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# Synthesis of HPr(Ser-P)(His~P) by Enzyme I of the Phosphoenolpyruvate: Sugar Phosphotransferase System of *Streptococcus salivarius*<sup>†</sup>

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**ABSTRACT:** HPr is a protein of the bacterial phosphoenolpyruvate:sugar phosphotransferase transport system (PTS). In Gram-positive bacteria, HPr can be phosphorylated on Ser<sub>46</sub> by HPr(Ser) kinase/phosphorylase (HPrK/P) and on His<sub>15</sub> by enzyme I (EI) of the PTS. In vitro studies have shown that phosphorylation on one residue greatly inhibits the second phosphorylation. However, streptococci contain significant amounts of HPr(Ser-P)(His~P) during exponential growth, and recent studies suggest that phosphorylation of HPr(Ser-P) by EI is involved in the recycling of HPr(Ser-P)(His~P). We report in this paper a study on the phosphorylation of *Streptococcus salivarius* HPr, HPr(Ser-P), and HPr(S46D) by EI. Our results indicate that (i) the specificity constant ( $k_{\text{cat}}/K_m$ ) of EI for HPr(Ser-P) at pH 7.9 was approximately 5000-fold smaller than that observed for HPr, (ii) no metabolic intermediates were able to stimulate HPr(Ser-P) phosphorylation, (iii) the rate of HPr phosphorylation decreased at pHs below 6.5, while that of HPr(Ser-P) increased and was almost 10-fold higher at pH 6.1 than at pH 7.9, (iv) HPr(S46D), a mutated HPr alleged to mimic HPr(Ser-P), was also phosphorylated more efficiently under acidic conditions, and, lastly, (v) phosphorylation of *Bacillus subtilis* HPr(Ser-P) by *B. subtilis* EI was also stimulated at acidic pH. Our results suggest that the high levels of HPr(Ser-P)(His~P) in streptococci result from the combination of two factors, a high physiological concentration of HPr(Ser-P) and stimulation of HPr(Ser-P) phosphorylation by EI at acidic pH, an intracellular condition that occurs in response to the acidification of the external medium during growth of the culture.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS),<sup>1</sup> first described in *Escherichia coli* in 1964 by Kundig et al. (1), detects, transports, and phosphorylates a variety of mono- and disaccharides and also serves as a signal transduction system that allows the cells to preferentially select rapidly metabolizable sugars (2). The

first step in the transport of all PTS sugars involves the general energy-coupling proteins EI and HPr. EI undergoes autophosphorylation at the expense of PEP and transfers its phosphoryl group to HPr on a histidyl residue at position 15, leading to HPr(His~P). Then, HPr(His~P) transfers its phosphoryl group (PO<sub>4</sub>) to sugar-specific EIIs, which translocate and phosphorylate incoming sugars (1). The EIIs are composed of three and sometimes four domains, namely, IIA, IIB, IIC, and IID, which can be on separate polypeptides or fused to form multidomain proteins (2, 3).

HPr(His~P) is not only involved in sugar transport but also regulates the activity of PRD-containing transcriptional activators and antiterminators by phosphorylation, which modulate the transcription of several genes involved in the metabolism of secondary carbon sources (4, 5). HPr(His~P) also controls glycerol kinase in some *Enterococcus* and *Bacillus* species (6–8) and the non-PTS lactose transporter LacS in *Streptococcus thermophilus* (9) by phosphorylation.

In Gram-positive bacteria, HPr can also be phosphorylated on a strictly conserved seryl residue at position 46 by an ATP-dependent bifunctional HPr(Ser) kinase/phosphorylase, (HPrK/P) (10, 11). Unlike the phosphoamidate bond of HPr(His~P), the phosphoester bond of HPr(Ser-P) is heat stable and cannot be used to mediate PTS transport. However, by interacting with the transcriptional regulator CcpA (12), which belongs to the LacI/GalR family of transcriptional repressors/activators, HPr(Ser-P) is involved in carbon catabolite repression and carbon catabolite activation (13,

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<sup>1</sup> Abbreviations: PTS, bacterial phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I; EI~P, phosphorylated enzyme I; HPr, heat-stable histidine-containing protein; Ser<sub>46</sub>, seryl residue at position 46; His<sub>15</sub>, histidyl residue at position 15; HPr(His~P), HPr phosphorylated at histidine 15; HPr(Ser-P), HPr phosphorylated at serine 46; HPr-P, HPr phosphorylated on one residue; HPr(Ser-P)(His~P) and HPr-P<sub>2</sub>, doubly phosphorylated HPr; HPr(S46D), HPr in which serine 46 is replaced by an aspartate; His<sub>6</sub>, six-histidine tag; CcpA, catabolite control protein A; cre, catabolite responsive element; IIAB<sup>Mnn</sup>, cytoplasmic domains A and B of the mannose:phosphotransferase system; IIC and IID, membrane-bound EII domains C and D; HPrK/P, HPr(Ser) kinase/phosphorylase; LacS, non-PTS lactose/H<sup>+</sup> symporter; LDH, lactate dehydrogenase; PRD, PTS regulation domain; GC, guanosine + cytosine; w/v, weight/volume; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid;  $\epsilon_{280}$ , molar extinction coefficient at 280 nm;  $k_{\text{cat}}$ , turnover number;  $K_m$ , Michaelis–Menten affinity constant;  $V_{\text{max}}$ , maximal velocity;  $k_{\text{cat}}/K_m$ , specificity constant.

Table 1: Strains and Plasmids

strain/plasmid	relevant genotypes and/or characteristic(s) <sup>a</sup>	source or ref
<b>strains</b>		
<i>E. coli</i> LMG194	F <sup>-</sup> , $\Delta$ lacX74, <i>galE</i> , <i>thi</i> , <i>rpsL</i> , $\Delta$ phoA, ( <i>pvuII</i> ), $\Delta$ ara714, <i>leu</i> ::Tn10 (Tet <sup>r</sup> )	Invitrogen
<i>E. coli</i> NM522	<i>SupE</i> , <i>thi</i> , $\Delta$ ( <i>lac-proAB</i> ), $\Delta$ hds5 ( <i>r<sup>-</sup>m<sup>-</sup></i> ), [ <i>F<sup>+</sup></i> , <i>proAB</i> , <i>lacI<sup>q</sup></i> ΔM15]	Stratagene
<i>E. coli</i> BL21(DE3)	F <sup>-</sup> , <i>ompT</i> , <i>hsdS<sub>B</sub></i> , ( <i>r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup></i> ), <i>dcm gal</i> , $\lambda$ (DE3)	Novagen
<b>plasmids</b>		
pET28a(+)	expression vector, Kan <sup>r</sup>	Novagen
pBAD/HisB	expression vector, Amp <sup>r</sup>	Invitrogen
pQE30	expression vector, Amp <sup>r</sup>	Qiagen
pHPW18	contains the <i>ptsH</i> gene of <i>S. salivarius</i> ATCC 25975 cloned into pBAD/HisB	35
pH46D	contains the <i>ptsH</i> gene of <i>S. salivarius</i> ATCC 25975 with a mutation replacing HPr S46 by D, cloned into pBAD/HisB	this work
pETI-16	contains the <i>ptsI</i> gene of <i>S. salivarius</i> ATCC 25975 cloned into pET28a(+)	27
pHPKHis	contains the <i>hprK</i> gene of <i>S. salivarius</i> ATCC 25975 cloned into pET28a(+)	35
pAG2	contains the <i>ptsH</i> gene of <i>B. subtilis</i> GM107-4 cloned into pQE30	J. Deutscher, INRA
pAG3	contains the <i>ptsI</i> gene of <i>B. subtilis</i> GM107-4 cloned into pQE30	J. Deutscher, INRA

<sup>a</sup> Kan<sup>r</sup>, kanamycin resistance; Amp<sup>r</sup>, ampicillin resistance; Tet<sup>r</sup>, tetracycline resistance.

14). The complex CcpA/HPr(Ser-P) binds to consensus DNA sequences called *cre* located in the promoter region of targeted operons, leading to transcriptional activation or repression, depending on the position of the *cre* sequence (12). In *Bacillus subtilis*, more than 300 genes are regulated by CcpA in a direct or indirect manner, and in several cases, the interaction of CcpA with HPr(Ser-P) is required (15, 16). In several bacteria, HPr(Ser-P) is also involved in the phenomenon of inducer exclusion, a physiological process that prevents the metabolism of several PTS and non-PTS sugars when a rapidly metabolizable sugar such as glucose is available (17–19).

Conceptual models of sugar metabolism regulation in low-GC Gram-positive bacteria involve the phosphorylation state of HPr, which is determined by environmental conditions. In these models, transport and regulatory PTS-related functions have been attributed to HPr(His~P) and HPr(Ser-P) exclusively (20–23). However, it is now established that rapidly growing streptococci (24–26) contain high levels of the doubly phosphorylated derivative HPr(Ser-P)(His~P). Moreover, recent studies have shown that this form of HPr can efficiently transfer its phosphate group to the IIA-like domain of the non-PTS LacS permeases of *Streptococcus salivarius* and *S. thermophilus* (26, 27) and to the *S. salivarius* IAB<sup>Man</sup> proteins (unpublished results), indicating that HPr(Ser-P)(His~P) can control the activity of proteins possessing a IIA-like domain and can participate in PTS-mediated sugar transport.

While functions can now be ascribed to HPr(Ser-P)(His~P), the way it is synthesized in vivo remains to be elucidated. Theoretically, the doubly phosphorylated HPr could be synthesized via the phosphorylation of HPr(Ser-P) by EI and/or via the phosphorylation of HPr(His~P) by HPrK/P. However, in vitro studies have shown that phosphorylation of one residue strongly reduces the rate of phosphorylation of the second residue (28, 29). It should be noted, however, that these observations result from studies that were conducted with PTS proteins from *Enterococcus faecalis* and *B. subtilis*, two Gram-positive bacteria that, unlike streptococci (24–26), do not accumulate HPr(Ser-P)(His~P) in vivo (30, 31).

To determine how streptococci synthesize high levels of HPr(Ser-P)(His~P), we studied in vitro the effect of glycolytic intermediates and pH on the rates of phosphorylation

of HPr, HPr(Ser-P), and HPr(S46D) by EI using recombinant proteins from *S. salivarius* ATCC 25975. Our results suggested that the high levels of HPr(Ser-P)(His~P) in streptococci result from the combination of two factors: a high physiological concentration of HPr(Ser-P) and stimulation of HPr(Ser-P) phosphorylation by EI at acidic pH, an intracellular condition that occurs in response to the acidification of the external medium during growth of the culture.

## EXPERIMENTAL PROCEDURES

**Strains, Plasmids, and Culture Conditions.** All strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown aerobically on a New Brunswick Co. rotatory shaker at 180 rpm and 37 °C in a 2 L Erlenmeyer flask containing 1 L of LB medium supplemented with appropriate antibiotics. For the production of large amounts of HPr, *E. coli* was grown aerobically at 37 °C in a Microferm laboratory fermentor (New Brunswick Scientifics) containing 10 L of LB medium supplemented with appropriate antibiotics, with the stirrer speed set at 200 rpm and the O<sub>2</sub> flux at 2 L/min.

**Gene Cloning.** Replacement of HPr Ser<sub>46</sub> by an aspartyl residue was carried out by PCR with pHW18 as template and the QuickChange site-directed mutagenesis kit (Stratagene). The PCR mixture contained 50 ng of pHW18, 0.2 mM deoxynucleoside triphosphate, 125 ng of the oligonucleotide primers ptsH46D-F (5'-AGTTAACCTTAAAGATATCATGGGTGTTAT-3') and ptsH46D-R (5'-ATAACACCATGATATCTTTAAGGTTAACT-3'), and 2.5 units of *Pfu* Turbo DNA polymerase (Promega). After a 30 s incubation at 95 °C, the amplification reaction was carried out for 16 cycles, each with a 30 s denaturing step at 95 °C, a 1 min annealing step at 45 °C, and a 8 min extension step at 68 °C. After digestion with *DpnI* and transformation of *E. coli* XL1 Blue (Stratagene) with the resulting mixture, we obtained the plasmid pH46D. This plasmid bore the same DNA fragment as pHW18, with a two-nucleotide substitution that replaced the serine at position 46 by an aspartate. When induced, cells harboring this plasmid overexpressed the recombinant HPr(S46D) protein, a mutated HPr previously demonstrated to be structurally and functionally similar to HPr(Ser-P) (32, 33).

**Protein Overexpression.** All proteins used in this study were overexpressed as recombinant proteins fused with an

N-terminal six-histidine tag sequence (His<sub>6</sub>). To overproduce HPr and HPr(S46D) from *S. salivarius* and HPr from *B. subtilis*, *E. coli* LMG194 (pHPW18 or pH46D) or *E. coli* NM522 (pAG2) cultures were induced at mid-log phase ( $OD_{600} \cong 0.4\text{--}0.7$ ) after growth at 37 °C in LB medium supplemented with ampicillin (50 µg/mL) or with ampicillin (50 µg/mL) and tetracycline (20 µg/mL) for strain LMG194. Inductions were carried out with 0.02% L-arabinose for 16 h for strain LMG194 or with 1 mM IPTG for 4 h for strain NM522. Cells were then harvested by centrifugation at 4 °C at 11000 rpm (20000g) for 5 min in a J2-21 centrifuge (Beckman). The cells (3 g) were resuspended using a vortex in 30 mL of cold Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 10% glycerol. Cell suspensions were supplemented with 0.5 mg/mL lysozyme (chicken egg white lysozyme, Sigma-Aldrich) and 1 mg of DNase I (Roche Applied Science) and were left at 4 °C with gentle agitation for 2 h. The cell suspensions were then sonicated ( $4 \times 30$  s) on ice using a Sonifier Cell Disruptor W-350 (Heat Systems Ultrasonics) at 50% duty cycle, using the pulse cycle mode and with the intensity set at 5. The sonication periods were separated by at least 1 min. Cell-free extracts were obtained by centrifugation at 4 °C for 60 min at 13000 rpm (22000g) in a J2-21 centrifuge followed by ultracentrifugation at 4 °C for 90 min at 35000 rpm (125000g) in an Optima LE-80K ultracentrifuge (Beckman). The cellular extracts were immediately passed through a column of Ni<sup>2+</sup>-affinity resin as described below or stored at -20 °C in the presence of 0.1 mM PMSF, 2 µM pepstatin A, 2 µM leupeptin, and 1 mM EDTA. For the purification of the recombinant enzymes HPrK/P and EI from *S. salivarius*, and for EI from *B. subtilis*, cell-free extracts from *E. coli* BL21(DE3) (pHPKHis or pETI-16) or *E. coli* NM522 (pAG3) were obtained as described above. However, kanamycin (30 µg/mL) was used instead of ampicillin for the growth of BL21(DE3) strains, and all inductions were performed with 1 mM IPTG for 4 h.

**Purification of Recombinant HPr and HPr(S46D).** *S. salivarius* HPr and HPr(S46D), as well as HPr from *B. subtilis*, were purified from cellular extracts obtained from 1 or 10 L of cultures as described above. The cellular extract obtained from 1 L of culture was passed through a 1 mL Ni<sup>2+</sup>-Sephacel HP column (Amersham Biosciences) equilibrated at 4 °C with 50 mM Tris-acetate, pH 7.5, 300 mM NaCl, and 10 mM imidazole. The column was washed with 5 mL of the same buffer. The His-tagged proteins were eluted by gravity flow with the same buffer containing 500 mM imidazole. Elution fractions were then pooled, 0.22 µm filtered (Millipore), and concentrated at 4 °C by ultrafiltration through a NanoSep 3K membrane (Pall). The concentrated fraction was applied to a Superdex 75GL 10/300 column (Amersham Biosciences) equilibrated with 25 mM HEPES, pH 7.5, and connected to an FPLC LKB Controller LCC-500 Plus (Pharmacia). The proteins were eluted at room temperature in the same buffer at 0.5 mL/min, and 0.5 mL fractions were collected and analyzed by SDS-PAGE on 15% acrylamide gels as described below. The elution volume of HPr and HPr(S46D) from *S. salivarius* was between 11 and 14 mL, whereas that of *B. subtilis* HPr was between 13 and 15 mL (data not shown). Fractions containing HPr or HPr(S46D) were pooled and stored at -20 °C in the presence of 10% glycerol. This method yielded approximately 5 mg

of recombinant HPr or HPr(S46D) per liter of culture with a degree of purity exceeding 95% as determined by SDS-PAGE (data not shown).

**Recombinant EI Purification.** *S. salivarius* and *B. subtilis* EI were purified by passing the cell-free extracts obtained from 1 L of culture through 1 mL Ni<sup>2+</sup>-Sephacel HP columns (Amersham Biosciences) at 4 °C following the same protocol used for HPr. Pooled fractions of EI were 0.22 µm filtered. They were first dialyzed at 4 °C for 16 h against 25 mM HEPES, pH 7.5, containing 5 mM MgCl<sub>2</sub>, 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 200 µM PEP. This step was carried out to optimize the dimerization of EI (34). The EI fractions were then dialyzed for another 24 h against the same buffer without PEP and EDTA. Dialyzed fractions were applied to another 1 mL Ni<sup>2+</sup>-Sephacel HP column equilibrated at 4 °C with 25 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl<sub>2</sub>, and 10 mM imidazole. The column was washed with 5 mL of the same buffer. Elution was carried out by gravity flow in the same buffer containing 500 mM imidazole. The pooled fractions were 0.22 µm filtered before being concentrated at 4 °C by ultrafiltration through a NanoSep 3K membrane. Concentrated fractions were then applied on a Superdex 200GL 10/300 column (Amersham Biosciences) equilibrated with 25 mM HEPES, pH 7.5, 150 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 1 mM DTT and operated with the same apparatus as described above. The column was calibrated with the high (HMW, lot 307915) and low molecular weight (LMW, lot 319694) gel filtration calibration kits from Amersham Biosciences. The following protein standards were used: ribonuclease A (from bovine pancreas, 13700 Da), chymotrypsinogen A (from bovine pancreas, 25000 Da), ovalbumin (from hen egg, 43000 Da), albumin (from bovine serum, 67000 Da), aldolase (from rabbit muscle, 158000 Da), catalase (from bovine liver, 232000 Da), and ferritin (from horse spleen, 440000 Da). The recombinant enzymes were eluted at room temperature in the same buffer at 0.5 mL/min, and 0.5 mL fractions were collected and analyzed by SDS-PAGE on 10% acrylamide gels as described below. The two EI enzymes were recovered in an elution volume between 13 and 15 mL (data not shown). Fractions containing EI were pooled and incubated with 2 mM PEP for 30 min at 37 °C and then extensively dialyzed at 4 °C against 25 mM HEPES, pH 7.5, containing 150 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 10% glycerol. Dialyzed fractions were 0.22 µm filtered before being stored at -80 °C. This method yielded about 10 mg of >95% homogeneous EI per liter of culture with high activity and stability (data not shown). Before experiments involving EI, the enzyme was diluted in 25 mM HEPES, pH 7.5, containing 5 mM MgCl<sub>2</sub>, 150 mM KCl, 1 mM EDTA, and 1 mM DTT, and the solution was preincubated for 15 min at 37 °C. Under these conditions, *S. salivarius* and *B. subtilis* recombinant EIs were stable for up to 1 h (data not shown). One unit of EI was defined as the amount of enzyme that phosphorylated 1 µmol of HPr/min at 37 °C.

**Purification of Recombinant HPrK/P.** *S. salivarius* HPrK/P was purified at 4 °C from *E. coli* BL21(DE3) (pHPKHis) cell-free extract as described previously (35) except that Ni<sup>2+</sup>-Sephacel HP was used instead of Ni-NTA Superflow (Qiagen). All of the material used for the purification of HPrK/P was siliconized, extensively washed with deionized



water, and autoclaved (11). After passage on the  $\text{Ni}^{2+}$  resin, the fractions of interest were pooled, dialyzed at 4 °C for 16 h against 50 mM Tris–acetate, pH 7.5, and supplemented with 10% glycerol, 0.1 mM PMSF, 2  $\mu\text{M}$  pepstatin A, 2  $\mu\text{M}$  leupeptin, and 1 mM EDTA for storage at –80 °C. This method yielded approximately 10 mg/L >5% homogeneous recombinant HPrK/P with high specific activity and stability (data not shown). The stock solution was diluted in 50 mM Tris–acetate buffer, pH 7.5, for maximal activity.

**Synthesis of [ $^{32}\text{P}$ ]PEP.** [ $^{32}\text{P}$ ]PEP was prepared according to the method of Mattoo and Waygood (36) using purified PEP carboxykinase from *E. coli* K-12 HFr 3000, which was kindly provided by A. H. Goldie (University of Saskatchewan).

**HPr(Ser-P) Synthesis and Purification.** HPr(Ser-P) was prepared from *S. salivarius* ATCC 25975 HPr and *B. subtilis* GM107-4 HPr as described previously (26, 27, 35) with some modifications. Briefly, the synthesis was carried out in a total volume of 15 mL containing 50 mM Tris–acetate, pH 7.5, 5 mM  $\text{MgCl}_2$ , 5 mg of purified HPr, and 150  $\mu\text{g}$  of purified HPrK/P from *S. salivarius*. After a 5 min preincubation at 37 °C, the reaction was initiated by adding 2 mM ATP and stopped after 60 min by placing the reaction mixture on ice. This first step yielded a mixture of recombinant HPr(Ser-P) and HPrK/P. The preparation was further purified by affinity and size exclusion chromatographies on a column of  $\text{Ni}^{2+}$  resin and a column of Superdex 75GL as described above for HPr. This step allowed HPrK/P to be separated from HPr(Ser-P) (data not shown). The fractions were then analyzed by PAGE and SDS–PAGE on 15% acrylamide gels as described below.

**Spectroscopy.** All spectrophotometric measurements were carried out in a DU-530 spectrophotometer (Beckman). For protein quantification at 280 nm, data were recorded at 25 °C. When using the EI-LDH-coupled assay (see below), the spectrophotometer was equipped with a Peltier temperature control module adjusted at 37 °C (Beckman).

**Protein Quantification and Electrophoretic and Densitometric Analyses.** The concentrations of HPrK/P and EI were determined spectrophotometrically at 280 nm. The following molar extinction coefficients ( $\epsilon_{280}$ ) were used: 31920  $\text{M}^{-1} \text{cm}^{-1}$  and 32430  $\text{M}^{-1} \text{cm}^{-1}$  for *S. salivarius* EI and HPrK/P, respectively, and 29360  $\text{M}^{-1} \text{cm}^{-1}$  for EI from *B. subtilis* GM107-4. The coefficients were calculated using the PepTidSort program of the GCG package (Genetic Computer Group) from the amino acid sequences of the His-tagged proteins. The HPr concentrations were determined using the spectrophotometric EI-LDH-coupled assay originally described by Waygood et al. (37, 38) with the following modifications. The reaction was carried out in a total volume of 0.6 mL containing 50 mM HEPES, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 250 nM EI, 20 units of LDH (from rabbit muscle; Sigma-Aldrich), different amounts of HPr, and 150 mM KCl, which was added for enzyme stability. The reaction mix was preincubated for 15 min at 37 °C before adding 100  $\mu\text{M}$  NADH and 10 mM PEP to initiate the reaction that reached completion within 10 s. HPr concentrations were then calculated from the  $\Delta A_{340}$  resulting from the NADH to  $\text{NAD}^+$  conversion ( $\epsilon_{340} = 6300 \text{ M}^{-1} \text{cm}^{-1}$ ). The HPr concentrations calculated using the LDH-coupled assay were used in the Beer–Lambert equation ( $A = \epsilon cl$ ) to estimate HPr  $\epsilon_{280}$ . For *S. salivarius* HPr,  $\epsilon_{280}$  was estimated at 3475  $\text{M}^{-1} \text{cm}^{-1}$ , whereas for *B. subtilis* HPr, the coefficient was

3250  $\text{M}^{-1} \text{cm}^{-1}$ . The *S. salivarius* and *B. subtilis* HPr coefficients ( $\epsilon_{280}$ ) determined as described above were also used for the quantification of HPr(Ser-P) and HPr(S46D). Electrophoretic analyses were all performed using a Bio-Rad Mini-Protean II apparatus under either native or denaturing conditions as described by Laemmli (39). Lastly, for densitometric analyses, gels were first Coomassie Blue stained for 30 min in a solution containing 50% methanol, 10% acetic acid, and 0.05% (w/v) Coomassie Blue (Brilliant Blue R-250, Sigma-Aldrich) and destained in 5% methanol/7% acetic acid for 16–24 h. Gels were then scanned using a Gel Doc 2000 UV transilluminator (Bio-Rad), and data were analyzed using Quantity One software (Bio-Rad).

**HPr(His~P) Phosphohydrolysis.** The spontaneous phosphohydrolysis of HPr(His~P) from *S. salivarius* was studied at 37 °C using the spectrophotometric assay described above, except that the EI concentration in the reaction mix was raised to 500 nM to ensure rapid and complete rephosphorylation of free HPr. This allowed the concentration of HPr(His~P) to remain virtually unchanged throughout the experiment, making it possible to calculate the phosphohydrolysis first-order rate constant.

**Kinetic Studies.** The kinetic constants  $K_m$ ,  $V_{\text{max}}$ ,  $k_{\text{cat}}$ , and  $k_{\text{cat}}/K_m$  were determined using the EI-LDH-coupled assay described above, except that the reactions were carried out in 50 mM MES, pH 5.5 or 6.5, or HEPES, pH 7.5. Experiments were conducted with various concentrations of HPr, HPr(Ser-P), HPr(S46D), and EI, as indicated in the figure legends. After a preincubation for 15 min at 37 °C, phosphotransfer reactions were initiated by adding 100  $\mu\text{M}$  NADH and 10 mM PEP, and reaction rates were measured by recording the absorbance at 340 nm for 4 min. The rates were subsequently corrected for NADH autoxidation. Under all conditions, the initial reaction rates were linear for up to 30 s with correlation coefficients ( $R^2$ )  $\geq 0.97$ . The final pH of all solution was determined after mixing all of the components.

**Kinetic Data Analysis.** The kinetics of HPr phosphorylation were analyzed with Kaleidagraph 3.51 (Synergy Software) using a Michaelis–Menten nonlinear regression curve function. The kinetics of HPr(Ser-P) and HPr(S46D) phosphorylation were analyzed by linear regression with Microsoft Excel 2000 (Microsoft Corp.).

## RESULTS

**Purification of EI.** The protocol used to purify *S. salivarius* and *B. subtilis* EI was optimized to preserve the activity of the enzymes during purification and storage. Analyses by size exclusion chromatography confirmed that the *S. salivarius* and *B. subtilis* EI preparations contained a single protein with a molecular mass of approximately 130 kDa (data not shown), which corresponds to the dimeric active form (40). Consistent with a previously published study on *S. salivarius* EI (41), and unlike *E. coli* and *Salmonella typhimurium* EI (38, 42), we never observed monomers in the purified preparations of recombinant EI used in this study. In addition, unlike EI from *E. coli* (38) and *E. faecalis* (43), EI from *S. salivarius* did not lose its activity when stored for several months at –80 °C in a solution at a pH near neutrality (data not shown). The purification procedure yielded *S. salivarius* EI preparations with specific activities

Table 2: Kinetic Constants of *S. salivarius* EI

substrate	pH <sup>a</sup>	K <sub>m</sub> (μM) <sup>b</sup>	V <sub>max</sub> (units/ mg of EI) <sup>b</sup>	k <sub>cat</sub> (s <sup>-1</sup> )	k <sub>cat</sub> /K <sub>m</sub> (M <sup>-1</sup> s <sup>-1</sup> ) <sup>c</sup>
HPr	6.1	12 ± 2	145 ± 8	157 ± 9	1.3 × 10 <sup>7</sup>
	7.0	20 ± 1	391 ± 11	424 ± 12	2.1 × 10 <sup>7</sup>
	7.9	39 ± 4	336 ± 15	365 ± 16	0.93 × 10 <sup>7</sup>
HPr(Ser-P)	6.1	ND	ND	ND	16 × 10 <sup>3</sup>
	7.9	ND	ND	ND	1.9 × 10 <sup>3</sup>
HPr(S46D)	6.1	ND	ND	ND	80 × 10 <sup>3</sup>
	7.9	ND	ND	ND	6.1 × 10 <sup>3</sup>

<sup>a</sup> The pHs of the reaction mixtures were determined after mixing all of the components including PEP, which was added at time 0. <sup>b</sup> The values (± standard error) are the means of three determinations. ND, not determined. See comments in the text about the K<sub>m</sub> of *S. salivarius* EI for HPr(Ser-P) and HPr(S46D). <sup>c</sup> The specificity constants for HPr were obtained using the Michaelis–Menten equation, while those for HPr(Ser-P) and HPr(S46D) were obtained using linear regressions. The values are the means of three determinations.

ranging from about 145 to 400 units/mg depending on the pH of the solution (Table 2).

**Purification of EI Substrates.** Determination of the kinetic constants of EI for its substrates required large amounts of HPr and HPr(S46D). For this purpose, HPr(S46D) was purified from several 1 L cultures and HPr from several 10 L cultures as described in the Experimental Procedures section. The substrate HPr(Ser-P) was produced by phosphorylation of HPr with HPrK/P and ATP before being further purified by size exclusion chromatography as described above. This method allowed the separation of the kinase from the phosphoserine HPr derivative and yielded HPr(Ser-P) preparations free of unphosphorylated HPr as demonstrated by native PAGE and silver staining. The absence of free HPr in the HPr(Ser-P) preparations was also confirmed as described previously (27) by incubating the purified preparation of HPr(Ser-P) with EI and [<sup>32</sup>P]PEP and detecting the reaction products by autoradiography after separation by native PAGE. Only <sup>32</sup>P~EI and HPr(Ser-P)-(His~<sup>32</sup>P) were detected. The absence of free HPr in the HPr(Ser-P) preparations was essential to prevent interference when measuring the rate of HPr(Ser-P) phosphorylation using the spectrophotometric assay.

**HPr(His~P) Phosphohydrolysis.** Because the N<sup>δ</sup><sub>1</sub>-P-histidyl residue of HPr(His~P) is unstable (44), we determined the rate of spontaneous hydrolysis of *S. salivarius* HPr(His~P) as described in the Experimental Procedures section using eight different concentrations of HPr(His~P) ranging from 20.4 to 52.9 μM. We found that the first-order reaction constant for *S. salivarius* HPr(His~P) spontaneous phosphohydrolysis was 0.07 ± 0.005 min<sup>-1</sup>. Thus, *S. salivarius* HPr(His~P) is rather unstable, as is the case for HPr(His~P) from *B. subtilis* (44), *Bacillus thuringiensis israelensis* (45), *E. coli* (46, 47), *E. faecalis* (48), *Staphylococcus aureus* (49), *Lactococcus lactis* (44), *Mycoplasma capricolum* (50), *Mycoplasma pneumoniae* (51), and *Staphylococcus carnosus* (52). The rate of spontaneous phosphohydrolysis was taken into account in determining the rate of HPr phosphorylation by EI, particularly at very low concentrations of HPr.

**HPr(Ser-P) Is a Poor Substrate for *S. salivarius* EI.** Since HPr(Ser-P)(His~P) is present in large amounts in streptococci (24, 25), it was expected that the phosphorylation rate of HPr(Ser-P) by *S. salivarius* EI would be higher than the rate observed with proteins isolated from bacteria that do

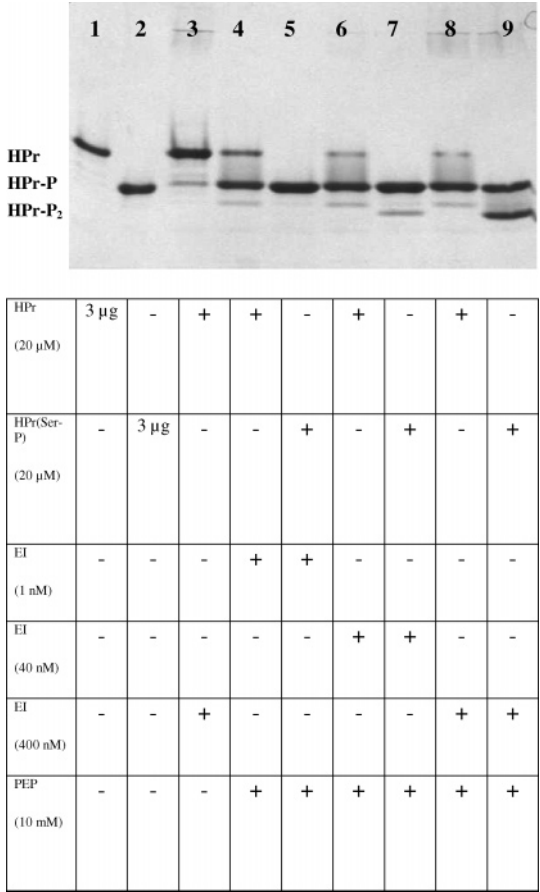


FIGURE 1: PEP-dependent phosphorylation of HPr and HPr(Ser-P) by *S. salivarius* EI. The reaction mixtures (40 μL) contained 50 mM HEPES, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 150 mM KCl, 25 μM HPr or 25 μM HPr(Ser-P), and 1, 40, or 400 nM EI. The final pH was 7.9. After a preincubation for 5 min at 37 °C, 10 mM PEP was added to initiate the reactions. After 30 min, 20 μL of 3× PAGE loading buffer was added to each reaction mixture, and 20 μL was loaded on a 15% nondenaturing polyacrylamide gel (PAGE). Lane 1: *S. salivarius* HPr (3 μg). Lane 2: *S. salivarius* HPr(Ser-P) (3 μg). The faint band that migrated as HPr(His~P) in the absence of PEP (lane 3) was likely the result of HPr phosphorylation by P~EI already present in the enzyme preparation.

not contain the doubly phosphorylated HPr (29, 31). We thus first compared the ability of *S. salivarius* EI to phosphorylate HPr and HPr(Ser-P) under identical conditions by carrying out in vitro phosphorylation experiments as described in the legends of Figures 1 and 2. The HPr isoforms were separated by PAGE. From the results presented in Figure 1, we evaluated that HPr(Ser-P) was approximately 1000 times less efficiently phosphorylated by EI than HPr. This is consistent with previous studies conducted with PTS proteins from *B. subtilis* and *E. faecalis* (28, 29). It should be noted that the presence of free HPr detected in lanes 4, 6, and 8 of Figure 1 was most likely the result of spontaneous phosphohydrolysis of HPr(His~P), as suggested by the results mentioned above. We then looked at whether the PEP-dependent phosphorylation of HPr(Ser-P) was stimulated in the presence of the following metabolic intermediates tested at various concentrations (53, 54): glucose 6-phosphate (0–1000 μM), fructose 1-phosphate (25 mM), fructose 6-phosphate (0–100 μM), fructose 1,6-bisphosphate (0–50 mM), glyceraldehyde 3-phosphate (0–600 μM), 2,3-bisphosphoglycerate (25 mM), 3-phosphoglycerate (0–2500 μM), 2-phospho-

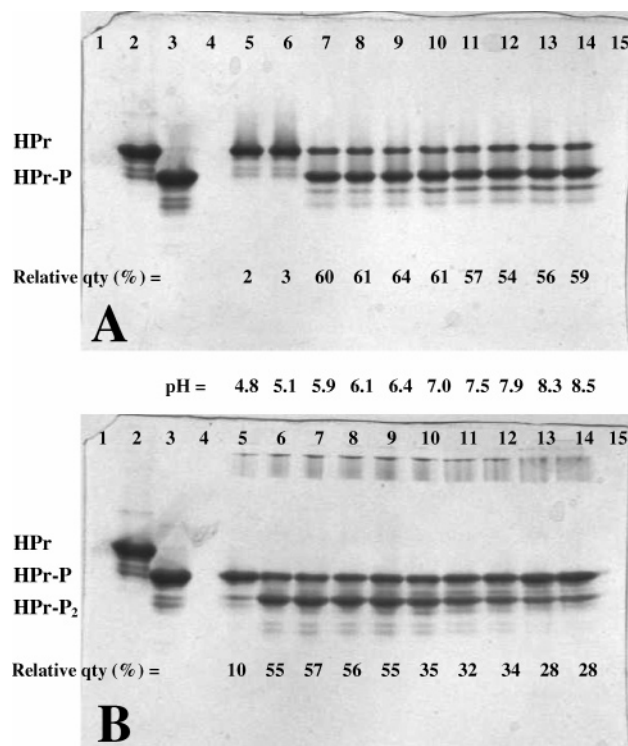


FIGURE 2: PEP-dependent phosphorylation of HPr and HPr(Ser-P) by *S. salivarius* EI as a function of pH. The reaction mixtures (40  $\mu$ L) contained 50 mM sodium acetate, pH 4.0, 4.5, and 5.0, MES, pH 5.5, 6.0, and 6.5, HEPES, pH 7.0 and 7.5, or tricine, pH 8.0 and 8.5, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 29  $\mu$ M HPr and 0.5 nM EI (A) or 29  $\mu$ M HPr(Ser-P) and 1  $\mu$ M EI (B). After a preincubation for 5 min at 37 °C, 10 mM PEP was added to initiate the reactions. After 30 min, 20  $\mu$ L of 3 $\times$  PAGE loading buffer was added to reaction mixtures, and 15  $\mu$ L was then loaded on a 15% nondenaturing polyacrylamide gel (PAGE). Lanes 1, 4, and 15 (A and B): nothing. Lane 2 (A and B): *S. salivarius* HPr (3  $\mu$ g). Lane 3 (A and B): *S. salivarius* HPr(Ser-P) (3  $\mu$ g). The relative quantities indicated as percentages represent in (A) the proportions of HPr(His~P) and in (B) the proportions of HPr(Ser-P)(His~P).

glycerate (0–500  $\mu$ M), pyruvate (0–2000  $\mu$ M), lactate (0–50 mM), ribulose 1,5-bisphosphate (25 mM), and dihydroxyacetone phosphate (0–2000  $\mu$ M). None of these had any effect on HPr or HPr(Ser-P) phosphorylation (data not shown).

**EI Activity as a Function of pH.** Unlike enterococci and bacilli (55, 56), the intracellular pH of streptococci decreases during growth in response to the acidification of the extracellular medium caused by metabolic production of organic acids (55, 57). This prompted us to study the effect of pH on HPr and HPr(Ser-P) phosphorylation. We first measured the relative quantities of histidine phosphate derivatives formed as a function of pH after separation of the reaction products by electrophoresis under native conditions. Results presented in Figure 2 clearly indicate that a decrease in the pH had a different effect on the ability of *S. salivarius* EI to phosphorylate HPr and HPr(Ser-P). Indeed, while the phosphorylation of HPr was strongly inhibited at pHs below 5.9, with less than 5% of HPr being phosphorylated (Figure 2, panel A, lanes 5 and 6), the phosphorylation of HPr(Ser-P) was enhanced at lower pHs. Indeed, HPr(Ser-P)(His~P) represented more than 55% of total HPr after the reaction had reached equilibrium at pHs between 5.1 and 6.4 (Figure 2, panel B, lanes 6–9), while the percentage dropped to less than 30% at higher pHs.

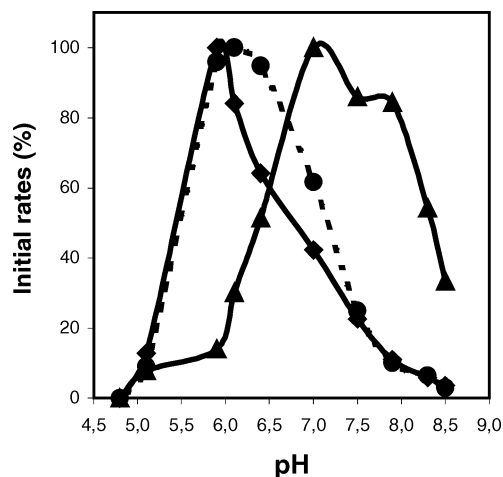


FIGURE 3: Initial rates of phosphotransfer reactions from *S. salivarius* P~EI to HPr, HPr(Ser-P), and HPr(S46D) as a function of pH. The reaction mixtures (600  $\mu$ L) contained 50 mM sodium acetate, pH 4.0, 4.5, and 5.0, MES, pH 5.5, 6.0, and 6.5, HEPES, pH 7.0 and 7.5, or tricine, pH 8.0 and 8.5, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 150 mM KCl, 29  $\mu$ M HPr and 1.5 nM EI ( $\blacktriangle$ ), 29  $\mu$ M HPr(Ser-P) and 1  $\mu$ M EI ( $\blacklozenge$ ), or 29  $\mu$ M HPr(S46D) and 1  $\mu$ M EI (dotted line), and 20 units of LDH. Data were collected in duplicate. Maximal reaction rates were measured at pH 7.0 for HPr ( $4.1 \times 10^{-3}$   $\mu$ mol min<sup>-1</sup>), pH 5.9 for HPr(Ser-P) ( $5.5 \times 10^{-3}$   $\mu$ mol min<sup>-1</sup>), and pH 6.1 for HPr(S46D) ( $1.8 \times 10^{-2}$   $\mu$ mol min<sup>-1</sup>). Because the amount of EI differed substantially with respect to the substrate, the results are expressed as relative reaction rates.

To determine more accurately how pH modifies the interaction of EI with its substrates, we measured the initial rates of phosphorylation of HPr, HPr(Ser-P), and HPr(S46D) at different pHs using a spectrophotometric EI-LDH-coupled assay. Results presented in Figure 3 clearly show that HPr was a very good phosphoryl acceptor at pHs above 6.5, with a maximal phosphorylation rate at pH 7.0, while HPr(Ser-P) was better phosphorylated below pH 6.5, with maximal rate at slightly below pH 6.0. The opposite effect of acidification on the phosphorylation of HPr and HPr(Ser-P) most likely came from the strong negative charge of the phosphoryl group linked to Ser<sub>46</sub> in HPr(Ser-P). This was confirmed by studying the phosphorylation of HPr(S46D), which also possesses a negative charge at position 46 owing to the replacement of Ser<sub>46</sub> by an aspartyl residue. This mutated HPr is structurally and functionally similar to HPr(Ser-P) (32, 33). Results shown in Figure 3 indicate that the variation of the phosphorylation rate of HPr(S46D) by EI as a function of the pH was similar to the pattern observed with HPr(Ser-P), except that HPr(S46D) was a better substrate than HPr(Ser-P) at all pH values.

**Determination of *S. salivarius* EI Kinetic Constants.** To determine how pH affects the kinetic properties of *S. salivarius* EI, we first measured the kinetic constants associated with the phosphorylation of HPr at three different pHs (6.1, 7.0, and 7.9). The results shown in Figure 4 were obtained by analyzing the data with the Michaelis–Menten equation. The affinity constants ( $K_m$ ), maximal velocities ( $V_{max}$ ), turnover numbers ( $k_{cat}$ ), and specificity constants ( $k_{cat}/K_m$ ) calculated from the computerized best fit curves are listed in Table 2. The  $K_m$  values of *S. salivarius* EI for HPr varied with respect to pH from 12 to 39  $\mu$ M with specificity constants ( $k_{cat}/K_m$ ) approaching diffusion-limited reaction rates. Interestingly, the kinetic constants varied with respect



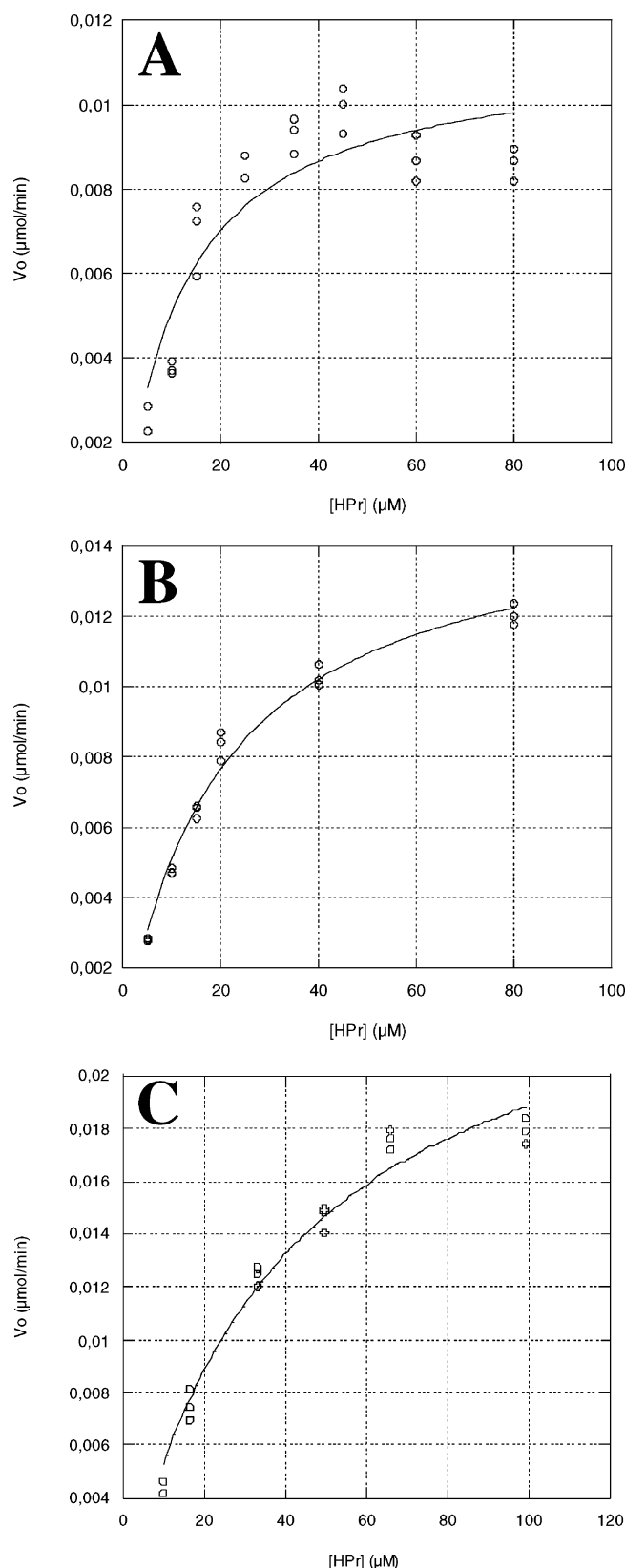


FIGURE 4: Kinetics of HPr phosphorylation by *S. salivarius* EI at three different pHs. The reaction mixtures (600  $\mu$ L) contained 50 mM MES, pH 5.5 (A), MES, pH 6.5 (B), or HEPES, pH 7.5 (C), 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 150 mM KCl, 20 units of LDH, various concentrations of HPr from 0 to 100  $\mu$ M, and 1 nM EI at pH 7.0 or 2 nM EI at pH 6.1 and 7.9. The final pHs were determined as 6.1 (A), 7.0 (B), and 7.9 (C). Data were collected at least in triplicate and analyzed by nonlinear regression. Correlation coefficients ( $R^2$ ) for these fits were all over 0.90.

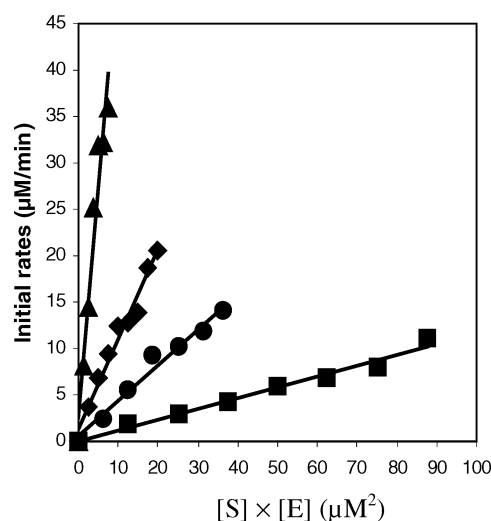


FIGURE 5: Initial rates of phosphotransfer reactions from *S. salivarius* P~EI to HPr(Ser-P) and HPr(S46D) at two different pHs. The reaction mixtures (600  $\mu$ L) contained 50 mM MES, pH 5.5, or HEPES, pH 7.5, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 150 mM KCl, 20 units of LDH, and various concentrations of HPr(Ser-P) (0–200  $\mu$ M) or HPr(S46D) (0–150  $\mu$ M). The final pHs were equal to 6.1 for the solutions containing the MES buffer and 7.9 for the solutions containing the HEPES buffer. The reactions with HPr(Ser-P) as the substrate were carried out in the presence of 500 nM EI at a final pH of 7.9 (■) and 100 nM EI at a final pH of 6.1 (◆) while the reactions with HPr(S46D) as the substrate were carried out in the presence of 250 nM EI at a final pH of 7.9 (●) and 50 nM EI at a final pH of 6.1 (▲). Data were collected at least in triplicate. Each value was the means of three determinations, and the qualities of the fits were assessed by inspecting the random distributions of the residuals (data not shown).

to pH at the most by a factor of 3. The affinity was highest at the lowest pH tested (pH 6.1). However, the  $k_{\text{cat}}$  was also reduced at this pH, being about 3 times lower than at pH 7.0 where maximal activity was observed. The combined effects gave a  $k_{\text{cat}}/K_m$  value less than 2-fold higher at pH 7.0 compared with the value obtained under acidic conditions.

Since the phosphorylation rate of HPr(Ser-P) was approximately 10-fold higher at pH 6.1 than at pH 7.9 (Figure 3), we attempted to determine the kinetic constants associated with the phosphorylation of HPr(Ser-P) and HPr(S46D) by *S. salivarius* EI at these two pH values. Unfortunately, we were unable to reach high enough concentrations of these two EI substrates (i.e., above the expected  $K_m$ ) to accurately calculate the  $K_m$  and  $V_{\text{max}}$  values with the Michaelis–Menten equation (58). For comparison purposes with the published  $K_m$  and  $V_{\text{max}}$  values of *B. subtilis* EI for HPr(Ser-P) and HPr(S46D), which have been extrapolated from experiments conducted with substrate concentrations below the estimated  $K_m$  values (29), we nonetheless extrapolated the  $K_m$  and  $V_{\text{max}}$  values for HPr(Ser-P) and HPr(S46D). The affinity constants and the maximal velocities estimated were similar to the values obtained with the *B. subtilis* proteins (data not shown). While the conditions used (substrate concentrations low compared to  $K_m$ ) do not allow precise determinations of the  $K_m$ ,  $V_{\text{max}}$ , and  $k_{\text{cat}}$  values, they do allow an accurate determination of the specificity constant ( $k_{\text{cat}}/K_m$ ) (58). This constant is equivalent to the  $k_{\text{cat}}$  when the enzymatic reaction proceeds via a ping-pong mechanism, which is the case for EI (41, 59). We thus determined the specificity constants (Figure 5 and Table 2) for the two substrates and found that the  $k_{\text{cat}}/K_m$  value for HPr(Ser-P) phosphorylation by *S.*



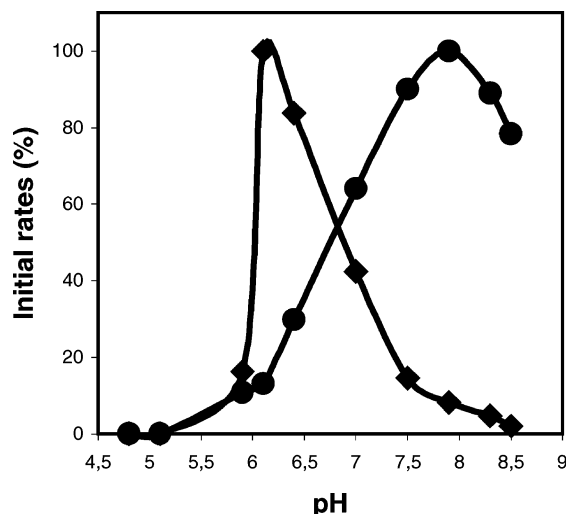


FIGURE 6: Initial rates of phosphotransfer reactions from *B. subtilis* P~EI to *B. subtilis* HPr and HPr(Ser-P) as a function of pH. The reaction mixtures (600  $\mu$ L) contained 50 mM sodium acetate, pH 4.0, 4.5, and 5.0, MES, pH 5.5, 6.0, and 6.5, HEPES, pH 7.0 and 7.5, or tricine, pH 8.0 and 8.5, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 20 units of LDH, 34  $\mu$ M HPr and 12 nM EI (●), or 34  $\mu$ M HPr(Ser-P), 400 mM KCl, and 4  $\mu$ M EI (◆). Data were collected in duplicate. Maximal reaction rates were measured at pH 7.9 for HPr ( $3.5 \times 10^{-3} \mu\text{mol min}^{-1}$ ) and at pH 6.1 for HPr(Ser-P) ( $1.1 \times 10^{-2} \mu\text{mol min}^{-1}$ ). Because the amount of EI differed substantially with respect to the substrate, the results are expressed as relative reaction rates.

*salivarius* EI was 8 times higher under acidic (pH 6.1) than alkaline (pH 7.9) conditions. As expected, the  $k_{\text{cat}}/K_m$  for HPr(S46D) also increased with a decrease in pH, the specificity constant being 13 times higher under acidic conditions.

**Effect of pH on the Phosphorylation of HPr(Ser-P) by *B. subtilis* EI.** The absence of HPr(Ser-P)(His~P) in *Bacillus* species (31) prompted us to determine whether phosphorylation of HPr(Ser-P) by *B. subtilis* EI was also enhanced at lower pHs. Results presented in Figure 6 show that the phosphorylation of *B. subtilis* HPr and HPr(Ser-P) was influenced by pH in the same manner as the *S. salivarius* proteins, except that the maximum rates of phosphotransfer were observed at slightly more alkaline pHs. Lastly, the availability of purified proteins from *S. salivarius* and *B. subtilis* enabled us to study the synthesis of HPr(Ser-P)(His~P) with heterologous systems. Our results showed that the phosphorylation of *S. salivarius* HPr(Ser-P) by *B. subtilis* EI as well as the phosphorylation of *B. subtilis* HPr(Ser-P) by *S. salivarius* EI occurred at similar rates and was activated at acidic pHs in a manner similar to that of the homologous systems (results not shown).

## DISCUSSION

In Gram-positive bacteria, HPr is phosphorylated at the expense of PEP on the catalytic histidine at position 15 by EI, leading to HPr(His~P), and on a seryl residue at position 46 by the ATP-dependent bifunctional enzyme HPrK/P, leading to HPr(Ser-P) (60). Studies conducted with proteins from *E. faecalis* and *B. subtilis* have shown that HPr(Ser-P) is a poor substrate for EI while HPr(His~P) is poorly recognized by HPrK/P (28, 29). In both cases, the phosphorylation rates of phosphorylated HPr are much lower than that of free HPr. Consistent with these results, only minute

amounts of the doubly phosphorylated HPr, HPr(Ser-P)(His~P), have been found in *B. subtilis* and *E. faecalis* (30, 31). These findings led to the suggestion that synthesis of HPr(Ser-P) is a physiological process aimed at regulating the transport of PTS sugars (17, 20).

Several studies conducted over the years have shown, however, that HPr(Ser-P)(His~P) may account for more than 70% of the total HPr in exponentially growing cells of a number of streptococci (24–26, 61). Furthermore, it was demonstrated that this form of HPr can efficiently transfer its phosphate group to the IIA-like domain of the lactose/ $\text{H}^+$  symporter LacS of *S. salivarius* and *S. thermophilus* (26, 27) and to *S. salivarius* IIAB<sup>Man</sup> (unpublished results), a protein involved in the transport of PTS sugars (62). These results clearly indicate that HPr(Ser-P)(His~P) is a physiologically important phosphoprotein.

The possible involvement of HPr(Ser-P)(His~P) in PTS-mediated sugar uptake requires recycling via phosphorylation of HPr(Ser-P) by EI at a rate sufficient to maintain the cellular pool high enough to maximize sugar transport. In this context, one would expect that the phosphorylation of HPr(Ser-P) by *S. salivarius* EI would be a rather efficient process. Surprisingly, we found that HPr(Ser-P) was a poor substrate for *S. salivarius* EI at neutral pH, a result similar to that obtained with proteins isolated from bacteria that do not synthesize large amounts of HPr(Ser-P)(His~P) (28, 29). Moreover, the addition of key metabolites to the reaction mixture did not improve the rate of HPr(Ser-P) phosphorylation, suggesting that cellular levels of HPr(Ser-P)(His~P) are not controlled by the cellular levels of specific central metabolic intermediates. However, while the phosphorylation of HPr(Ser-P) by EI was not efficient at a pH near the neutrality, the initial rates of phosphotransfer determined using a quantitative spectrophotometric assay unequivocally showed that HPr(Ser-P), unlike free HPr, was phosphorylated more efficiently at acidic pH. Indeed, the phosphorylation rate measured with the serine phosphate derivative was almost 10-fold higher under acidic conditions (see Figure 3).

Previous studies have indicated that the phosphorylation of the seryl 46 residue does not significantly modify the structure of HPr (63, 64). The inability of EI to phosphorylate HPr(Ser-P) results mostly from disruption of hydrophobic interactions between the enzyme and its substrate caused by electrostatic repulsion between the dianionic charge of the phosphoserine and a glutamyl residue in EI (Glu<sub>84</sub> in *E. coli* EI) (64). This repulsion does not occur with unphosphorylated HPr, as the free hydroxyl group of the seryl 46 residue is actually hydrogen-bonded to this glutamate (65). Also, it was suggested that the electrostatic repulsion between EI Glu<sub>84</sub> and the phosphoryl group on HPr Ser<sub>46</sub> may be reduced by a slight rotation of the Ser<sub>46</sub> C $\alpha$ –C $\beta$  bond. This local change is believed to disturb the hydrophobic interaction between EI and HPr (64). We thus propose that the stimulation of HPr(Ser-P) phosphorylation by EI at acidic pH is, to some extent, caused by a partial neutralization of the negative charges of Ser<sub>46</sub> of HPr(Ser-P) and/or of the glutamyl residue of EI. Neutralization of these negative charges should partially overcome the electrostatic repulsion between the enzyme and its substrate and decrease the distortion of the Ser<sub>46</sub> C $\alpha$ –C $\beta$  bond. This in turn should stabilize the EI/HPr(Ser-P) complex and allow the efficient

synthesis of HPr(Ser-P)(His~P). However, the rotation of the Ser<sub>46</sub> C<sup>α</sup>–C<sup>β</sup> bond caused by phosphorylation of HPr on Ser<sub>46</sub> may also be caused by sterical hindrance (64). This would explain why acidification of the intracellular lumen did not restore the phosphorylation of HPr(Ser-P) by EI to a level similar to that for HPr. The hypothesis that the stimulation of HPr(Ser-P) phosphorylation by EI at low pHs results from the protonation of the phosphate group and/or the glutamyl residue is supported by experiments conducted with HPr(S46D). Indeed, because the aspartyl residue is a weaker acid than the phosphate group of HPr(Ser-P), one would expect that HPr(S46D) would be a better substrate than HPr(Ser-P) for EI and that charge repulsion would be weakened at a higher pH. We indeed found that HPr(S46D) was phosphorylated almost 5 times faster than HPr(Ser-P) under all conditions tested and that its maximal phosphorylation rate occurred at a pH higher than that of HPr(Ser-P).

From our results, a scenario that explains how rapidly growing streptococci synthesize high levels of HPr(Ser-P)(His~P) began to emerge. The amount of HPr in exponentially growing streptococci can account for 3–4% of the total cytoplasmic proteins, which represents HPr concentrations between 0.5 and 1 mM (66). Additionally, HPr(Ser-P) can represent more than 70% of the total HPr pool in exponentially growing cells (24–26). Thus, the concentrations of the phosphoserine derivative can be roughly estimated at 250–500 μM. These concentrations might even be much higher locally according to the phenomena of macromolecular crowding and metabolite channeling that allow metabolite sequestration and concentrations of cytoplasmic proteins up to 400 mg/mL (67, 68), thus favoring the formation of stable protein complexes. It is noteworthy that the existence of such stable complexes involving several PTS proteins and some of their substrates has already been proposed (69, 70). Accordingly, our results suggest that the cellular concentrations of HPr(Ser-P) in streptococci are high enough to favor phosphorylation of HPr(Ser-P) over HPr, which is virtually absent in growing cells (24). Moreover, several studies have indicated that streptococci do not maintain their intracellular pH near the neutrality in response to a decrease in extracellular pH (55, 71). According to our results, the decrease in the intracellular pH during cellular growth should enhance HPr(Ser-P)(His~P) synthesis by promoting the interaction of HPr(Ser-P) with EI, while phosphorylation of HPr becomes unlikely. These results suggested that the high levels of HPr(Ser-P)(His~P) in streptococci result from the combination of two factors: a high physiological concentration of HPr(Ser-P) and stimulation of HPr(Ser-P) phosphorylation by EI following acidification of the cellular lumen.

*B. subtilis* and *E. faecalis* contain only trace amounts of HPr(Ser-P)(His~P) (30, 31). Surprisingly, we found that the rate of phosphorylation of HPr(Ser-P) by *B. subtilis* EI varied as a function of pH in a manner similar to that observed with the *S. salivarius* proteins. However, unlike streptococci, bacilli and enterococci maintain an intracellular pH near neutrality under conditions of low extracellular pH (55, 56). This property may explain why these bacterial species do not synthesize large amounts of HPr(Ser-P)(His~P) during growth.

The succession of physiological events that occur in streptococcal cells after inoculation of a glucose-containing culture medium can be described as follows. Glucose is first

rapidly taken up via the constitutive mannose/glucose PTS, which may function initially with HPr(His~P) (72). The rapid metabolism of glucose via the Emden–Meyerhoff pathway causes an increase in the levels of cellular metabolites that are indicators of a high cellular energy status (54, 71). Under these conditions, the kinase activity of HPrK/P is stimulated while its phosphorylase activity is repressed, allowing the synthesis of large amounts of HPr(Ser-P) (25, 35). The cells are then able to prevent the entry of secondary energy sources by HPr(Ser-P)-mediated inducer exclusion (17–19) and to inhibit the expression of genes under the control of the CcpA/HPr(Ser-P) complex (73). The intense cellular glycolytic activity generates large amounts of lactic acid, which is excreted into the external medium (74). As the acidification of the external milieu is accompanied by a decrease in intracellular pH (57), the synthesis of HPr(Ser-P)(His~P) is stimulated and the doubly phosphorylated HPr becomes the principal EI/IIA substrate, ensuring optimal PTS-mediated sugar uptake. Consistent with this hypothesis is the recent finding that HPr(Ser-P)(His~P) can efficiently transfer its PO<sub>4</sub> to *S. salivarius* IIA<sup>B<sub>Man</sub></sup> (unpublished results), a PTS protein involved in the transport of glucose, fructose, and mannose (62). At this stage, the cellular levels of free HPr and HPr(His~P) are virtually nil (24). This should prevent the activation of transcriptional activators and antiterminators containing PRD domains. This hypothesis is supported by recent studies suggesting that *B. subtilis* HPr(Ser-P)(His~P) is unable to phosphorylate the PRD-containing antiterminator LicT (75). According to this model, control of sugar metabolism in exponentially growing streptococcal cells would be mediated by HPr(Ser-P) via CcpA-dependent transcription activation and repression as well as inducer exclusion while PTS sugar transport would be ensured by HPr(Ser-P)(His~P).

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