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Biochemistry

ATP-dependent protein kinase-catalyzed phosphorylation of a seryl residue in HPr, a phosphate carrier protein of the phosphotransferase system in *Streptococcus pyogenes*

[phospho(seryl)HPr/HPr kinase/phospho(seryl)HPr phosphatase/glycolytic intermediates]

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ABSTRACT **HPr, a phosphate carrier protein of the streptococcal phosphotransferase system, is phosphorylated at the N-1 position of a single histidyl residue in a reaction requiring phospho*enol*pyruvate (*P-e*Prv), Mg^{2+}, and enzyme I (*P-e*Prv–HPr phosphotransferase, EC 2.7.3.9). We demonstrate that in addition to this reaction, a seryl residue within HPr can be phosphorylated in an ATP-dependent process. This reaction is catalyzed by a protein kinase with an approximate M_r of 20,000. In whole cells the kinase activity is stimulated by glucose, whereas in crude extracts the activity is stimulated by glycolytic intermediates such as glucose 6-phosphate, fructose 1,6-diphosphate, and 2-phosphoglycerate. *P*-(Ser)-HPr cannot transfer its phosphate group via enzyme II to a sugar as does the *P*-(His)-HPr. Instead, a phosphatase (M_r = 70,000) was found to hydrolyze the phosphate group of P-(Ser)-HPr. The phosphatase reaction is strongly inhibited by the addition of *P-e*Prv and enzyme I. Protein kinase-catalyzed phosphorylation of the enzyme constituents of the phosphotransferase system in *Escherichia coli* has also been demonstrated. These observations lead us to suggest that phosphorylation of a seryl residue in HPr is involved in the regulation of sugar transport in the bacteria cell.**

HPr is one of the phosphate transfer enzymes of the bacterial phospho*enol*pyruvate (*P-e*Prv)-dependent phosphotransferase system (1–3). It has a M_r of 7,500 in *Staphylococcus aureus* (4) and of approximately the same value in *Streptococcus faecalis*, *Streptococcus lactis*, and *Bacillus subtilis* (5). The M_r of HPr is slightly higher in *Escherichia coli* (2, 6) and *Salmonella typhimurium* (7), with a value of 9,600. For the sugar uptake reaction and the concomitant phosphorylation of the sugar, HPr has been shown to be phosphorylated by P-enzyme I at the N-1 position of a single histidyl residue [His-15 in the sequences of *S. aureus* (4) and *S. typhimurium* (7)]. P-HPr then transfers its phosphate group to either an enzyme III or directly via the membrane-bound enzyme II to the sugar (8).

In addition to the role of HPr in the *P-e*Prv-dependent uptake of sugars, genetic data indicate that it functions in the regulation of adenylate cyclase and sugar uptake. Thus, *ptsH* mutants of *S. typhimurium* and *E. coli* that are defective for HPr are hypersensitive to catabolic repression (9) and show very low levels of cellular cyclic AMP (10). This paper provides direct biochemical evidence that HPr, one component of the bacterial phosphotransferase system, is phosphorylated on a seryl residue at the expense of ATP. HPr phosphorylation is catalyzed by an HPr kinase, of low molecular weight, and the activity of this enzyme is stimulated by metabolites of the glycolytic pathway *in vitro*. The P-(Ser)-HPr bond is hydrolyzed by a P-(Ser)-HPr phosphatase that is inhibited in the presence of *P-e*Prv and enzyme I. These secondary ATP-dependent phosphorylation reactions are presumed to be involved in the regulation of sugar transport.

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MATERIALS AND METHODS

Bacteria. *S. pyogenes* type 12 was employed for most experiments. Cells were grown in complex medium as described previously (11). Strain S797A of *S. aureus* (*ptsH lac* constitutive) was grown in LB medium.

Protein Purification. HPr of *Streptococcus pyogenes* was purified by ion-exchange chromatography on DEAE-cellulose and chromatography on Sephadex G-75. The [^{32}P]P-(Ser)-HPr was purified in the same way. In both cases the protein was homogeneous according to its behavior on NaDodSO$_4$/polyacrylamide gels. HPr activity was measured by the mutant complementation assay using the *ptsH* mutant S797A of *S. aureus* (12). HPr kinase was partially purified by chromatography on Sephadex G-75. In crude extracts it was membrane associated but could be released from the membrane with high salt (100 mM phosphate buffer). The P-(Ser)-HPr phosphatase was partially purified by chromatography on Sephadex G-75 and ion-exchange chromatography on DEAE-cellulose.

^{32}P Labeling of HPr. The *in vivo* phosphorylation of HPr with [^{32}P]phosphate was conducted employing a published procedure (13). HPr kinase activity could be stimulated *in vivo* by addition of glucose (10 mM) to the cell suspension. For the *in vitro* experiments [γ-^{32}P]ATP (purchased from Amersham) was used. The concentration of ATP in the assay mixture was 0.1 mM (specific activity = 800 μCi/μmol; 1 Ci = 3.7 × 10^{10} Bq). The same assay conditions were used when partially purified HPr kinase was employed. Concentrations of the other assay components were as follows: HPr, 25 μM; metabolites of the glycolytic pathway, 1–5 mM; Mg^{2+}, 0.1 mM. After a 5-min incubation period at 37°C, the reaction was terminated by addition of NaDodSO$_4$ (6% solution). A NaDodSO$_4$/polyacrylamide gel was run as described (14), and phosphorylated proteins were detected by autoradiography.

P-(Ser)-HPr Phosphatase Assay. Purified [^{32}P]P-(Ser)-HPr was used for the phosphatase assay. Five micrograms of the phosphorylated protein (10,000 cpm) was incubated for 15 min with the phosphatase in the presence of 0.5 mM Mg^{2+} at 37°C. The reaction was stopped by addition of NaDodSO$_4$, and phosphatase activity was estimated after NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography by measuring the loss of label from the [^{32}P]P-(Ser)-HPr band.

Abbreviations: HPr, phosphate carrier protein of the streptococcal phosphotransferase system; *P-e*Prv, phospho*enol*pyruvate.

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Separation of P-Ser, P-Thr, and P-Tyr. Separation of the three acid-stable phosphoamino acids was accomplished as described (15). One-dimensional electrophoresis gave satisfactory separation of the three phosphoamino acids. Ten micrograms of hydrolyzed [^{32}P]P-(Ser)-HPr and 1 μg of standard P-Tyr, P-Thr, and P-Ser were applied on a cellulose plate for electrophoresis. The standards were detected with a ninhydrin spray (0.2% ninhydrin in acetone).

RESULTS

Previous results in this laboratory have suggested that in species of *Streptococcus*, ATP-dependent protein phosphorylation might control the rates of sugar transport (11, 16–18). Therefore, attempts were made to demonstrate protein phosphorylation *in vivo*. In whole cells of *S. pyogenes*, a protein of low molecular weight was found to be phosphorylated when the cells were preloaded with [^{32}P]phosphate. Phosphorylation was dependent on the presence of glucose in the incubation mixture. Glucose stimulated the phosphorylation of this protein about 10-fold (17).

In crude extracts of *S. pyogenes* the phosphorylation of the low molecular weight protein was dependent on ATP. The protein could be labeled in the presence of [γ-^{32}P]ATP. Glucose 6-phosphate, fructose 1,6-diphosphate, and 2-phosphoglycerate stimulated its phosphorylation *in vitro* (Fig. 1). Other glycolytic intermediates tested elicited little or no stimulatory effect.

To gain more information about the phosphorylated protein, a crude extract derived from 7 g (wet weight) of *S. pyogenes* cells was incubated for 10 min with [γ-^{32}P]ATP (25 μM, 1.6 mCi/μmol) in the presence of 5 mM glucose 6-phosphate and 5 mM 2-phosphoglycerate. The reaction mixture was then loaded onto a DEAE-cellulose column. After washing most of the ATP from the column, proteins were eluted by employing a linear gradient (0–0.5 M NaCl). A major radioactive peak eluted at about 0.15 M NaCl. Only one radioactive band was visible by autoradiography after NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2). After staining and destaining with Coomassie brilliant blue [0.25% Coomassie blue in methanol/H$_2$O/acetic acid, 4:4:1 (vol/vol)], several protein bands were visualized, and the single radioactive band coincided with a low molecular weight protein. The staining and destaining procedure did not remove the ^{32}P label from the protein, suggesting that an acid-stable phosphoamino acyl residue, (P-Ser, P-Thr, or P-Tyr) must be present in the phosphorylated protein.

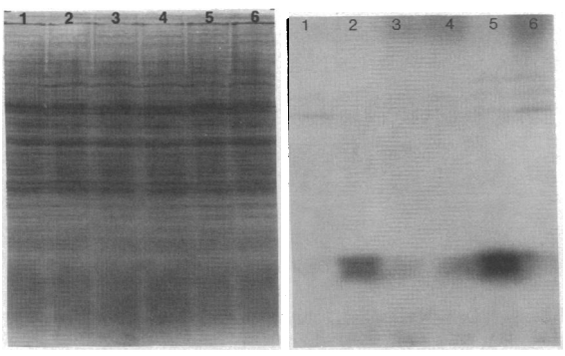

FIG. 1. Phosphorylation of a small protein by [γ-^{32}P]ATP in response to stimulation by various glycolytic intermediates. (*Left*) NaDodSO$_4$/polyacrylamide gel of a *S. pyogenes* crude extract (50 μl, 1 g of wet cells in 6 ml). The extract was incubated with [γ-^{32}P]ATP, Mg^{2+}, and glucose or different glycolytic intermediates. Lane 1, 5 mM glucose; lane 2, 5 mM glucose 6-phosphate; lane 3, 5 mM fructose 6-phosphate; lane 4, 2 mM fructose 1,6-diphosphate; lane 5, 2 mM 2-phosphoglycerate; and lane 6, 2 mM glyceraldehyde-3-phosphate. (*Right*) Autoradiogram of *Left* after an exposure time of 21 hr; the best stimulation of ATP-dependent phosphorylation was achieved with glucose 6-phosphate and 2-phosphoglycerate. Fructose 1,6-diphosphate stimulated slightly.

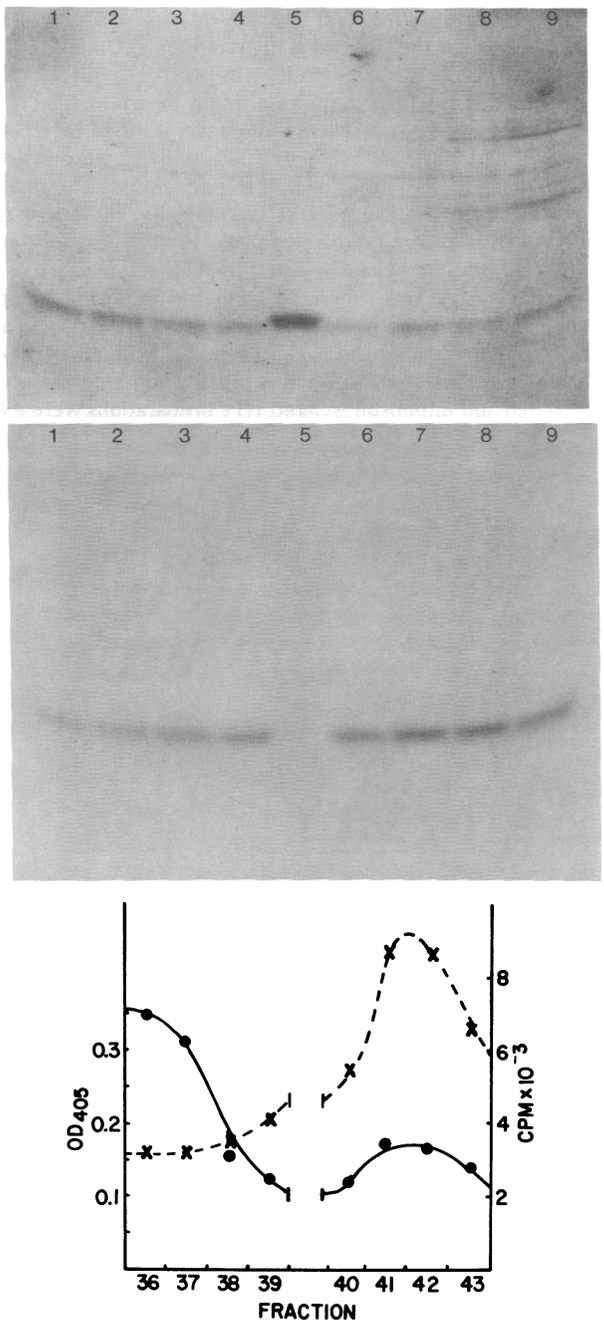

FIG. 2. Identification of the phosphorylated protein as HPr based on its elution profile from DEAE-cellulose. (*Top*) NaDodSO$_4$/polyacrylamide gel of fractions 36–45 (left to right) after elution of proteins from a *S. pyogenes* crude extract from a DEAE-cellulose column employing a linear gradient of 0–0.5 M NaCl. Purified *S. faecalis* HPr (10 μg) had been applied on lane 5. The crude extract had been incubated with [γ-^{32}P]ATP, Mg^{2+}, glucose 6-phosphate, and 2-phosphoglycerate (see text). (*Middle*) Autoradiogram of *Top* after an exposure time of 8 hr. The autoradiogram shows only one ^{32}P-labeled protein band. (*Bottom*) Distribution of radioactivity (---) and HPr activity (——) in the above fractions. Two peaks of HPr activity are shown, one with its maximum in fraction 36 and the other with its maximum in fraction 41. The latter coincides with the peak of radioactivity.

The size, properties, and quantity of the phosphorylated protein as well as the fact that it migrated on $NaDodSO_4$/polyacrylamide gels in the same position as purified *S. faecalis* HPr suggested that the phosphoprotein might be a new type of P-HPr. Therefore, the radioactive protein was purified by DEAE-cellulose, and fractions containing radioactivity were assayed for HPr activity. Two peaks of HPr activity eluted. The smaller peak coincided with the peak of radioactivity; the larger one eluted in front of the radioactive peak (Fig. 2). Possibly, part of the HPr became phosphorylated by ATP and because of its additional charge was separated from the unphosphorylated protein. Therefore, both peaks of HPr activity were pooled and run separately on a Sephadex G-75 column. Both eluted at exactly the same position. After this purification step the phosphorylated and unphosphorylated HPr preparations were electrophoretically pure. They migrated to the same position in $NaDodSO_4$/polyacrylamide gels, and autoradiography revealed that the phosphorylated HPr showed a radioactive band coinciding with the protein band.

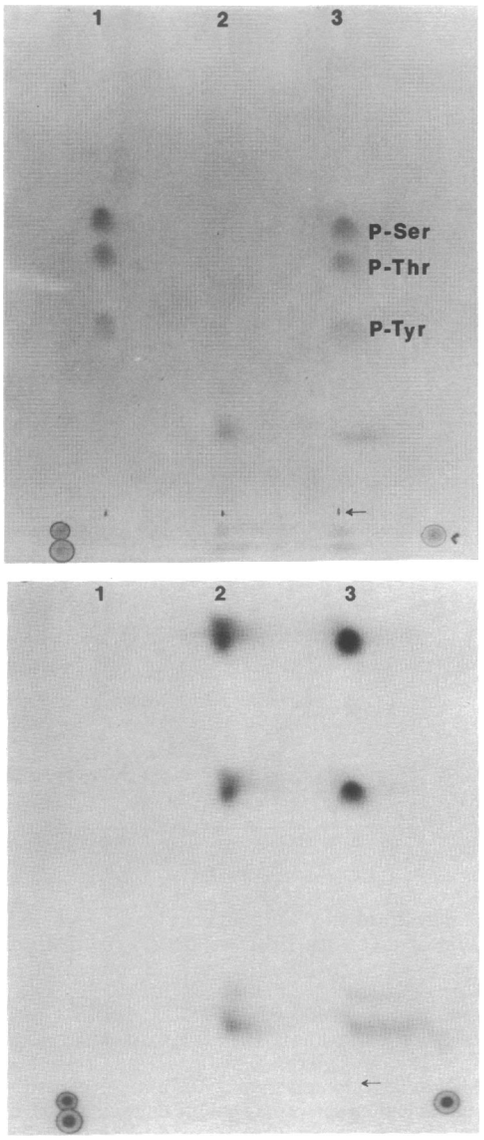

FIG. 3. Identification of the phosphorylated amino acyl residue in ^{32}P-labeled HPr as a phosphoseryl residue. (*Upper*) Electrophoretogram of ^{32}P-labeled HPr after a 2.5-hr hydrolysis in 6 M HCl at 110°C. Lane 1, standards (1 μg each of, P-Tyr, P-Thr, and P-Ser); Lane 2, hydrolyzed [^{32}P]P-(Ser)-HPr; lane 3, a mixture of both. The origin is indicated by the arrow. (*Lower*) Autoradiogram of *Upper*. The hydrolysate of ^{32}P-labeled HPr contains only phosphoserine. Free phosphate that is produced by hydrolysis of the phosphoprotein is the fast moving spot; the slow moving spots are HPr fragments that result from incomplete hydrolysis.

Because of its acid stability during staining and destaining of the gels, the phosphate group could not be linked to a histidyl residue in the protein. P-(His)-HPr has been shown to be acid labile (2, 19). To further test the acid stability of the phosphorylated protein, aliquots of the isolated ^{32}P-labeled HPr were brought to different pH values (2.9, 4.3, 7.5, and 11) for $1\frac{1}{2}$ hr at 42°C. Under no condition was loss of radioactive label from ^{32}P-labeled HPr observed. Therefore, the HPr had to contain an acid-stable phosphoamino acyl residue, P-Ser, P-Thr, or P-Tyr.

To distinguish between these three possibilities, total hydrolysis of ^{32}P-labeled HPr was performed at 110°C in 6 M HCl for $2\frac{1}{2}$ hr (15). Electrophoresis of the hydrolysate and of the three standard phosphoamino acids was performed, and the standards were detected with ninhydrin. Autoradiography of the electrophoretogram revealed that the new P-HPr contained a P-seryl residue, but phosphorylated threonine and tyrosine were not detected (Fig. 3).

To determine if P-(Ser)-HPr was capable of transferring its phosphate group to a sugar molecule via enzyme III and enzyme II, the following experiment was performed. [^{32}P]P-(Ser)-HPr was incubated with 1 mM $MgCl_2$ and 20 mM *P-e*Prv in the presence of a crude extract of *S. aureus* strain S797A. A second assay solution contained, in addition to these constituents, 10 mM *o*-nitrophenyl-β-D-galactoside, a substrate of the lactose *P-e*Prv–phosphotransferase system, and, consequently, a potential phosphate acceptor. Both assay solutions were incu-

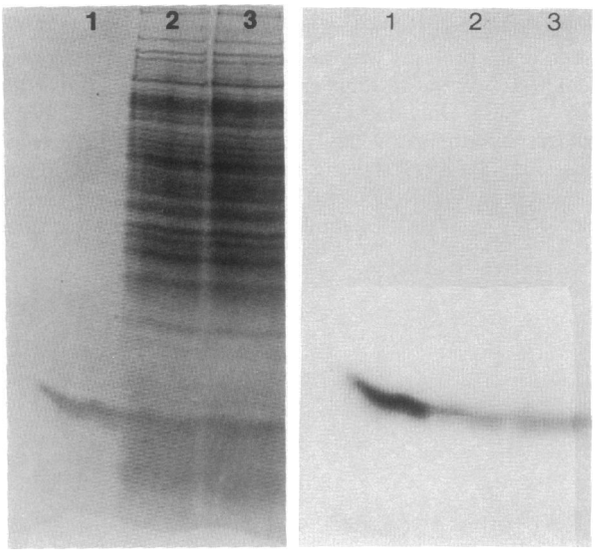

FIG. 4. (*Left*) $NaDodSO_4$/polyacrylamide gel of the following samples: lane 1, 10 μg of [^{32}P]P-(Ser)-HPr; lane 2, 10 μg of [^{32}P]P-(Ser)-HPr, 1 mM $MgCl_2$, 20 mM *P-e*Prv, 10 μl of *S. aureus* S797A crude extract (1 g of wet cells in 2 ml of buffer); lane 3, components present in the sample of lane 2 and 10 mM *o*-nitrophenyl-1-β-D-galactopyranoside. The samples were incubated for 10 min at 37°C before being subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. (*Right*) Autoradiogram of *Left* after an 8-hr exposure period. The autoradiogram shows the same amount of ^{32}P bound to HPr in lanes 2 and 3. This fact indicates that the sugar does not accelerate loss of ^{32}P from P-(Ser)-HPr and that there is no transfer of the phosphate group of P-(Ser)-HPr to the sugar. Loss of ^{32}P from HPr in lanes 2 and 3 in comparison with the amount of label in [^{32}P]P-(Ser)-HPr that had not been exposed to S797A crude extract (lane 1) reflects the presence of P-(Ser)-HPr phosphatase activity in the S797A crude extract.

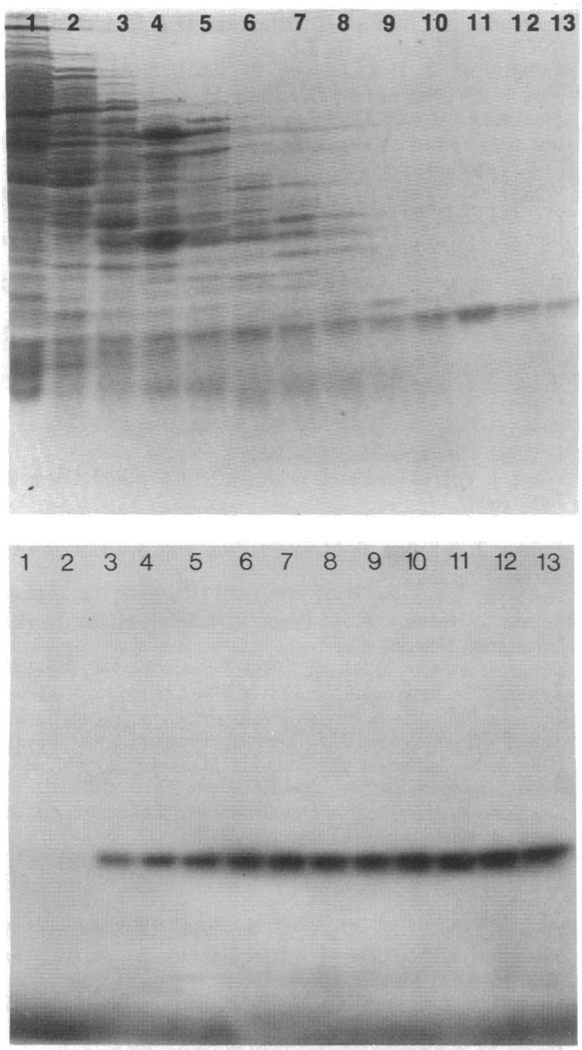

FIG. 5. Characterization of P-(Ser)-HPr phosphatase activity in *S. pyogenes* after elution from a Sephadex G-75 column. (*Upper*) P-(Ser)-HPr phosphatase was assayed after passage of an *S. pyogenes* crude extract through a Sephadex G-75 column; 20 μl of every other fraction from 21 to 39 and of fractions 42 and 45 was assayed for phosphatase activity before separation of the proteins by $NaDodSO_4$/polyacrylamide gel electrophoresis. Lane 13 contains purified ^{32}P-(Ser)-HPr only. (*Lower*) Autoradiogram of *Upper* after an exposure time of 26 hr. The autoradiogram shows maximal phosphatase activity in fractions 21 and 23 with a lesser amount of activity in fraction 25. In a separate experiment the first fractions containing protein were assayed; fraction 18 contained no phosphatase activity, whereas fraction 19 contained slight activity. Therefore, the P-(Ser)-HPr phosphatase eluted from the Sephadex G-75 (superfine) column immediately after the void volume with an M_r of ≈70,000.

bated until the latter reaction mixture was yellow due to phosphorylation and hydrolysis of the glycoside. The reaction was then terminated by addition of $NaDodSO_4$, and $NaDodSO_4$/polyacrylamide gel was run (Fig. 4). Autoradiography revealed the same amount of [^{32}P]P-(Ser)-HPr in the presence and absence of *o*-nitrophenyl-β-D-galactoside. Therefore, loss of the phosphate group from P-(Ser)-HPr had not been accelerated by incubation with the sugar.

In comparison with the amount of [^{32}P]P-(Ser)-HPr originally present, (Fig. 4, lane 1) almost half of the ^{32}P label had been hydrolyzed in both assay mixtures during the incubation, indicating that the *S. aureus* S797A crude extract contained P-(Ser)-HPr phosphatase activity. The phosphatase from *S. pyogenes* was found to be soluble (100,000 × *g* for 2 hr) (Fig. 5). It eluted from Sephadex G-75 near the void volume, suggesting a M_r of ≈70,000. On DEAE-cellulose it eluted at a relatively high salt concentration (0.55 M NaCl). In high salt it rapidly lost its activity, and further purification was not possible. An interesting observation was that the phosphatase activity was inhibited about 80% by addition of *P-e*Prv (10 mM) and purified enzyme I from *S. faecalis* to the incubation mixture (data not shown). Enzyme I or *P-e*Prv alone exerted no influence over phosphatase activity. Possibly *P-e*Prv and enzyme I phosphorylate the histidyl residue in P-(Ser)-HPr, and the doubly phosphorylated HPr is a poor substrate for the phosphatase. It is not known if the phosphatase is specific for P-(Ser)-HPr.

The HPr kinase has been partially purified. It was membrane associated in low salt (20 mM Tris·HCl, pH = 7.5, containing 0.1 mM dithiothreitol) but, after a 24-hr exposure to 100 mM phosphate buffer (pH = 7.5) containing 0.1 mM dithiothreitol, >90% of the kinase activity was released in a soluble form. The solubilized kinase seemed to be a small protein that eluted from a Sephadex G-75 column with an apparent M_r of 20,000. In the partially purified state, the HPr kinase activity was not stimulated by glucose 6-phosphate but showed some stimulation by 2-phosphoglycerate. The possibility of a membrane-associated regulatory subunit requires further investigation. The possibility of proteolytic release from the membrane has not been eliminated.

DISCUSSION

Previous reports from this and other laboratories have shown that the uptake of sugars into bacterial cells is regulated by at least five distinct mechanisms (20–22). Moreover, rapid efflux of preaccumulated sugar from the cytoplasm of the cell can be effected under certain physiological conditions (11, 16–18). Although inhibition of sugar uptake has been equated with the phenomenon of inducer exclusion (21), stimulation of sugar efflux has been shown to be related to inducer expulsion (11, 23), and therefore both regulatory phenomena function to control the rates of transcription of carbohydrate catabolic enzyme systems.

In studies aimed at elucidating the mechanism of inducer expulsion in species of *Streptococcus*, observations were made that led to the suggestion that ATP-dependent protein phosphorylation might control the process. Stimulation of TMG efflux was dependent both on a cellular source of ATP and a source of glycolytic intermediates (17). These observations led to an *in vitro* search for a protein kinase that was subject to allosteric activation by glycolytic intermediates. The present report provides (*i*) a preliminary description of this protein kinase, (*ii*) characterization of the principal protein target of this kinase, and (*iii*) identification of the protein phosphate phosphatase that reverses the action of the kinase.

In crude extracts of *S. pyogenes*, the kinase is membrane associated and is subject to stimulation by glucose 6-phosphate and other glycolytic intermediates. After its extraction from the membrane with high salt, a small protein (M_r = 20,000) can be purified that retains kinase activity but has lost sensitivity to glucose 6-phosphate stimulation. We postulate that a regulatory subunit, conferring sensitivity to glycolytic intermediates, is present as an integral constituent of the membrane. Possibly the kinase binds to the membrane through this regulatory subunit.

The principal protein product of the kinase reaction has been unequivocally identified as HPr of the phosphotransferase system. This phosphate carrier protein had previously been shown to be phosphorylated on a histidyl residue of the protein as an intermediate in the *P-e*Prv-dependent phosphorylation of sugar. By contrast, ATP-dependent kinase-catalyzed phosphorylation

of the protein results in the formation of a seryl phosphate that is not in equilibrium with the histidyl phosphate of the same protein. Incubation of P-(Ser)-HPr with sugar and the other enzyme constituents of the *P-e*Prv–sugar phosphotransferase system did not result in dephosphorylation. Instead, a soluble protein phosphate phosphatase (M_r = 70,000) catalyzed hydrolysis of the seryl phosphate bond. We postulate that ATP-dependent protein phosphorylation, demonstrated previously in bacteria (24–28) and of general applicability to the regulation of eukaryotic metabolism (29, 30), functions in bacteria specifically to control the rates of sugar transport and metabolism (17, 20–22). In this regard it is interesting to note that the only target of a bacterial protein kinase so far identified is isocitrate dehydrogenase in *E. coli* (31), a key enzyme in the Krebs cycle.

A cursory search of other bacteria has revealed that kinases probably phosphorylate proteins of the phosphotransferase system in *E. coli*, *S. faecalis*, and *S. aureus* (unpublished data). Interestingly, two principal *P-e*Prv–sugar phosphotransferase system protein targets of protein kinase action in the Gram-negative bacteria have been identified: HPr and the enzyme III^{glc} of the phosphotransferase system (unpublished data). Although P-Ser-HPr is acid and base stable, the phospho-enzyme III^{glc} formed from [γ-^{32}P]ATP is both acid and base sensitive. Because of the implication of the enzyme III^{glc} as a central regulatory protein for the control of inducer exclusion and cyclic AMP synthesis (21, 22, 32, 33), the physiological significance of this observation may prove to be most intriguing. Because HPr kinase activity is stimulated by glycolytic intermediates, the inhibition of sugar uptake by intracellular carbohydrate phosphates (20, 21, 34) can be postulated to be a consequence of P-(Ser)-HPr formation.

Protein kinases have long been recognized as highly important regulatory catalysts for the control of cellular anabolic, catabolic, and differentiative processes in higher organisms (29, 30). The present communication serves to provide a hint as to the possible functions of these enzymes in bacteria. The implications with respect to the control of cellular carbon and energy metabolism are likely to be far reaching.

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