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Stimulation of Dihydroxyacetone and Glycerol Kinase Activity in *Streptococcus faecalis* by Phosphoenolpyruvate-Dependent Phosphorylation Catalyzed by Enzyme I and HPr of the Phosphotransferase System

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Recently we reported the phosphoenolpyruvate (PEP)-dependent phosphorylation of a 55-kilodalton protein of *Streptococcus faecalis* catalyzed by enzyme I and histidine-containing protein (HPr) of the phosphotransferase system (J. Deutscher, FEMS Microbiol. Lett. 29:237–243, 1985). The purified 55-kilodalton protein was found to exhibit dihydroxyacetone kinase activity. Glycerol was six times more slowly phosphorylated than dihydroxyacetone. The K_ms were found to be 0.7 mM for ATP, 0.45 mM for dihydroxyacetone, and 0.9 mM for glycerol. PEP-dependent phosphorylation of dihydroxyacetone kinase stimulated phosphorylation of both substrates about 10-fold. Fructose 1,6-diphosphate at concentrations higher than 2 mM inhibited the activity of phosphorylated and unphosphorylated dihydroxyacetone kinase in a noncompetitive manner. The rate of PEP-dependent phosphorylation of dihydroxyacetone kinase was about 200-fold slower than the phosphorylation rate of III proteins (also called enzyme III or factor III), which so far have been considered the only phosphoryl acceptors of histidyl-phosphorylated HPr. *P*-Dihydroxyacetone kinase was found to be able to transfer its phosphoryl group in a backward reaction to HPr. Following [^{32}P]PEP-dependent phosphorylation and tryptic digestion of dihydroxyacetone kinase, we isolated a labeled peptide composed of 37 amino acids, as determined by amino acid analysis. The single histidyl residue of this peptide most likely carries the phosphoryl group in phosphorylated dihydroxyacetone kinase.

The phosphotransferase system (PTS) catalyzes the uptake and concomitant phosphorylation of carbohydrates in anaerobic and facultatively anaerobic bacteria (14, 19, 28). Usually, the PTS consists of four protein components forming a phosphorylation chain. Histidine-containing protein (HPr) becomes phosphorylated at the N-1 position of His-15 in a phosphoenolpyruvate (PEP)-dependent reaction catalyzed by enzyme I (3, 13). Histidyl-phosphorylated HPr (P-His-HPr) transfers the phosphoryl group to different III proteins. P-III proteins carry the phosphoryl group bound to the N-3 position of a single histidyl residue (7, 10, 16). Each P-III protein interacts with a certain sugar-specific enzyme II, the membrane-bound component of the PTS. Enzyme II catalyzes the transfer of the phosphoryl group from P-III to the sugar and its concomitant uptake.

Recently, the PEP-dependent phosphorylation of a 55-kilodalton (kDa) protein of *Streptococcus faecalis*, catalyzed by enzyme I and HPr of the PTS, has been reported (6). As this protein was isolated from glucose-grown cells, it was thought to be III^{glc}. However, no III activity could be attributed to this protein. Here we report that the 55-kDa protein is capable of catalyzing the ATP-dependent phosphorylation of dihydroxyacetone (DHA) and, at a lower rate, of glycerol. PEP-dependent phosphorylation of DHA kinase stimulates the rate of phosphorylation of both its substrates about 10-fold.

MATERIALS AND METHODS

Purification of proteins. Enzyme I (1) and HPr (17) of *S. faecalis* were purified as described previously. Serine-phosphorylated HPr (P-Ser-HPr) was obtained as described by Deutscher et al. (9). III^{lac} of *Staphylococcus aureus* was obtained from strain S305A (7). DHA kinase was purified from *S. faecalis* cells grown on either glucose, glycerol, or mannitol as described previously (6). As the enzyme contained one or two minor protein contaminants after purification, 0.7-mg portions were further purified by high-pressure liquid chromatography (HPLC) on a Bio-Sil TSK DEAE-3SW column (7.5 by 75 mm; Bio-Rad Laboratories, Richmond, Calif.). Proteins were eluted by applying a linear gradient of 0 to 55% buffer B (1.5 M NaCl in 10 mM for 55 min potassium phosphate, pH 6.7). Buffer A was 10 mM potassium phosphate, pH 6.7. The flow rate was 0.8 ml/min and the temperature was 22°C. Dihydroxyacetone kinase eluted at 0.62 M NaCl.

Assay systems. DHA kinase activity was measured by a coupled photometric assay. The assay mixture contained 50 mM Tris hydrochloride (pH 7.5), 1.5 mM ATP, 3 mM $MgCl_2$, 0.5 mM NADH, 1.5 mM DHA, 4 μg of glycerol 3-phosphate dehydrogenase, and 5 to 50 μl of sample in 320 μl. Activity of the enzyme was measured at 25°C by following the decrease in absorption at 334 nm. Glycerol kinase activity was measured in a similar way. The assay mixture contained 100 mM glycine-hydrazine, pH 8.8, 1.5 mM ATP, 3 mM $MgCl_2$, 0.5 mM NAD, 1.5 mM glycerol, 4 μg of glycerol 3-phosphate dehydrogenase, and 5 to 50 μl of sample in 320 μl. Glycerol kinase activity was also measured with [^{14}C]glycerol. The assay mixture contained 30 mM NH_4HCO_3 (pH 8.3), 5 mM $MgCl_2$, 2.5 mM ATP, 0.1 mM [^{14}C]glycerol (2 μCi/μmol), and 5 to 50 μl of sample in 100 μl. After 15 min of incubation at 37°C, glycerol and glycerol 3-phosphate were separated on a Dowex AG 1-X2 column (Bio-Rad Laboratories) as previously described (24). When the activity of phosphorylated DHA kinase was measured, the sample was first incubated for 30 min at 37°C in 25 mM

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NH_4HCO_3 (pH 8.3) together with 250 nmol of PEP, 1 μmol of $MgCl_2$, 1 μg of enzyme I, and 1 μg of HPr.

[^{32}P]PEP synthesis. [^{32}P]PEP was synthesized and prepared by the method of Alpert et al. (1).

Native polyacrylamide gels. Native polyacrylamide gels contained 0.4 M Tris-glycine, pH 9.3, 7.5% acrylamide, and 0.13% methylenebisacrylamide (7).

Separation of peptides from ^{32}P-labeled DHA kinase. An 0.7-mg amount of DHA kinase was dissolved in 300 μl of 50 mM NH_4HCO_3 (pH 8.3) and incubated together with 10 μg of enzyme I, 20 μg of HPr, 0.1 μCi of [^{32}P]PEP,and 5 mM $MgCl_2$. After 5 min of incubation at 37°C, unlabeled PEP was added to give a final concentration of 50 μM. This allowed almost complete phosphorylation of DHA kinase after a further 30-min incubation period. Then, 30 μg of trypsin was added. After 5 h of tryptic digestion at 37°C, the sample was loaded on a Nucleosil C-8 reverse-phase column (250 by 4.6 mm; Bischoff, Leonberg, Federal Republic of Germany). Peptides were eluted by applying a gradient of 0 to 40% buffer B for 40 min, 40 to 50% buffer B for 20 min, and 50 to 60% buffer B for 10 min. Buffer A was 0.1% trifluoroacetic acid, pH 2.0, and buffer B was 85% acetonitrile in 0.1% trifluoroacetic acid, pH 2.0. The main labeled peptide was collected and lyophilized before it was used for amino acid analysis.

Alkaline hydrolysis of ^{32}P-labeled proteins. For the phosphorylation of enzyme III specific for lactose (III^{lac}) from *S. aureus* (100 μg) and DHA kinase (300 μg), immobilized HPr of *S. faecalis* bound to Affi-Gel 15 (Bio-Rad) was used. The binding of HPr to Affi-Gel 15 was carried out as described by the manufacturer; 5 μg of enzyme I, 20 nmol of $MgCl_2$, and 0.1 μCi of [^{32}P]PEP (carrier-free) were dissolved in 20 μl of 50 mM NH_4HCO_3 (pH 8.3) and loaded onto a 200 μl column of Affi-Gel 15 carrying immobilized HPr. After 10 min of incubation at ambient temperature, enzyme I and unreacted [^{32}P]PEP were washed from the column. Subsequently, III^{lac} or DHA kinase, dissolved in 30 μl of NH_4HCO_3, was loaded onto the column and incubated for 10 min at ambient temperature. Phosphorylated III^{lac} and phosphorylated DHA kinase were washed from the column with 50 mM NH_4HCO_3 and collected in 60 μl fractions. To the fraction with the highest amount of radioactivity, 30 μl of 9 M KOH was added. For the phosphorylation of HPr, 100 μg was dissolved in 40 μl of 50 mM NH_4HCO_3 and incubated for 10 min at 37°C together with 2 μg of enzyme I, 0.1 μCi of [^{32}P]PEP, and 0.1 mM $MgCl_2$. Then 25 μl of 9 M KOH was added directly to the reaction mixture.

Alkaline hydrolysis of the different ^{32}P-labeled proteins was performed at 105°C for 4 h. Under these conditions the P-histidine (P-His) bond is fairly stable, whereas P-Ser, P-threonine (P-Thr), and P-tyrosine (P-Tyr) are rapidly hydrolyzed (26). After cooling, the pH of the samples was adjusted to between 9 and 10 with concentrated $HClO_4$. $KClO_4$ precipitated out of solution and was removed by centrifugation. Portions (2 to 10 μl) of the samples were spotted on electrophoresis paper (Whatmann 3MM). High-voltage paper electrophoresis was performed with 50 mM ammonium acetate buffer (pH 8.4), which allows the separation of 1- and 3-phosphohistidine (13).

Separation of P-DHA kinase from PEP, enzyme I, and HPr. DHA kinase (0.7 mg) was dissolved in 300 μl of 50 mM NH_4HCO_3 (pH 8.3) and incubated together with 20 μg of enzyme I, 20 μg of HPr, 5 mM $MgCl_2$, and 2.5 mM PEP for 30 min at 37°C. The sample was loaded on a Bio-Sil TSK DEAE-3SW column and run under the same conditions as described for unphosphorylated DHA kinase. PEP and HPr eluted at 0.22 M NaCl, enzyme I at 0.46 M NaCl, and P-DHA kinase at 0.62 M NaCl, at the same position as unphosphorylated DHA kinase.

Phosphoryl transfer reaction from P-DHA kinase to HPr. DHA kinase (0.7 mg) was phosphorylated and separated from PEP, enzyme I, and HPr as described above. The pH of the 0.7-ml fractions was adjusted to 8.3 with 50 μl of 1 M NH_4HCO_3. HPr (0.2 mg) was added to the fraction with the highest DHA kinase activity. The sample was incubated at 21°C, and after different time intervals 75-μl portions were taken and immediately frozen at −18°C. Two 75-μl samples were taken before HPr was added. One was incubated at 37°C for 40 min, and the other was kept frozen at −18°C. Samples were loaded on native polyacrylamide gels. HPr and P-His-HPr were separated by polyacrylamide gel electrophoresis, and in this way phosphoryl group transfer from P-DHA kinase to HPr could be demonstrated.

As the transfer reaction was too fast for measuring time dependence with this method, different amounts of HPr were added to the assay mixtures (500 μl total volume) used to measure DHA kinase activity and containing 15 μg of isolated P-DHA kinase. The phosphoryl group transfer could be followed by the decrease in DHA kinase activity. DHA kinase had a 10-fold-lower activity than P-DHA kinase.

Photometric assay of PEP-dependent phosphorylation of DHA kinase. The reaction mixture was placed in a semimicrocuvette and contained 600 pmol of enzyme I, 20 nmol of DHA kinase, 400 pmol of lactate dehydrogenase (rabbit muscle; Boehringer Mannheim), 2.5 mM $MgCl_2$, 2.5 mM PEP, 0.4 mM NADH, and 50 mM Tris hydrochloride, pH 7.5, in 200 μl. After a 5-min incubation at 20°C, the reaction was started by adding 300 pmol of HPr or P-Ser-HPr. The decrease in NADH was followed at 334 nm.

Amino acid analysis. Individual peptides were hydrolyzed in 6 M HCl in vacuo at 110°C for 24 h. Amino acid analysis was performed on a Bio Cal BC 200 amino acid analyzer equipped with a Durrum DC 6A column (0.4 by 23 cm) with a modified lithium citrate buffer system (2).

RESULTS

Glycerol and DHA kinase activity associated with a 55-kDa protein. Recently we reported the purification and PEP-dependent phosphorylation of a 55-kDa protein from *S. faecalis* (6). This protein was believed to carry out regulatory functions similar to those demonstrated for III^{glc} from *Escherichia coli* and *Salmonella typhimurium* (21, 22, 24, 25). When we tried to measure the influence of the 55-kDa protein on glycerol kinase from *S. faecalis*, we found that the purified 55-kDa protein contained glycerol kinase and DHA kinase activity. We therefore measured glycerol kinase and DHA kinase activity during isolation of the 55-kDa protein following the purification procedure described previously (6). We used photometric as well as radioactivity assays to determine glycerol and DHA kinase activity. After each column step, the kinase activity and the 55-kDa protein eluted at exactly the same position. As the 55-kDa protein, isolated by the above procedure, contained one minor protein contaminant, we purified 0.7-mg portions by HPLC on a Bio-Sil TSK DEAE-3SW column. The 55-kDa protein and DHA kinase activity comigrated again (Fig. 1). The 55-kDa protein was now electrophoretically pure, suggesting that it was DHA kinase.

Substrate specificity and physical properties of DHA kinase. DHA kinase phosphorylated glycerol and DHA. However, at pH 8.3 glycerol was found to be phosphorylated about six times more slowly than DHA. The K_ms were found to be

0.45 mM for DHA and 0.9 mM for glycerol. Phosphorylation of glucose, fructose, fructose 1-phosphate, fructose 6-phosphate, gluconate, and glyceraldehyde was not catalyzed by DHA kinase. Besides ATP, 3 mM CTP and UTP could also function as phosphoryl donors, although at a five times slower rate. GTP showed no activity. When ATP and DHA were used as substrates, the highest molecular activity (number of molecules transformed per minute by a single enzyme molecule) of the enzyme we measured was 122 for the enzyme in the unphosphorylated form and 1,180 for the enzyme in the phosphorylated form. The K_m for ATP was 0.7 mM.

As previously described (6), DHA kinase consists of a single polypeptide chain with a molecular weight of 55,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by size exclusion chromatography on a TSK G-2000 SW column. The enzyme was stored lyophilized at 4°C. Under these conditions the enzyme lost its activity very slowly. When it was kept in solution at −18°C, total loss of activity occurred within 2 to 3 weeks. Addition of dithiothreitol slowed the loss of activity. Phosphorylation of DHA kinase was not impaired by loss of activity. Even when the enzyme had totally lost its activity, it could still be completely phosphorylated. However, phosphorylation of inactive DHA kinase did not restore DHA kinase activity.

Stimulation of DHA kinase activity by PEP-dependent phosphorylation and inhibition by fructose 1,6-diphosphate. Enzyme I and HPr have been shown to phosphorylate the 55-kDa protein in a PEP-dependent reaction (6). We now found that phosphorylation of this protein led to a 10-fold increase in DHA kinase activity. This increase was observed when either DHA or glycerol was used as substrate. As with the phosphorylation of DHA kinase, stimulation of its activity could only be observed when PEP, enzyme I, and HPr were present together. To demonstrate that the activity of P-DHA kinase is also elevated in the absence of P-enzyme I, P-HPr, and PEP, we phosphorylated 0.7 mg of DHA kinase and separated it from P-enzyme I, P-HPr, and PEP by HPLC on a TSK DEAE-3SW column. P-DHA kinase eluted at the same position as unphosphorylated DHA kinase (Fig. 1A and 2A). Following the separation, phosphorylation of DHA kinase was judged by native polyacrylamide gel electrophoresis (Fig. 1B and 2B). Even though P-DHA kinase and DHA kinase eluted at the same position from the DEAE column, P-DHA kinase ran markedly faster on native polyacrylamide gels than unphosphorylated DHA kinase (6). It is clear that P-DHA kinase had about 10-fold-higher activity than unphosphorylated DHA kinase (Fig. 1A and 2A). There was almost no loss of the phosphoryl group from P-DHA kinase during electrophoresis at pH 9.3 (Fig. 2B). Similarly, when the pH of the P-DHA kinase-containing fractions was adjusted to 8.3 with 1 M NH_4HCO_3, P-DHA kinase could be stored for 3 weeks at 4°C and lose only about 15% of the phosphoryl group. However, after storage for 24 h at pH 6.7 at 4°C, about 80% of the phosphoryl bonds were hydrolyzed in P-DHA kinase, and the enzyme activity was reduced about ninefold.

When fructose 1,6-diphosphate was present in the assay mixture, it was found to inhibit DHA kinase activity at concentrations above 2 mM. The unphosphorylated as well as the phosphorylated enzyme was inhibited in a noncompetitive manner. The K_i of fructose 1,6-diphosphate was found to be 7 mM. The enzyme activity was reduced about fourfold in the presence of 20 mM fructose 1,6-diphosphate, a concentration which has been shown to occur in streptococcal cells during growth on glucose (30).

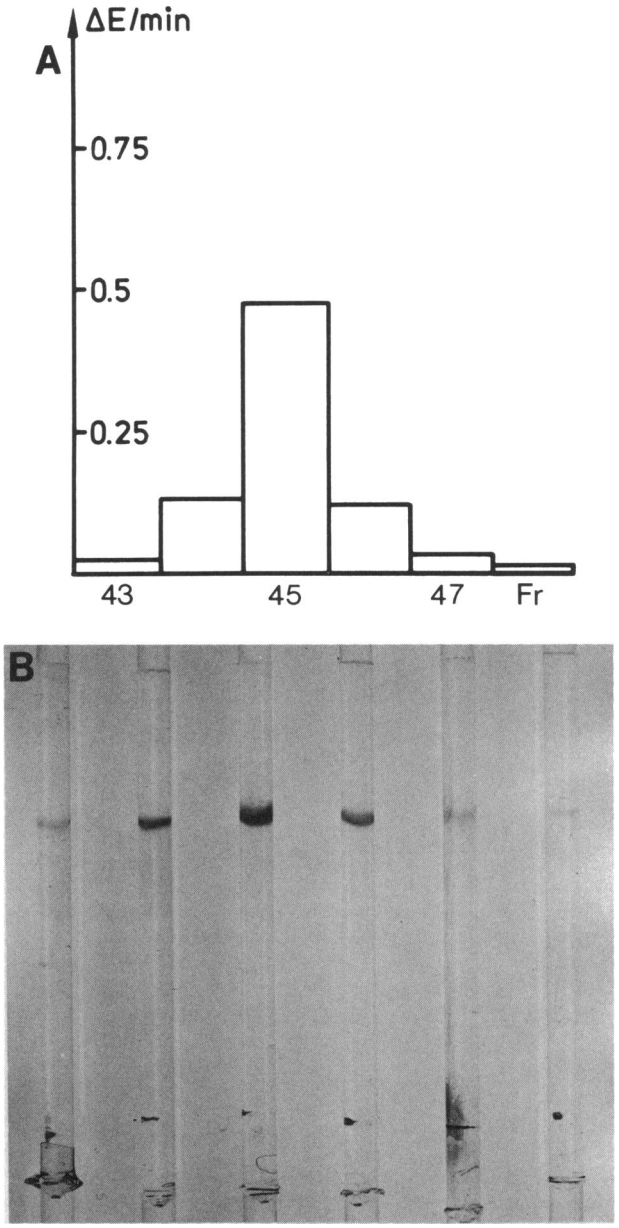

FIG. 1. HPLC of DHA kinase on a Bio-Sil TSK DEAE-3SW column. DHA kinase (0.7 mg) was loaded on a DEAE column and eluted with a linear gradient of NaCl as described in Materials and Methods. Portions (100 μl) of the 0.7-ml fractions were used to measure DHA kinase activity (A), and 50-μl portions were run on native polyacrylamide gels (B). The bars and fraction numbers in panel A correspond, from left to right, to the lanes in panel B.

Rate of DHA kinase phosphorylation. To measure the rate of PEP-dependent phosphorylation of DHA kinase, we used a coupled photometric assay. The pyruvate, formed during phosphorylation, was reduced by lactate dehydrogenase in an NADH-dependent reaction. The rate of phosphorylation of DHA kinase was measured in the presence of HPr or P-Ser-HPr. Interestingly, phosphorylation of DHA kinase was about four times faster in the presence of P-Ser-HPr than in the presence of HPr (Fig. 3). During the PTS-mediated sugar uptake reaction, enzyme I and HPr catalyze the PEP-dependent phosphorylation of either III proteins or enzyme II. We therefore compared the rate of phosphoryla-

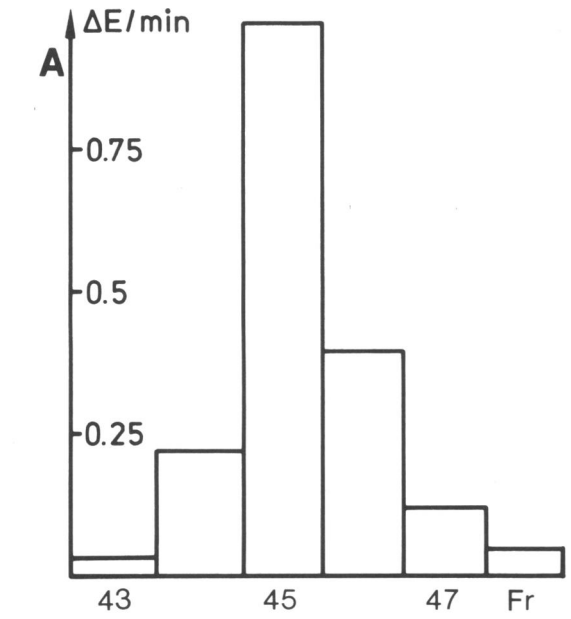

FIG. 2. HPLC of phosphorylated DHA kinase on a Bio-Sil TSK DEAE-3SW column. DHA kinase (0.7 mg) was phosphorylated by PEP, enzyme I, and HPr. The incubation mixture was separated on a DEAE column under the conditions described in the legend to Fig. 1; 20-μl portions of the fractions were used to measure DHA kinase activity (A), and 50-μl portions were run on native polyacrylamide gels (B). The bars and fraction numbers in panel A correspond, from left to right, to the lanes in panel B.

tion of DHA kinase with that of III^{lac} from *S. aureus*. III^{lac} was phosphorylated in the presence of either 15 pmol of HPr or 300 pmol of P-Ser-HPr. Phosphorylation of III^{lac} in the presence of HPr was about 200 times faster than that of DHA kinase, whereas the rate of phosphorylation of III^{lac} in the presence of P-Ser-HPr was only two times faster than that of DHA kinase in the presence of P-Ser-HPr (Fig. 3).

Phosphorylation of HPr by P-DHA kinase. DHA kinase (0.7 mg) was phosphorylated with PEP, enzyme I, and HPr, and P-DHA kinase was isolated by HPLC on a DEAE column as

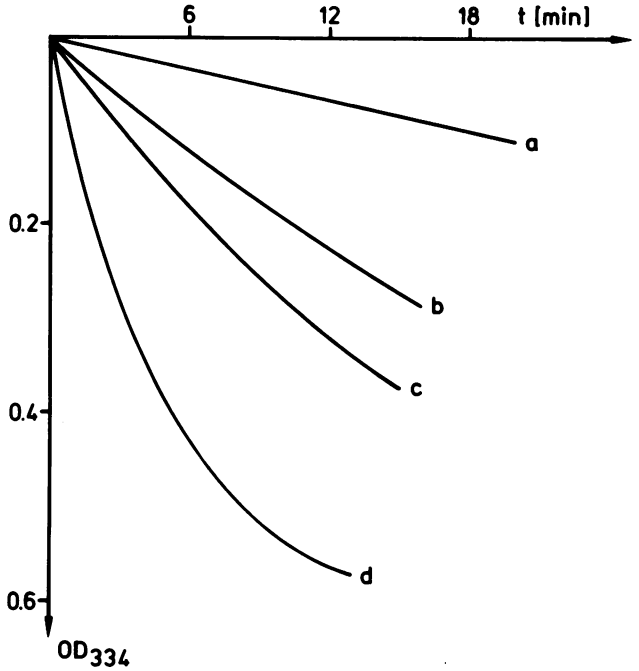

FIG. 3. Rate of PEP-dependent phosphorylation of DHA kinase and III^{lac} in the presence of either HPr or P-Ser-HPr. The formation of pyruvate from PEP was measured at 334 nm by a coupled photometric assay with lactate dehydrogenase and NADH as described in Materials and Methods. Phosphorylation of DHA kinase was determined in the presence of (a) 300 pmol of HPr or (b) 300 pmol of P-Ser-HPr, and phosphorylation of III^{lac} of *S. aureus* was determined in the presence of (c) 300 pmol of P-Ser-HPr or (d) 15 pmol of HPr.

described in Materials and Methods. HPr (0.2 mg) was added to the 0.7-ml fraction containing the highest amount of DHA kinase activity. Phosphorylation of HPr was demonstrated by electrophoresis of 75-μl portions on native polyacrylamide gels. P-DHA kinase was dephosphorylated and a small amount of P-His-HPr was formed (Fig. 4, lane c). However, the reaction was too fast to allow determination of the rate of the phosphoryl group transfer reaction with this method. The phosphoryl group transfer could also be demonstrated when different amounts of HPr (50 to 200 μg) were added to the assay mixture used to measure DHA kinase activity, which contained P-DHA kinase isolated as described above. At 30 s after the reaction had been started and had reached a constant rate, HPr was added. The reaction rate slowed down immediately and reached a new constant level, corresponding to the activity of unphosphorylated DHA kinase (Fig. 5). In the presence of 40 μM HPr, dephosphorylation of P-DHA kinase was complete within 1 min.

Isolation of a ^{32}P-labeled tryptic peptide from [^{32}P]DHA kinase. DHA kinase (0.7 mg) was phosphorylated with [^{32}P]PEP, enzyme I, and HPr as described in Materials and Methods. Subsequently, trypsin was added to digest phosphorylated DHA kinase. Peptides were separated by HPLC on a Nucleosil C-8 reverse-phase column. Radioactivity and UV absorbance (230 nm) of the effluent were monitored by a radioactivity detector (Berthold LB 504) connected to an Apple IIe computer and by a UV monitor (Jasco Uvidec 100 III). Peaks of radioactivity were integrated for quantitative evaluation. Besides the PEP-phosphate peak eluting at 4 min, there were two minor peaks

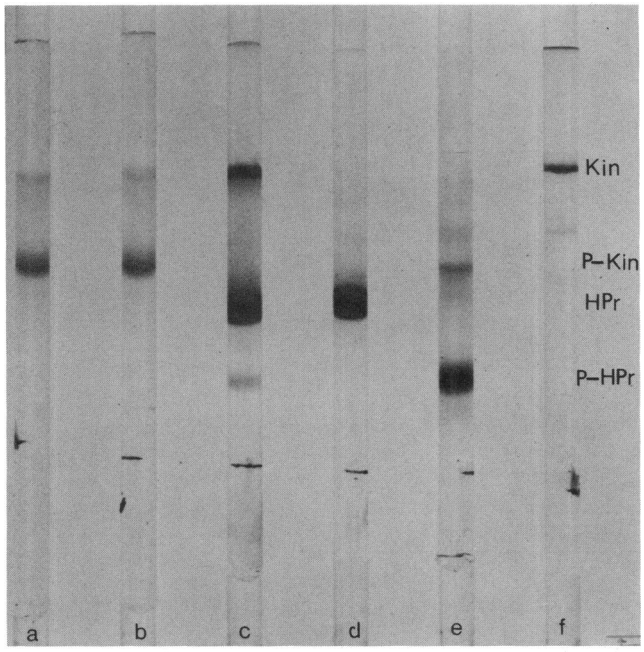

FIG. 4. Phosphoryl transfer from P-DHA kinase (P-Kin) to HPr demonstrated by polyacrylamide gel electrophoresis. The following samples were loaded onto the gels: (a) 4 μg of P-DHA kinase kept at −18°C for 5 min; (b) 4 μg of P-DHA kinase kept at 37°C for 40 min; (c) 10 μg of P-DHA kinase incubated with 20 μg of HPr for 1 min at 21°C; (d) 20 μg of HPr; (e) 20 μg of P-His-HPr (HPr incubated with 2 μg of enzyme I, 10 mM PEP, and 5 mM $MgCl_2$ for 10 min at 37°C); and (f) 3 μg of DHA kinase (Kin).

of radioactivity and one major peak (Fig. 6). The major labeled peptide revealed the following amino acid composition: 3 Asx, 1 Thr, 2 Ser, 5 Glx, 3 Pro, 5 Gly, 5 Ala, 2 Val, 2 Met, 1 Ile, 2 Leu, 2 Tyr, 2 Phe, 1 Lys, and 1 His. The single histidyl residue most likely carried the phosphoryl group in phosphorylated DHA kinase.

Nature of the phosphoryl bond in [^{32}P]DHA kinase. [^{32}P]DHA kinase, [^{32}P]III^{lac} from *S. aureus*, and [^{32}P]His-HPr from *S. faecalis* were prepared and subjected to alkaline hydrolysis as described in Materials and Methods. The two latter phosphoproteins were run as standards in subsequent high-voltage paper electrophoresis. P-III^{lac} has been shown by nuclear magnetic resonance to contain N-3-phosphohistidine (16), whereas P-His-HPr was found to contain N-1-phosphohistidine (13). After autoradiography, the major radioactive spot of hydrolyzed [^{32}P]DHA kinase comigrated with 3-phosphohistidine, whereas a second radioactive spot ran between 1- and 3-phosphohistidine, possibly caused by phosphoryl transfer during hydrolysis. Only a minute amount of P_i was present in the hydrolysate (Fig. 7).

DISCUSSION

There is genetic and biochemical evidence that the PEP-dependent phosphorylation of the components of the PTS is used not only for the sugar uptake reaction, but also for the regulation and control of carbohydrate uptake systems. *E. coli* and *S. typhimurium crr* mutants (catabolite repression resistant) were found to have low levels of cyclic AMP (4, 11). Recently, III^{glc} of the PTS has been shown to be encoded by the *crr* gene (20), suggesting that P-III^{glc} is involved in stimulation of adenylate cyclase activity. III^{glc} was also found to bind to the lactose permease of *E. coli* and to glycerol kinase of *S. typhimurium*, inhibiting both enzymes, whereas P-III^{glc} had no effect on these two enzymes (21, 22, 24, 25). Here we report for the first time that PEP-dependent phosphorylation of a non-PTS protein, catalyzed by enzyme I and HPr, is used to regulate the enzymatic activity of this protein. The previously described PEP-dependent phosphorylation of DHA kinase (6) was found to stimulate the ATP-dependent phosphorylation of DHA and glycerol about 10-fold. In bacterial cells, glycerol

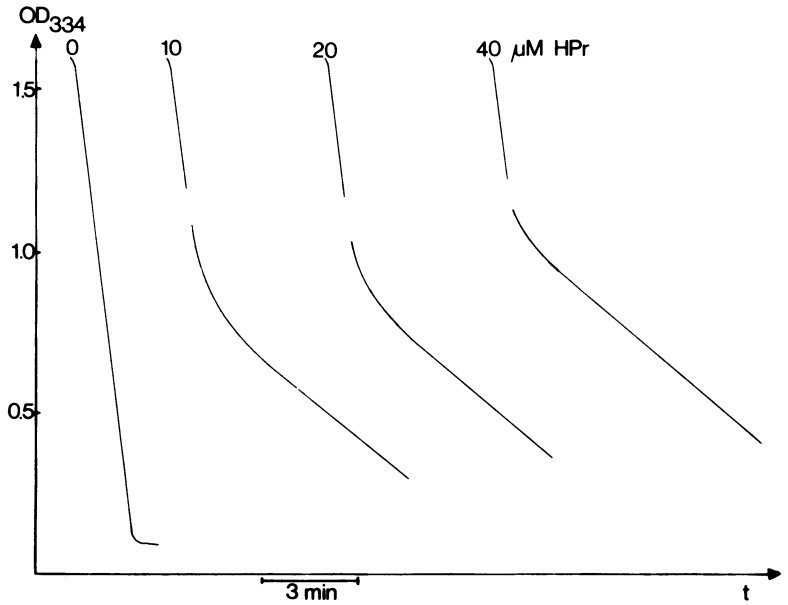

FIG. 5. Phosphoryl transfer from P-DHA kinase to HPr followed by a decrease in enzyme activity. DHA kinase activity was measured as described in Materials and Methods. At 30 s after the reaction had been started, different amounts of HPr (0, 50, 100, and 200 μg) were added to assay mixtures containing 10 μg of P-DHA kinase. After addition of HPr, the reaction slowed, indicating dephosphorylation of P-DHA kinase in the presence of HPr. The unphosphorylated enzyme had a 10-fold lower activity than the phosphorylated enzyme.

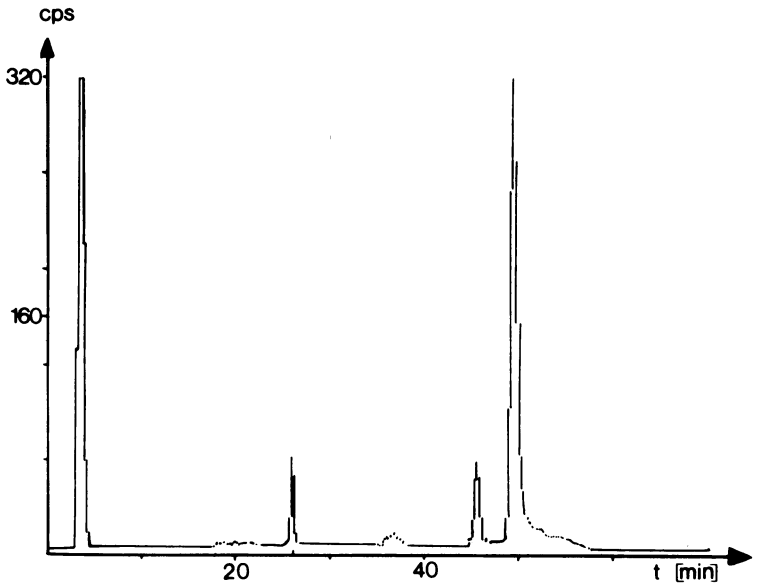

FIG. 6. Separation of peptides by HPLC on a C-8 reverse-phase column after tryptic digestion of 0.7 mg of ^{32}P-labeled DHA kinase. Following phosphorylation of DHA kinase with [^{32}P]PEP and tryptic digestion, peptides were separated by HPLC as described in Materials and Methods. The figure shows the distribution of radioactivity in the effluent.

dissimilation can be achieved by two chemical modes: following uptake via the glycerol facilitator, glycerol is either first phosphorylated by glycerol kinase and subsequently oxidized to DHA phosphate by glycerol 3-phosphate dehydrogenase, or it is first oxidized by glycerol dehydrogenase to DHA and subsequently phosphorylated to DHA phosphate by an ATP-dependent DHA kinase (18). In *E. coli*, DHA was found to be phosphorylated in a PEP-dependent reaction. The uptake and concomitant phosphorylation of DHA in *E. coli* are catalyzed by the PTS (15). In *E. coli* and most of the other bacteria investigated, the pathway involving ATP-dependent glycerol phosphorylation as the first step was found to be the main route of glycerol dissimilation (5). However, some bacteria such as *Bacillus subtilis* and *Klebsiella aerogenes* were found to possess the enzymes for both modes of glycerol dissimilation (12, 18). In *K. aerogenes*, glycerol kinase was found to phosphorylate glycerol at a higher rate than DHA, whereas DHA kinase phosphorylated DHA faster than glycerol (29). However, besides the DHA kinase described here, no other kinase phosphorylating glycerol or DHA could be detected in *S. faecalis* cells grown on glycerol. Similar to the results obtained with *ptsI* and *ptsH* mutants of *S. typhimurium* (4), *B. subtilis* mutants with a defective PTS seemed to be impaired in the inducibility of their glycerol facilitator and glycerol kinase (23, 27). In gram-negative bacteria glycerol kinase was found to be allosterically regulated by interaction with III^{glc} of the PTS, a regulatory event termed inducer exclusion (22, 25). Despite an intensive search, the existence of III^{glc} in gram-positive bacteria has not been proven. According to the results presented here, it is possible that glycerol uptake and metabolism in streptococci is regulated by PEP-dependent protein phosphorylation catalyzed by enzyme I and HPr. DHA kinase competes with III proteins for the common phosphoryl donor P-His-HPr. However, phosphorylation of DHA kinase was found to be slower than that of III proteins. Thus, P-DHA kinase will only be formed when no PTS sugar is taken up by the cell. In the absence of a PTS sugar, glycerol will be 10 times more rapidly metabolized by streptococcal cells due to the formation of P-DHA kinase. In the presence of a PTS sugar only the less active, unphosphorylated form of DHA kinase will be present in the cell. Dephosphorylation of P-DHA kinase most likely does not require a phosphoprotein phosphatase. P-DHA kinase was found to be able to transfer its phosphoryl group at a high rate to HPr. The uptake of a PTS sugar which leads to dephosphorylation of P-His-HPr will also lead to dephosphorylation of P-DHA kinase and thus slow down the metabolism of glycerol. This model is in agreement with the observation that glycerol uptake in *B. subtilis* mutants with low enzyme I activity is more severely repressed by PTS sugars than in cells with normal enzyme I activity (27). The lower enzyme I activity leads to stronger competition for P-His-HPr. However, a possible involvement of III^{glc} in the regulation of glycerol uptake in *B. subtilis* is also in agreement with the above observation. In vitro, DHA kinase was found to be phosphorylated four times faster in the presence of P-Ser-HPr than in the presence of HPr. However, the physiological importance of this observation is questionable. Fructose 1,6-diphosphate, which stimulates the formation of P-Ser-HPr (8, 26), was found to inhibit the activity of phosphorylated and unphosphorylated DHA kinase. Proteins involved in glycerol uptake and metabolism were always found to be inducible, and growth on glucose severely repressed the synthesis of these proteins (18). The DHA kinase described here was isolated in similar amounts from cells grown on either glucose, mannitol, or glycerol. This enzyme therefore seems to be expressed constitutively. Growth on glucose did not affect its expression.

As in phosphorylated III proteins (7, 10, 16), the phosphoryl group in P-DHA kinase is most likely bound to the N-3 position of a histidyl residue. After alkaline hydrolysis of [^{32}P]DHA kinase and subsequent paper electrophoresis, the major radioactive spot comigrated with 3-phosphohistidine. As III proteins and DHA kinase are phosphorylated by P-His-HPr, a similarity in phosphorylation sites could be expected. The amino acid sequence around the site of phosphorylation in III^{gct}, III^{mtl}, and DHA kinase of *S. faecalis* is under investigation. PEP-dependent phosphorylation of a protein with unknown function,

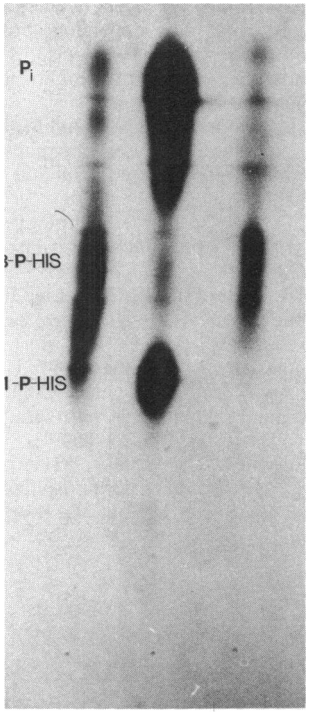

FIG. 7. High-voltage paper electrophoresis of hydrolyzed [^{32}P]DHA kinase. Phosphorylation and subsequent hydrolysis of III^{lac}, HPr, and DHA kinase was carried out as described in Materials and Methods. In a subsequent paper electrophoresis, hydrolyzed [^{32}P]III^{lac} and [^{32}P]His-HPr were run as standards for 3-phosphohistidine (3-P-His) (right-hand lane) and 1-phosphohistidine (1-P-His) (middle lane), respectively. [^{32}P]His-HPr was not separated from [^{32}P]PEP after phosphorylation, and the hydrolysate therefore contained large amounts of P_i, which was almost absent from hydrolyzed [^{32}P]III^{lac} and [^{32}P]DHA kinase. The main radioactive spot in hydrolyzed [^{32}P]DHA kinase comigrated with 3-phosphohistidine.

catalyzed by enzyme I and HPr, has been reported (31). The protein had a molecular weight of 40,000 and was observed in *S. typhimurium* and *E. coli*. The amount of this protein in the cells was dependent on the growth conditions, mainly the carbohydrate source. The gene locus of this protein is not within the *pts* operon, as the protein was also found in an *S. typhimurium* strain with an extensive deletion in the *pts* operon.

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