Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System: P-Ser-HPr and Its Possible Regulatory Function†

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ABSTRACT: HPr of the bacterial phosphotransferase system is a histidine-containing phospho-carrier protein. It is phosphorylated at a single histidyl residue with phosphoenolpyruvate (PEP) and enzyme I and transfers the histidyl-bound phosphoryl group to a variety of factor III proteins. Recently, we described an HPr phosphorylated at a seryl residue (P-Ser-HPr), which is formed in an adenosine 5′-triphosphate dependent reaction catalyzed by a protein kinase [Deutscher, J., & Saier, M.-H., Jr. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6790–6794]. Now we demonstrate that this P-Ser-HPr is an altered substrate of phosphorylated enzyme I and factor III proteins compared to unphosphorylated HPr. Thus, P-Ser-HPr of *Streptococcus lactis* is phosphorylated about 5000 times slower by PEP and enzyme I than HPr. The slow phosphorylation by PEP and enzyme I can be overcome when factor III protein specific for gluconate (factor III^{Gct}) of *Streptococcus faecalis* is added. Most likely, a complex of P-Ser-HPr and factor III^{Gct} is formed which then becomes phosphorylated as fast as free HPr. Factor III protein specific for lactose (factor III^{Lac}) of *Staphylococcus aureus* also enhances the phosphorylation of P-Ser-HPr by enzyme I and PEP, but its effect is lower. Thus, P-Ser-HPr is phosphorylated 70–100-fold slower in the presence of factor III^{Lac} than in the presence of factor III^{Gct}. The described interaction of P-Ser-HPr with enzyme I in the presence of different factor III proteins could account for the regulation of sugar uptake within the phosphotransferase system. Some of the phosphoenolpyruvate-dependent phosphotransferase system sugars like glucose are known to be taken up in preference to others, for example, lactose.

The bacterial phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system consists of four components (Simoni et al., 1968). Enzyme I and HPr are the two common nonspecific proteins, whereas factor III and the membrane-bound enzyme II are specific for a certain sugar. A reaction scheme is shown for lactose uptake in *Staphylococcus aureus* (Kalbitzer et al., 1981):

$$\text{PEP} + \text{enzyme I} \overset{Mg^{2+}}{\rightleftharpoons} \text{P-enzyme I} + \text{pyruvate}$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I}$$

$$\text{P-HPr} + \text{FIII}^{Lac} \rightleftharpoons \text{P-FIII}^{Lac} + \text{HPr}$$

$$\text{P-FIII}^{Lac} + \text{lactose}_{out} \xrightarrow{\text{enzyme II}^{Lac},\ Mg^{2+}} \text{FIII}^{Lac} + \text{lactose-6-P}_{in}$$

HPr has been shown to be phosphorylated by P-enzyme I at the N-1 position of a sinlge histidyl residue. This histidyl residue was found to be His-15 in the sequence of *S. aureus* (Beyreuther et al., 1977), of *Salmonella typhimurium* (Weigel et al., 1982), and of *Streptococcus faecalis* and *Bacillus subtilis* (Muss, 1982). HPr then transfers the phospho group to the N-3 position of His-82 in $FIII^{Lac}$ (Deutscher et al., 1982; K. Stüber et al., unpublished results). An additional, ATP-dependent phosphorylation of HPr has been found (Deutscher & Saier, 1983). It occurs at a single seryl residue, the position of which is not yet identified within the HPr primary structure and requires a protein kinase. As the ATP-dependent phosphorylation of HPr in whole cells is largely stimulated by glucose in the growth medium and by glucose 6-phosphate and 2-phosphoglycerate in the crude extract, it was considered to have a regulatory function. In 1967, McGinnis and Paigen showed that glucose exerts an immediate and reversible inhibition on the uptake and utilization not only of non-PTS sugars but also of PTS sugars. Later they showed that the inhibitory effect of glucose is due to diminished carbohydrate uptake rates (McGinnis et al., 1973). Five mechanisms were discussed for this kind of inhibition (Dills et al., 1980). One of these was the competition of enzyme II complexes of FIII proteins for the common phosphoryl donor protein P-His-HPr. Here we demonstrate that P-Ser-HPr, the formation of which is dependent on a metabolizable sugar in the medium, most likely contributes to this kind of regulatory mechanism.

Materials and Methods

Bacterial Strains. Streptococcus lactis 11454 (Streptococcenzentrale Kiel) was used for the isolation of HPr and P-Ser-HPr. Cells were grown in a 100-L Chemap fermenter at 37 °C to late log phase in complex medium containing 1000 g of yeast extract (Ohly, Hamburg), 200 g of tryptone (Difco), 100 g of peptone casein [tryptic digested (Merck)], and 1000 g of glucose. The pH was kept at 7 by addition of 10% NaOH. *S. aureus* S305A and *S. faecalis* 26487 were also grown in a 100-L Chemap fermenter in complex medium containing 1000 g of yeast extract (Ohly, Hamburg), 200 g of tryptone (Difco), and 250 g of Na_2HPO_4, and for *S. faecalis* 1000 g of gluconate in 100 L.

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phospho-carrier protein; P-Ser-HPr, HPr phosphorylated at a seryl residue; P-His-HPr, HPr phosphorylated at a histidyl residue; $FIII^{Gct}$, factor III protein specific for gluconate; $FIII^{Lac}$, factor III protein specific for lactose; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; ATP, adenosine 5′-triphosphate; $FIII^{Glc}$, factor III protein specific for glucose; $FIII^{Mtl}$, factor III protein specific for mannitol; EI, enzyme I; P_2HPr or (P-Ser, P-His)-HPr, HPr phosphorylated at a histidyl and a seryl residue; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; P-enzyme I, phosphorylated enzyme I; Man, mannose; Suc, sucrose.

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Enzyme I. Enzyme I was purified from *S. faecalis* and *S. lactis* according to C. A. Alpert and W. Hengstenberg (unpublished results).

Factor III^{Lac}. Factor III^{Lac} was purified from *S. aureus* S305A according to Deutscher et al. (1982).

Factor III^{Gct}. Factor III^{Gct} was isolated from *S. faecalis* as described by Bernsmann et al. (1982).

P-Ser-HPr Phosphatase. P-Ser-HPr phosphatase was partially purified from *S. faecalis* by ion-exchange chromatography on DEAE-cellulose (Whatman DE-23, 12 × 30 cm) with an 8-L linear gradient of 0–1.0 M NaCl in standard buffer (0.05 M Tris-HCl, pH 7.5, 10^{-4} M DTT, 10^{-4} M PMSF, and 10^{-4} M EDTA), fractionated ammonium sulfate precipitation (35–55% saturation), acid precipitation at pH 5.3 (phosphatase activity in the supernatant), chromatography on Sephadex G-150, and a second ion-exchange chromatography on DEAE-cellulose (Whatman DE-52, 1.6 × 27 cm) with a 600-mL linear greadient of 0–0.3 M NaCl in standard buffer. To assay the phosphatase activity, either we used [^{32}P]P-Ser-HPr according to Deutscher & Saier (1983) or 10 μg of P-Ser-HPr was incubated with a sample in 50 mM Tris-HCl, pH 7.5, and 1 mM $MgCl_2$ for 15 min. Then the assay mixture was loaded on a native polyacrylamide gel and the amount of P-Ser-HPr converted to HPr was estimated visually after the gels were stained and destained. HPr and P-Ser-HPr were separated on native polyacrylamide gels. An unusual loss of activity during purification, described for the phosphatase of *Streptococcus pyogenes* (Deutscher & Saier, 1983), was not observed.

^{32}P-Labeled Phosphoenolpyruvate. [^{32}P]PEP was synthesized and prepared by using a modified method of Lauppe et al. (1972). [^{32}P]Orthophosphate was used carrier free (purchased from Amersham, 10 mCi/mL), and we therefore obtained carrier-free [^{32}P]PEP. The exact reaction conditions for the small-scale synthesis are described by C. A. Alpert and W. Hengstenberg (unpublished results).

Polyacrylamide Gel Electrophoresis. The tube gels contained 7.5% acrylamide, 0.13% methylenebis(acrylamide), and 0.4 M Tris–glycine, pH 9.3. Gels were stained with Coomassie Brilliant Blue. The destaining solution contained 7.5% acetic acid and 5% methanol (v/v).

Urea Gels. Urea gels were always freshly prepared. Urea (12 g), acrylamide (0.9 g), Tris (0.6 g), glycine (94 mg), methylenebis(acrylamide) (24 mg), and ammonium persulfate (11 mg) were mixed together. To this mixture were added 9 mL of H_2O, 9 mL of 0.8 M Tris–glycine, pH 9.3, and 20 μL of TEMED.

PEP-Dependent Phosphorylation of Proteins. Different reaction mixtures were used for the phosphorylation of the PTS proteins. They are described in the text. For the phosphorylation of FIII proteins with [^{32}P]PEP, the assay mixture was separated by HPLC on a TSK-125 column (30 × 0.75 cm) (Bio-Rad) at 21 °C and at a flow rate of 1 mL/min in 100 mM potassium phosphate buffer, pH 6.8. Radioactivity and UV absorption (230 nm) of the effluent were monitored by a radioactivity detector (Berthold LB 504) connected to an Apple II computer and by a UV monitor (Jasco Uvidec 100 III). The peaks of radioactivity were integrated for quantitative evaluations of protein phosphorylation. On the TSK-125 column, HPr and PEP were poorly separated. Native gels were therefore used to detect the PEP-dependent phosphorylation of HPr and P-Ser-HPr. In both cases, the PEP-dependent phosphorylation caused a significant shift of the protein band in the direction of the anode. When FIII proteins were phosphorylated, they migrated to the same position on native gels as the unphosphorylated proteins. To detect simultaneously the PEP-dependent phosphorylation of HPr, P-Ser-HPr, and FIII proteins, urea gels were run. On urea gels, all proteins investigated were shifted in the direction of the anode following phosphorylation.

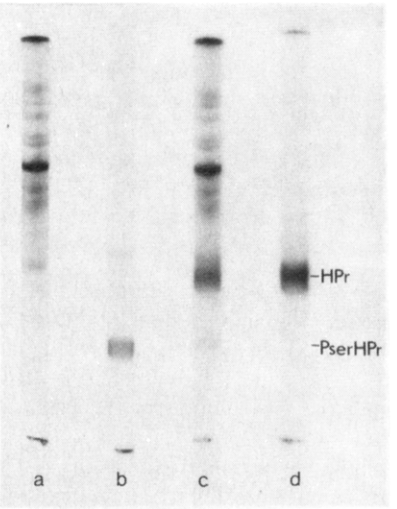

FIGURE 1: Native polyacrylamide gels. Lane a shows partially purified protein phosphatase, lane b 10 μg of P-Ser-HPr, lane c 10 μg of P-Ser-HPr after a 15-min incubation at 37 °C in the presence of phosphatase, and lane d 20 μg of HPr. Through the action of protein phosphatase, most of the P-Ser-HPr is converted to unphosphorylated HPr.

Photometric Assay of FIII^{Lac} *Phosphorylation.* The assay mixture for the photometric assay was placed in a semimicrocuvette and had a total volume of 200 μL. It contained 300 pmol of enzyme I (*S. lactis*), 8.6 nmol of FIII^{Lac}, 400 pmol of LDH (hog muscle, Boehringer), 2.5 mM $MgCl_2$, 2.5 mM PEP, 0.4 mM NADH, and 50 mM Tris-HCl, pH 7.5. This mixture was preincubated 5 min at 30 °C before the reaction was started by adding different amounts of HPr or P-Ser-HPr. The decrease of NADH was followed at 334 nm.

Results

Two HPr species were isoalted from *S. lactis* 11454 (Streptococcenzentrale Kiel) after growth on glucose. On native polyacrylamide gels, they migrated to different positions. The faster moving HPr migrated to the same position as P-His-HPr. Since P-His-HPr is known to be unstable and to rapidly lose its phosphoryl group (Anderson et al., 1971), we considered it to be the recently described P-Ser-HPr (Deutscher & Saier, 1983). To purify the two HPr proteins, we used the same procedure as described by Kalbitzer et al. (1982). By the last purification step, ion-exchange chromatography on DEAE-cellulose (Whatman DE-52), HPr and P-Ser-HPr were not separated completely. To isolate P-Ser-HPr absolutely free of HPr, it was run twice on DEAE-cellulose. With 300 g of cells (wet weight), the final yield was 15 mg of P-Ser-HPr and 40 mg of HPr. P-Ser-HPr contained a minor protein contaminant, which was not HPr, as it was not affected by the phosphorylation experiments and ran at a different position than HPr on urea gels. To demonstrate that the second HPr species is indeed P-Ser-HPr, we incubated it with the partially purified protein phosphatase from *S. faecalis* which we call P-Ser-HPr phosphatase. After a 15-min incubation, the assay mixture was loaded on a native polyacrylamide gel. The results are presented in Figure 1. Lane a shows the P-Ser-HPr phosphatase, lane b 10 μg of P-Ser-HPr, lane c the incubation mixture with 5 μg of P-Ser-HPr, and land d 20 μg of HPr. From lane c, we can see that through

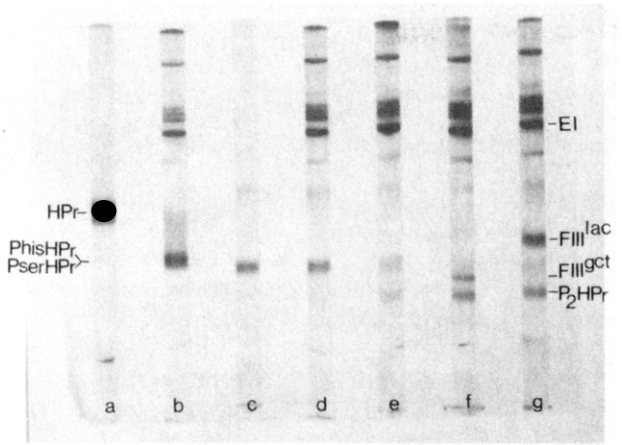

FIGURE 2: PEP-dependent phosphorylation of HPr and P-Ser-HPr, demonstrated on native polyacrylamide gels. The phosphorylation mixture contained 5 mM $MgCl_2$, 5 mM PEP, 10 μg of enzyme I of *S. lactis*, and 10 μg of HPr or P-Ser-HPr. It was incubated 3 min (or 25 min for lane e) at 21 °C. The pH was 7.5 in 50 mM Tris-HCl buffer. The reaction was stopped, cooling the samples to 4 °C. The standards are 20 μg of HPr for lane a and 10 μg of P-Ser-HPr for lane c. The migration position of enzyme I is known from homogeneous enzyme I of *S. lactis*. Lane b shows the phosphorylation of HPr, and lane d shows the phosphorylation of P-Ser-HPr. For lane e, the incubation time was extended to 25 min. Lanes f and g demonstrate the influence of FIII proteins on the phosphorylation of P-Ser-HPr. For lane f, 1 μg of FIII^{Gct} was added to the reaction mixture; for lane g, 5 μg of FIII^{Lac} was added. The conditions were otherwise the same as those for lane d.

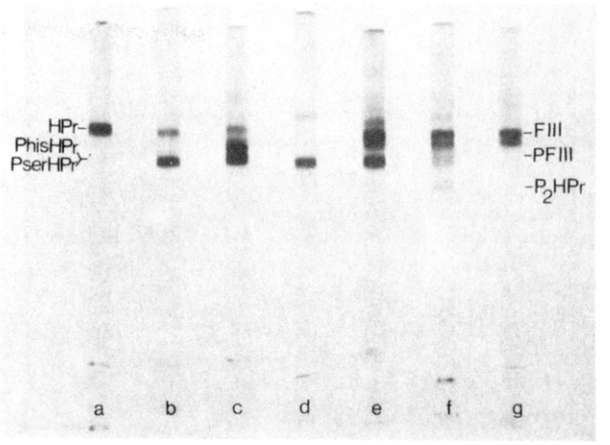

FIGURE 3: PEP-dependent phosphorylation of HPr or P-Ser-HPr and FIII^{Lac}, demonstrated on urea–polyacrylamide gels. The phosphorylation mixture had the same composition as in Figure 2, except that the amount of enzyme I was 100-fold reduced. The incubation time was 30 s (or 3 min for lane f) at 21 °C. The reaction was stopped by adding 100 μL of 8 M urea. The standards are 20 μg of HPr in lane a, 10 μg of P-Ser-HPr in lane d, and 5 μg of FIII^{Lac} in lane g. Lane b shows the phosphorylation of HPr and lane c the simultaneous phosphorylation of HPr and FIII^{Lac}. Lane e shows the phosphorylation of P-Ser-HPr in the presence of FIII^{Lac}. For lane f, the incubation time was extended 6-fold (3 min), and the amount of enzyme I was enhanced 5-fold. The amount of P-Ser-HPr was 2 μg.

the action of the phosphatase most of P-Ser-HPr is converted to a protein which migrates to the same position as free HPr. This indicates that we had indeed isolated P-Ser-HPr, as [^{32}P]P-Ser-HPr, prepared according to Deutscher & Saier (1983), gave the same result after incubation with phosphatase.

During the PEP-dependent phospho-transfer reaction, HPr is phosphorylated at a single histidyl residue (Beyreuther et al., 1977; Weigel et al., 1982). We tried now to investigate if P-Ser-HPr is phosphorylated by PEP and enzyme I at the same rate as HPr. The reaction mixture for the phosphorylation experiments contained $MgCl_2$ (5 mM), PEP (5 mM), enzyme I of *S. faecalis* (0.15 nmol), and HPr or P-Ser-HPr (1.2 nmol). The reaction mixture was incubated for 3 min (or 25 min in lane e) at 21 °C. Then it was cooled to 4 °C and loaded on native polyacrylamide gels. The results are shown in Figure 2. On lane b, HPr was completely phosphorylated by PEP and enzyme I. The P-His-HPr band is shifted in the direction of the anode compared to unphosphorylated HPr (2.4 nmol) in lane a. The smear above the P-His-HPr band, which tails to the position of the HPr band, is most likely due to slow dephosphorylation during electrophoresis (about 1.5 h). When the same experiment was conducted with P-Ser-HPr, only a small amount was phosphorylated to (P-Ser, P-His)-HPr as can be seen by comparison of lane c (1.2 nmol of P-Ser-HPr) and lane d. When the incubation time was extended to 25 min, approximately half of the P-Ser-HPr was converted to (P-Ser, P-His)-HPr (lane e). We also assayed PEP-dependent phosphorylation of P-Ser-HPr in the presence of two different FIII proteins. We added FIII^{Gct} (0.02 nmol) and FIII^{Lac} (0.14 nmol). The reaction conditions were otherwise the same as stated below. From lanes f and g of Figure 2, it can be seen that in the presence of FIII proteins P-Ser-HPr is phosphorylated much faster by PEP and enzyme I.

For a more quantitative description of HPr and P-Ser-HPr phosphorylation in the absence and presence of FIII proteins, we used urea gels. The incubation time was more accurate as the reaction was stopped by adding urea to the assay mixture. The amount of enzyme I was 100-fold reduced (1.5 pmol), and the amount of FIII^{Gct} was increased to 0.11 nmol. The incubation time was 30 s at 21 °C unless otherwise stated. All the other conditions were the same as those described for the experiments in Figure 2. Figure 3, lane a, shows HPr. In lane b, most of the HPr is phosphorylated to P-His-HPr by PEP and enzyme I. By comparison with the result for P-Ser-HPr, shown in lane e of Figure 2 (50-fold incubation time, 100-fold more enzyme I), we calculated that P-Ser-HPr is roughly 5000 times more slowly phosphorylated by PEP and enzyme I than is HPr. For the experiment shown in Figure 3, lane c, FIII^{Lac} was added to the incubation mixture. P-His-HPr and P-FIII^{Lac} migrate almost to the same position on urea gels. Both proteins are almost completely phosphorylated as can be seen by comparison with lane g, where unphosphorylated FIII^{Lac} was loaded on the gel. The two bands for FIII^{Lac} are probably due to incomplete denaturation by urea. After heating to 60 °C, we got only one band. Lane d of Figure 3 shows P-Ser-HPr. Under the reaction conditions used for the phosphorylation of HPr in lane b, no (P-Ser, P-His)-HPr could be detected (data not shown). The same was true when FIII^{Lac} was added to the reaction mixture (lane e). Only after the incubation time was extended to 3 min and the amount of enzyme I was increased 5-fold, (P-Ser, P-His)-HPr could be seen (lane f). For a better separation of P-Ser-HPr and FIII^{Lac}, the amount of P-Ser-HPr was reduced to 0.04 nmol. Under these conditions, half of the P-Ser-HPr and about the same amount of FIII^{Lac} were phosphorylated. The same experiments were conducted with FIII^{Gct} instead of FIII^{Lac}. The results are shown in Figure 4. The standards HPr (lane a), P-His-HPr (lane b), and P-Ser-HPr (lane d) are the same as in Figure 3. Lane c of Figure 4 shows a phosphoarylation experiment with FIII^{Gct} and HPr. As in the experiment with FIII^{Lac} (Figure 3, lane c), both proteins are almost completely phosphorylated. But the same was true when P-Ser-HPr was used instead of HPr, as can be seen from Figure 4, lane e. (P-Ser, P-His)-HPr migrates to the same position as FIII^{Gct}. The faster moving band, slightly separated,

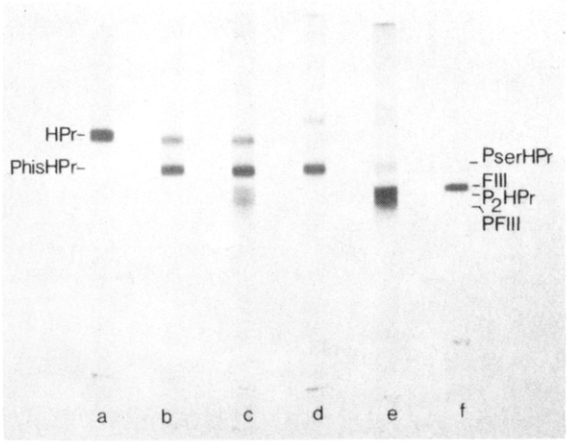

FIGURE 4: PEP-dependent phosphorylation of HPr or P-Ser-HPr and $FIII^{Gct}$, demonstrated on urea–polyacrylamide gels. The reaction conditions are the same as those for Figure 3. The standards are 20 μg of HPr in lane a, 10 μg of P-Ser-HPr in lane d, and 5 μg of $FIII^{Gct}$ in lane f. Lane b shows the phosphorylation of HPr; lane c shows the phosphorylation of HPr and $FIII^{Gct}$. Lane e shows the simultaneous phosphorylation of P-Ser-HPr and $FIII^{Gct}$.

Table I: PEP-Dependent Phosphorylation of $FIII^{Lac}$ and $FIII^{Gct}$ [a]

	radioactivity (cps)			% of total counts		
expt	EI	FIII	PEP	EI	FIII	PEP
1	212	1581	1836	5.8	43.7	50.5
2	341	108	3584	8.5	2.9	88.6
3	376	602	2507	10.8	17.5	65.2
4	80	1895	1340	2.4	57.2	40.4
5	46	1293	1463	1.7	46.2	52.2

[a] The assay mixture contained 5 mM $MgCl_2$, 80 pmol of enzyme I (*S. faecalis*), 12 pmol of HPr or P-Ser-HPr, 2.8 nmol of $FIII^{Lac}$ or 2.2 nmol of $FIII^{Gct}$, and 60 μM PEP (45 μCi/μmol) in a total volume of 80 μL. The pH was 7.5 in 50 mM Tris-HCl buffer. The incubation time was 15 s (or 7 min in experiment 3) at 21 °C. The reaction was stopped with 400 μL of ice-cold buffer and then loaded on a TSK-125 gel filtration column. Elution conditions are described under Materials and Methods. Beside enzyme I, the following proteins were included in the assay mixture: experiment 1, HPr and $FIII^{Lac}$; experiment 2, P-Ser-HPr and $FIII^{Lac}$; experiment 3, P-Ser-HPr and $FIII^{Lac}$, 7-min incubation period; experiment 4, HPr and $FIII^{Gct}$; experiment 5, P-Ser-HPr and $FIII^{Gct}$. The radioactive peaks were integrated, and the radioactivity (in counts per second) as well as the percentage of the total counts is listed.

represents P-$FIII^{Gct}$. In the case of $FIII^{Lac}$, the incubation time had to be extended 6-fold, and the amount of enzyme I had to be increased 5-fold to phosphorylate a much smaller amount of P-Ser-HPr and $FIII^{Lac}$.

To characterize the different influence of $FIII^{Gct}$ and $FIII^{Lac}$ on P-Ser-HPr more closely, we followed FIII phosphorylation using [^{32}P]PEP. Experiments were conducted as described in Table I. After the reaction was stopped, proteins were separated by HPLC on a TSK-125 gel filtration column. Radioactivity and UV absorption were detected as described under Materials and Methods. The results are listed in Table I. Under the conditions used in experiment 1, about 50% of [^{32}P]PEP is used to phosphorylate $FIII^{Lac}$. If P-Ser-HPr is used instead of HPr (experiment 2), almost no phosphorylation of $FIII^{Lac}$ can be observed. Even when the reaction time was extended to 7 min, only 17% of [^{32}P]PEP is used to phosphorylate $FIII^{Lac}$. From the data, we calculated that $FIII^{Lac}$ is phosphorylated 75–100 times more slowly by P-Ser-HPr than by HPr.

The same experiments were conducted with $FIII^{Gct}$ instead of $FIII^{Lac}$. In experiment 4, HPr was used, and it can be seen that $FIII^{Gct}$ is phosphorylated with approximately the same rate as $FIII^{Lac}$. In the presence of P-Ser-HPr (experiment 5), again about 50% of [^{32}P]PEP is used to phosphorylate $FIII^{Gct}$. Other than in the case of $FIII^{Lac}$, P-Ser-HPr did not alter the phosphorylation rate of $FIII^{Gct}$ compared to HPr.

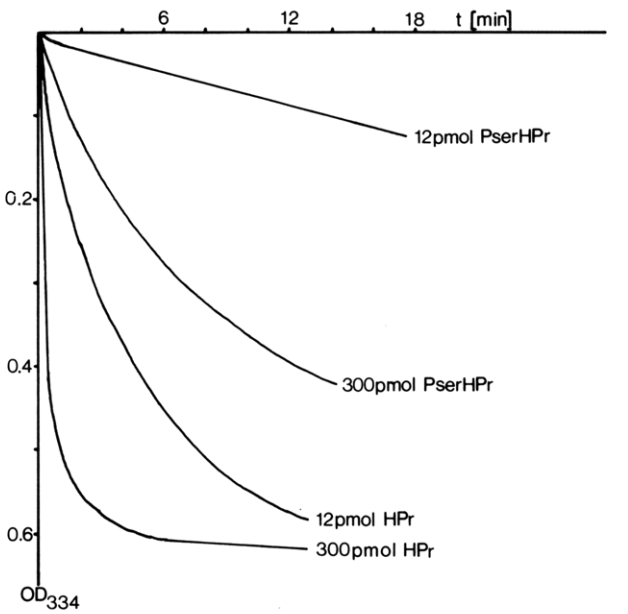

FIGURE 5: Photometric assay of $FIII^{Lac}$ phosphorylation. The reaction mixture was placed in a semimicrocuvette and contained in 200 μL 300 pmol of enzyme I (*S. lactis*), 8.6 nmol of $FIII^{Lac}$, 400 pmol of LDH (hog muscle, Boehringer), 2.5 mM $MgCl_2$, 2.5 mM PEP, 0.4 mM NADH, and 50 mM Tris-HCl, pH 7.5. After a 5-min incubation at 30 °C, the reaction was started by adding different amounts (300 or 12 pmol) of HPr or P-Ser-HPr. The decrease of NADH was followed at 334 nm.

$FIII^{Gct}$ was not contaminated with P-Ser-HPr phosphatase, which could have been the reason that we did not find a difference in phosphorylation of $FIII^{Gct}$ by HPr and P-Ser-HPr. P-Ser-HPr (10 μg) was incubated together with $FIII^{Gct}$ (20 μg) for 1 h at 37 °C. The reaction mixture was loaded on native polyacrylamide gels. No HPr could be detected after the gels were stained and destained.

PEP-dependent phosphorylation of $FIII^{Lac}$ was also followed by a photometric assay. The formed pyruvate was reduced to lactate by LDH and NADH. Experiments were conducted as described under Materials and Methods. The decrease of NADH was followed at 334 nm. HPr and P-Ser-HPr were used at different concentrations. The results are shown in Figure 5. It is obvious that PEP-dependent phosphorylation of $FIII^{Lac}$ is much slower in the presence of P-Ser-HPr than in the presence of HPr. From the initial rates, we calculated that $FIII^{Lac}$ is phosphorylated 85 times more slowly by P-Ser-HPr than by HPr.

Discussion

Phosphorylation–dephosphorylation reactions of proteins as regulatory events are well documented in higher organisms for almost all kinds of cellular processes (Rubin & Rosen, 1975; Krebs & Beavo, 1979). In contrast, in bacterial cells, little is known about protein phosphorylation as a regulatory mechanism. The only bacterial protein, identified as a target of an ATP-dependent protein kinase, is isocitrate dehydrogenase, the enzyme of the branch point between the tricarboxylic acid cycle and the glyoxylate bypass (Garnak & Reeves, 1979). This enzyme is of special interest, as the enzymatic activity of its phosphorylation and dephosphorylation is reported to be associated with one and the same polypeptide chain (La Porte & Koshland, 1982).

HPr now is the second bacterial protein for which an ATP-dependent phosphorylation has been found. To our

knowledge, it is also the only protein ever reported to be phosphorylated by two different enzymes using two different phosphoryl donors. The PEP-dependent phosphorylation by enzyme I at a histidyl residue is part of the reactions of the bacterial phosphotransferase system, first described by Kundig et al. (1964). The ATP-dependent phosphorylation by a membrane-associated protein kinase at a seryl residue is a regulatory event to control carbohydrate uptake. Thus, formation of P-Ser-HPr is largely stimulated by glucose in the growth medium.Lactose exerts only a slight stimulation (unpublished results). The effect of other sugars has to be tested. In cell-free extracts, the glycolytic intermediates glucose-6-P and 2-phosphoglycerate stimulate the ATP-dependent HPr kinase (Deutscher & Saier, 1983).

As a consequence of the ATP-dependent phosphorylation of HPr at a seryl residue, the PEP-dependent phosphorylation by enzyme I at a histidyl residue is slowed down by a factor of 5000 in in vitro experiments. FIII proteins are able to relieve the inhibition of PEP-dependent phosphorylation. But the capability to enhance the PEP-dependent phosphorylation by enzyme I is different for different sugar-specific FIII proteins. In the presence of $FIII^{Gct}$ of *S. faecalis*, the phosphorylation of P-Ser-HPr by PEP and enzyme I has the same velocity as the phosphorylation of HPr. The inhibition, caused by the phosphorylation at a seryl residue of HPr, is completely relieved by $FIII^{Gct}$. If the same reaction is carried out in the presence of $FIII^{Lac}$ of *S. aureus*, the phosphorylation velocity of P-Ser-HPr is 100 times slower but still 50-fold enhanced compared to the reaction velocity in the absence of $FIII^{Lac}$. As a consequence, in the presence of P-Ser-HPr, $FIII^{Lac}$ is phosphorylated 100 times more slowly than $FIII^{Gct}$. We do not have any evidence whether the FIII protein exerts its influence on either enzyme I or HPr, even though an interaction with HPr as the phosphoryl donor for FIII proteins in the PTS reaction is most likely. Evidence for a strong interaction of HPr and $FIII^{Lac}$ from NMR data (Kalbitzer et al., 1981) has been shown. This interaction is abolished as soon as the proteins are phosphorylated by PEP and enzyme I. It is interesting to note that some authors suggest a single multienzyme membrane-bound complex for the PTS (Saier et al., 1982; Robillard, 1982; Kaback, 1968). In the case of the fructose-specific FPr of *Escherichia coli* (Saier et al., 1976; Waygood, 1980), which we have purified to almost homogeneity, we found a complex of two proteins with molecular weights of 10000 and 40000, respectively (M. Dörschug and J. Deutscher, unpublished results). It is most likely that the smaller protein is the HPr-like protein specific for fructose and that the bigger one is $FIII^{Fru}$. However, despite this evidence for an HPr–FIII interaction, we did not notice an influence of the FIII proteins on the phosphorylation velocity of HPr using PEP and enzyme I. This influence could only be seen when P-Ser-HPr was phosphorylated at a histidyl residue. According to our results, the physiological function of P-Ser-HPr may be to regulate the uptake of PTS sugars. Glucose or any other metabolizable sugar, which stimulates the formation of P-Ser-HPr, is also known to exert an inhibitory effect on the uptake of other PTS sugars (McGinnis & Paigen, 1967, 1973). According to our results, the formation of P-Ser-HPr will result in a slower phosphorylation of some FIII proteins, like $FIII^{Lac}$, whereas others like $FIII^{Gct}$ will be phosphorylated with the same velocity as in the presence of HPr. This postulates an unaltered uptake rate for gluconate, whereas the uptake of lactose should be reduced to 1% after the formation of P-Ser-HPr. Competition of FIII proteins for the common phosphoryl donor P-His-HPr has already been discussed as a possible mechanism for the effect, which glucose exerts on the uptake of PTS sugars (Dills et al., 1980). However, after phosphorylation of a seryl residue in HPr, different rates of phosphorylation of FIII proteins are observed. It seems that different FIII proteins do not compete for the phosphoryl donor (P-Ser, P-His)-HPr but that most likely P-Ser-HPr, in complex with different FIII proteins, is phosphorylated with different velocities by PEP and enzyme I. We are aware that the postulated physiological significance of our findings is speculative. The interpretation of our results has to be further substantiated, extending the experiments to other FIII proteins, such as $FIII^{Mtl}$ of *S. aureus* or $FIII^{Glc}$ of *S. faecalis*. Further experiments would be off more physiological significance if P-Ser-HPr and FIII proteins would be from the same organism. P-Ser-HPr was discovered in connection with another regulatory event, called inducer expulsion. Preaccumulated, nonmetabolizable sugar phosphates are expelled from the cell after addition of glucose (Reizer & Panos, 1980; Thompson & Saier, 1981). The expulsion of 1-thiogalactoside 6-phosphate from *S. pyogenes* cells could be split into two events, an intracellular dephosphorylation of sugar phosphate and the following expulsion of free sugar. The intracellular sugar phosphate phosphatase activity was found to be dependent on ATP (Reizer et al., 1983). A sugar phosphate phosphatase, possibly involved in inducer expulsion, was isolated and characterized by Thompson & Chassy (1983). Searching for a protein kinase reaction, we found that under all conditions where expulsion occurred P-Ser-HPr was formed (Reizer et al., 1983). Even though we did not find a stimulation of sugar phosphate phosphatase in crude extracts of *S. pyogenes* after addition of P-Ser-HPr, it cannot be excluded that secondary events of the formation of P-Ser-HPr lead to such a stimulation in whole cells.

A similar expulsion mechanism is described for 2-deoxyglucose-6-P in *S. lactis* K1 cells (Thompson & Chassy, 1982). An interesting observation was than when 2-deoxyglucose, a substrate of the enzyme II^{Man}, was added to cells growing on sucrose, taken up by the enzyme II^{Suc}, growth of the cells was transiently halted as a consequence of utilization of PEP via the mannose PTS, caused by a wasteful futile cycle. 2-Deoxyglucose-6-P is intracellularly converted to 2-deoxyglucose, expelled from the cell, and taken up again. Within minutes, however, a regulatory response occurs which decreases the intracellular level of 2-deoxyglucose-6-P, establishes normal concentrations of glycolytic intermediates, and leads to a resumption of growth. The most striking event is that PEP is no longer used preferentially for the uptake of 2-deoxyglucose but for the translocation of sucrose (Thompson & Chassy, 1984). The authors could not give a satisfactory explanation for this event. The formation of P-Ser-HPr after addition of 2-deoxyglucose and its different interaction with the two sugar-uptake systems could possibly account for the described phenomenon.

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Staphylococcal Phosphoenolpyruvate-Dependent Phosphotransferase System: Purification and Characterization of a Defective Lactose-Specific Factor III Protein†

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ABSTRACT: Factor III protein specific for lactose ($FIII^{Lac}$) is part of the lactose-specific phosphotransferase system of *Staphylococcus aureus*. It is phosphorylated by the phosphorylated histidine-containing phospho-carrier protein (P-HPr). Phosphorylated $FIII^{Lac}$ (P-$FIII^{Lac}$) transfers the phosphoryl group via the enzyme II^{Lac} to the sugar during its uptake. The defective $FIII^{Lac}$ ($FIII^{Lac*}$) described here, isolated from *Staphylococcus aureus* strain S714G, showed most of the properties found for active $FIII^{Lac}$. It could still be phosphorylated by phosphoenolpyruvate, enzyme I, and HPr. As reported for the active protein, phosphorylation caused a dramatic structural change leading to increased hydrophobicity of the phosphorylated protein. As a consequence, P-$FIII^{Lac*}$, like P-$FIII^{Lac}$, bound to detergent micelles. But still, P-$FIII^{Lac*}$ was not able to transfer its phosphoryl group to lactose, indicating that the interaction with enzyme II^{Lac} is prohibited. To assay $FIII^{Lac*}$ during the purification procedure, we therefore used Ouchterlony tests with antibodies raised against $FIII^{Lac}$. The amino acid sequence of $FIII^{Lac}$ and of the first 56 amino acids of $FIII^{Lac*}$ revealed a difference for only one position: glycine in position 18 of $FIII^{Lac}$ is changed to glutamic acid in $FIII^{Lac*}$. This result, the exchange of an amino acid in the N-terminus of $FIII^{Lac}$, is in agreement with our previous findings that the N-terminal part of $FIII^{Lac}$ provides the binding domain for enzyme II.

The uptake of lactose in staphylococcal cells is achieved by the phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system (Hengstenberg et al., 1967; Simoni et al., 1973; Simoni & Roseman, 1973). This system consists of four proteins. Enzyme I and HPr are the two common nonspecific proteins, whereas FIII and enzyme II carry the sugar specificity. The following phosphoryl transfer reactions are involved in the uptake of lactose (Kalbitzer et al., 1981):

$$\text{PEP} + \text{enzyme I} \xrightleftharpoons{Mg^{2+}} \text{P-enzyme I} + \text{pyruvate}$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I}$$

$$\text{P-HPr} + FIII^{Lac} \rightleftharpoons \text{P-}FIII^{Lac} + \text{HPr}$$

$$\text{P-}FIII^{Lac} + \text{lactose}_{out} \xrightarrow{\text{enzyme } II^{Lac},\ Mg^{2+}} FIII^{Lac} + \text{lactose-6-P}_{in}$$

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