of the cell.

In summary, we feel that the HPr kinase, characterized in this study, plays a central role in the regulation of carbohydrate transport and metabolism in streptococci and possibly in other gram-positive bacteria. In view of this working hypothesis the details of its mechanism, its physiological role, and its regulation will prove of crucial significance to an understanding of carbon metabolism in these organisms.

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