BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 10765–10774 10765
DOI: 10.1021/bi901512b

Kinetic Studies of HPr, HPr(H15D), HPr(H15E), and HPr(His∼P) Phosphorylation by the *Streptococcus salivarius* HPr(Ser) Kinase/Phosphorylase†

Israël Casabon,‡ Manon Couture,§ Katy Vaillancourt,‡ and Christian Vadeboncoeur*,‡

‡*Groupe de recherche en écologie buccale (GREB), Faculté de Médecine Dentaire, and Département de Biochimie et de Microbiologie, Faculté des Sciences et de Génie, Université Laval, Quebec City, Quebec, Canada, and* §*Centre de recherche sur la fonction, la structure et l'ingénierie des protéines (PROTEO), Département de Biochimie et de Microbiologie, Faculté des Sciences et de Génie, Université Laval, Quebec City, Quebec, Canada*

Received August 28, 2009; Revised Manuscript Received September 29, 2009

ABSTRACT: HPr is a central protein of the phosphoenolpyruvate:sugar phosphotransferase transport system (PTS). In streptococci, HPr can be phosphorylated at His_{15} at the expense of PEP by enzyme I (EI) of the PTS, producing HPr(His∼P). HPr can also be phosphorylated at Ser_{46} by the ATP-dependent HPr(Ser) kinase/phosphorylase (HprK/P), producing HPr(Ser-P). Lastly, HPr can be phosphorylated on both residues, producing HPr(Ser-P)(His∼P) (HPr-P2). We report here a study on the phosphorylation of *Streptococcus salivarius* HPr, HPr(H15D), HPr(H15E), and HPr(His∼P) by HprK/P to assess the involvement of HprK/P in the synthesis of HPr-P2 in streptococcal cells. We first developed a spectrophotometric method for measuring HprK/P kinase activity. Using this assay, we found that the K_m of HprK/P for HPr at pH 7.4 and 37 °C was approximately 110 μM, with a specificity constant (k_{cat}/K_m) of 1.7×10^4 M^{-1} s^{-1}. The specificity constants for HPr(H15D) and HPr(H15E) were ∼13 times lower. Kinetic studies conducted under conditions where HPr(His∼P) was stable (i.e., pH 8.6 and 15 °C) showed that HPr(His∼P) was a poorer substrate for HprK/P than HPr(H15D), the k_{cat}/K_m for HPr(H15D) and HPr(His∼P) being approximately 9 and 26 times lower than that for HPr, respectively. Our results suggested that (i) the inefficiency of the phosphorylation of HPr(His∼P) by HprK/P results from the presence of a negative charge at position 15 as well as from other structural elements and (ii) the contribution of streptococcal HprK/P to the synthesis of HPr-P2 in vivo is marginal.

The PTS[1] is a transport and signal transduction system that plays a crucial role in the control of sugar metabolism in several bacteria (*1*). The primary functions of the PTS are to detect, transport, and phosphorylate a variety of mono- and disaccharides (*1*). Transport of sugars by the PTS begins with the autophosphorylation of EI at the expense of PEP to form P∼EI, which 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 to a variety of sugar-specific EIIs, which translocate and phosphorylate incoming sugars (*1*, *2*). EIIs are composed of three and sometimes four domains, namely, IIA, IIB, IIC, and IID, which can have different configurations on separate polypeptides or in multidomain proteins (*1*, *3*).

In low-GC Gram-positive bacteria, HPr can be phosphorylated at two residues, His_{15} and Ser_{46} (*1*). HPr(His∼P) is involved in PTS-mediated sugar uptake and regulates, by phosphorylation, enzymes, transporters, and several PRD-containing transcriptional regulators involved in the metabolism of secondary energy sources (*1*, *4*). HPr is phosphorylated at Ser_{46} by the ATP-dependent bifunctional HPr(Ser) kinase/phosphorylase (HprK/P) (*5*). The resulting product, HPr(Ser-P), cannot be used to mediate sugar transport since its phosphoester bond does not possess a high-energy phosphotransfer potential (*6*). However, in many low-GC Gram-positive bacteria, HPr(Ser-P) is involved in inducer exclusion (*1*, *7*) and in CCR and CCA by interacting with the pleiotropic transcriptional regulator CcpA (*8*). Recently, *Bacillus subtilis* HPr(Ser-P) has been proposed to interact with the transcriptional regulator RbsR and glyceraldehyde-3-phosphate dehydrogenase (*9*, *10*).

The bacterial HprK/P belongs to a new family of serine/threonine protein kinases that recognize the tertiary rather than the primary structure of their substrates (*11*−*13*). Most HprK/Ps are homohexameric enzymes in the native state and contain six HPr binding sites (*5*, *11*, *14*). The bifunctional HPr(Ser) kinase/phosphorylase (EC 2.7.11... and EC 2.7.4...) catalyzes, via a bi-bi sequential mechanism (*15*, *16*), the ATP- and PP_i-dependent phosphorylation of HPr as well as the P_i-dependent

†This work was supported by the Canadian Institutes of Health Research (MOP 36338). I.C. is a Ph.D. student in biochemistry supported by the Fonds québécois de la recherche sur la nature et les technologies (FQRNT) (96071) and the Natural Sciences and Engineering Research Council of Canada (NSERC) (6792-254666-2002).

*To whom correspondence should be addressed: Groupe de recherche en écologie buccale (GREB), Faculté de Médecine Dentaire, Université Laval, Quebec City, Quebec, Canada G1V 0A6. Phone: (418) 656-2319. Fax: (418) 656-2861. E-mail: Christian.Vadeboncoeur@bcm.ulaval.ca.

[1]Abbreviations: PTS, bacterial phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I; P∼EI, phosphorylated enzyme I; EII, enzyme II; HPr, heat-stable histidine-containing protein; Ser_{46}, seryl residue at position 46; His_{15}, histidyl residue at position 15; HPr(His∼P), HPr phosphorylated at histidine 15; HPr(Ser-P), HPr phosphorylated at serine 46; HPr(Ser-P)(His∼P) and HPr-P2, doubly phosphorylated HPr; HPr(H15D), HPr in which histidine 15 has been replaced with an aspartate; HPr(H15E), HPr in which histidine 15 has been replaced with a glutamate; CcpA, catabolite control protein A; CCR, carbon catabolite repression; CCA, carbon catabolite activation; $IIAB^{Man}$, cytoplasmic domains A and B of the mannose:phosphotransferase system; IIC and IID, membrane-bound EII domains C and D, respectively; HprK/P, HPr(Ser) kinase/phosphorylase; FBP, fructose 1,6-bisphosphate; LacS, lactose/H^+ symporter; LDH, lactate dehydrogenase; PK, pyruvate kinase; PRD, PTS regulatory domain; RbsR, ribose operon repressor in *Bacillus subtilis*; P_i, inorganic phosphate; PP_i, pyrophosphate.

Published on Web 10/13/2009

phosphorolysis of HPr(Ser-P) (see Scheme 1) (*5*). In *B. subtilis*, the HprK/P kinase activity is stimulated by glycolytic intermediates, the most potent being FBP (*1*, *17*). However, the kinase activity of HprK/Ps from enterococci, streptococci, and mycoplasma is not or is only marginally enhanced by FBP. Nonetheless, in most cases, FBP prevents the inhibition of kinase activity by P_i (*14*, *15*, *18*, *19*).

So far, most HPr-related functions in low-GC Gram-positive bacteria have been attributed to HPr(His∼P) and HPr(Ser-P) (*1*). However, rapidly growing streptococci and lactococci contain high levels of HPr(Ser-P)(His∼P) (HPr-P2) (*20*, *21*). HPr-P2 has also been detected in *Mycoplasma pneumoniae*, *B. subtilis*, and *Listeria monocytogenes* under specific growth conditions (*22*−*24*). Recent studies have shown that HPr-P2 can efficiently transfer its phosphoryl group to the IIA-like domain of the non-PTS LacS permeases of *Streptococcus thermophilus* and *Streptococcus salivarius* (*25*, *26*) and the *S. salivarius* $IIAB^{Man}$ protein (*20*). These results suggest that HPr-P2 controls the activity of proteins possessing a IIA-like domain and participates in PTS-mediated sugar transport. This is consistent with the observation that many streptococcal and lactococcal strains are virtually devoid of HPr(His∼P) but contain large amounts of HPr-P2 during growth on glucose (*20*).

We previously showed that streptococcal HPr-P2 can be synthesized via the phosphorylation of HPr(Ser-P) by EI (*27*). However, no data to date make it possible to assess the involvement of HprK/P in the synthesis of HPr-P2 in streptococci. Recently, it was found that HPr(His∼P) from *M. pneumoniae* can be phosphorylated in vitro by HprK/P (*28*). It was also shown that *L. monocytogenes* HprK/P can phosphorylate HPr(H15D) (*29*), a mutant protein used to mimic HPr(His∼P). These results suggest that the doubly phosphorylated HPr can be synthesized via ATP-dependent phosphorylation of HPr(His∼P) in some low-GC Gram-positive bacteria.

Scheme 1: Reactions Catalyzed by HprK/P

$$\text{HPr} + \text{ATP} \longrightarrow \text{HPr(Ser-P)} + \text{ADP}$$

$$\text{HPr} + \text{PP}_i \longrightarrow \text{HPr(Ser-P)} + \text{P}_i$$

$$\text{HPr(Ser-P)} + \text{P}_i \longrightarrow \text{HPr} + \text{PP}_i$$

The objective of this work was to determine whether streptococcal HprK/P can participate in the synthesis of HPr-P2. We thus determined, using a spectrophotometric assay, kinetic constants for HPr, the primary substrate of HprK/P, and for HPr(H15D) and HPr(H15E), two structural analogues that are believed to mimic HPr(His∼P). Then, we report results obtained with the second natural putative substrate of HprK/P, namely HPr(His∼P). Our results suggested that the presence of a negative charge as well as other structural elements prevents HprK/P from phosphorylating HPr(His∼P) at a high rate, which makes the involvement of HprK/P in the accumulation of HPr-P2 in the cells during growth unlikely.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions. All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown as described previously (*27*).

Gene Cloning. Replacement of HPr His_{15} with an aspartyl or a glutamyl residue was conducted as described previously for the replacement of HPr Ser_{46} with an aspartyl residue (*27*). The oligonucleotide primers used were ptsH15D-F (5′-CAGAAACAGGTATCGATGCACGTCCAGCTA-3′) and ptsH15D-R (5′-TAGCTGGACGTGCATCGATACCTGTTTCTG-3′) for the histidyl-to-aspartyl replacement or ptsH15E-F (5′-CAGAAACAGGTATCGAAGCACGTCCAGCTA-3′) and ptsH15E-R (5′-TAGCTGGACGTGCTTCGATACCTGTTTCTG-3′) for the histidyl-to-glutamyl replacement. Plasmids pH15D1 and pH15E were amplified in *E. coli* TOP10 (Invitrogen) and *E. coli* XL1-Blue (Stratagene), respectively.

Table 1: Strains and Plasmids

strain or plasmid	relevant genotypes and/or characteristic(s)[a]	source or reference
strains		
E. coli XL1 Blue	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac*[F′, *proAB, lacI*^{q}ZΔM15, Tn*10* (Tet^R)]	Stratagene
E. coli TOP10	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), *ϕ*80*lacZ*ΔM15, Δ*lacX74, recA1, araD139*, Δ(*ara-leu*)*7697, galU, galK, rpsL* (Str^R), *endA1, nupG*	Invitrogen
E. coli LMG194	F^-, Δ*lacX74, galE, thi, rpsL* (Str^R), Δ*phoA*, (*pvu*II), Δ*ara714, leu*::Tn*10* (Tet^R)	Invitrogen
E. coli BL21(DE3)	F^-, *ompT, hsdS*$_B$, (r_B^- m_B^-), *dcm, gal,* (DE3)	Novagen
plasmids		
pET28a(+)	expression vector, Kan^R	Novagen
pBAD/HisB	expression vector, Amp^R	Invitrogen
pHPW18	contains the *ptsH* gene of *S. salivarius* ATCC 25975 cloned into pBAD/HisB	*14*
pH15D1	contains the *ptsH* gene of *S. salivarius* ATCC 25975 with a mutation replacing HPr His_{15} by D, cloned into pBAD/HisB	this work
pH15E	contains the *ptsH* gene of *S. salivarius* ATCC 25975 with a mutation whereby HPr His_{15} is replaced with E, cloned into pBAD/HisB	this work
pETI-16	contains the *ptsI* gene of *S. salivarius* ATCC 25975 cloned into pET28a(+)	*26*
pHPKHis	contains the *hprK* gene of *S. salivarius* ATCC 25975 cloned into pET28a(+)	*14*

[a] Amp^R, ampicillin resistance; Kan^R, kanamycin resistance; Str^R, streptomycin resistance; Tet^R, tetracycline resistance.

Protein Overexpression. All proteins used in this study were overexpressed as recombinant proteins fused with an N-terminal six-histidine tag sequence (6×His), except for (6×His)HPr-(His∼P) synthesis where *S. salivarius* ATCC 25975 native EI was used. Recombinant *S. salivarius* HprK/P and EI enzymes were overproduced as described previously (*27*). The *S. salivarius* HPr, HPr(H15D), and HPr(H15E) substrates were overproduced using 1 or 10 L of *E. coli* LMG194 (pHPW18, pH15D1, or pH15E), as described previously for HPr (*27*).

Purification of EI and HprK/P. Native *S. salivarius* EI was purified as described previously (*30*). Recombinant *S. salivarius* EI was purified and used under previously described conditions (*27*). *S. salivarius* recombinant HprK/P (GenBank accession number AAD12781) was purified as described previously (*14*, *27*), with the following modifications. After passage on the Ni^{2+} resin, fractions containing the enzyme were pooled and dialyzed at 4 °C for 16 h against 25 mM Tris-HCl buffer (pH 7.9) and then loaded on a Mono-Q 5/50 GL column (Amersham Biosciences), which was first equilibrated at room temperature with 20 mM Tris-HCl (pH 7.5) containing 10% glycerol. The recombinant enzyme was eluted in the same buffer with a 0 to 350 mM KCl gradient at 1 mL/min for 35 min followed by a 5 min step with 500 mM KCl. Fractions (1 mL) were collected and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 12.5% acrylamide gels. Fractions of interest were pooled, dialyzed at 4 °C for 16 h against 25 mM Tris-acetate (pH 7.8) and 150 mM KCl, supplemented with 10% glycerol, 0.1 mM PMSF, 2 μM pepstatin A, 2 μM leupeptin, and 1 mM EDTA, and stored at −80 °C until they were used. This method yielded approximately 10 mg/L of culture of highly stable, homogeneous recombinant HprK/P as determined using Coomassie Blue-stained SDS−PAGE (results not shown). For experiments involving HprK/P, the enzyme was diluted in cold 50 mM Tris-acetate buffer (pH 7.8) containing 100 mM KCl and was left for 75 min on ice before being used. Under these conditions, *S. salivarius* recombinant HprK/P was stable for at least 2 h (data not shown). The HprK/P concentrations indicated throughout this work are those of the active sites (six per enzyme).

Purification of Recombinant HPr, HPr(H15D), and HPr(H15E). *S. salivarius* HPr, HPr(H15D), and HPr(H15E) were purified from cellular extracts obtained from 1 or 10 L of culture as described previously for HPr (*27*), with the following modifications. Before the elution, the Ni^{2+} column was washed with 15 mL of cold 50 mM Tris-acetate buffer (pH 7.8) containing 300 mM NaCl and 20 mM imidazole. The His-tagged proteins were then eluted with the same buffer containing 250 mM imidazole. Fractions containing the recombinant proteins were pooled and dialyzed at 4 °C for 16 h against 25 mM HEPES (pH 7.7) containing 50 mM NaCl, filtered through 0.20 μm pore filters (Millipore), and concentrated using NanoSep 3K membranes (Pall). KCl, at a final concentration of 100 mM, was added to the HEPES buffer used for the Superdex 75 10/300 GL column (Amersham Biosciences). This purification procedure yielded approximately 5 mg of recombinant HPr, HPr(H15D), or HPr(H15E) per liter of culture, with a degree of purity exceeding 98% as determined using Coomassie Blue-stained SDS−PAGE (results not shown). The three substrates also appeared to be homogeneous when analyzed by Coomassie Blue-stained PAGE (results not shown).

HPr(His∼P) Synthesis and Purification. HPr(His∼P) was synthesized by EI-catalyzed PEP-dependent phosphorylation of recombinant *S. salivarius* HPr in a total volume of 4.5 mL of 100 mM Tris-HCl (pH 8.7) containing 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 6% glycerol, 5 mM PEP, and 16.5 mg HPr. Native *S. salivarius* EI (200 μg) was added to initiate the reaction after a 5 min preincubation period at 15 °C. After 30 min at 15 °C, the reaction mixture was put on ice. All subsequent steps were performed at 4 °C. The solution was deposited on a Ni^{2+}-Sepharose HP resin (1 mL) (Amersham Biosciences) column, equilibrated with 50 mM Tris-HCl (pH 8.8) containing 10% glycerol, 300 mM KCl, and 10 mM imidazole, and the supernatant was removed by gravity flow. The resin was then washed with 24 mL of the same buffer. Lastly, HPr(His∼P) was eluted in the same buffer containing 200 mM KCl and 250 mM imidazole. Fractions (1 mL) were collected and immediately stored at −80 °C. After analysis by PAGE on 15% nondenaturing acrylamide gels, fractions of interest were pooled and dialyzed for 8 h at 4 °C against 2 × 2 L of 50 mM Tris-HCl (pH 8.8) containing 15% glycerol and 200 mM KCl, with a buffer change after 4 h. The resulting fraction was then stored at −80 °C until it was used. The final preparations of HPr(His∼P) contained approximately 25% unphosphorylated HPr, which is designated as endogenous HPr in this paper. HPr(His∼P) preparations can be kept at −80 °C for at least 3 months without significant loss due to phosphohydrolysis (results not shown).

Spectroscopy. All spectrophotometric measurements were taken using a DU-530 spectrophotometer (Beckman). For protein quantifications at 280 nm, data were recorded at 25 °C. When using the EI-LDH- or HprK/P-PK-LDH-coupled assay (see below), the spectrophotometer was equipped with a Peltier temperature control module (Beckman) adjusted to 15 or 37 °C.

Protein Quantification. The concentrations of HprK/P, EI, and HPr were determined spectrophotometrically as described previously (*27*). The *S. salivarius* HPr molar extinction coefficient (ε_{280} = 3475 M^{-1} cm^{-1}) (*27*) was used for the quantification of HPr(H15D) and HPr(H15E). The concentration of HPr(His∼P) was measured as follows. First, the concentration of endogenous HPr in the HPr(His∼P) preparation was determined by spectrophotometry using the EI-LDH-coupled assay as previously described (*27*), with the following modifications to prevent spontaneous phosphohydrolysis of HPr(His∼P). Briefly, the reaction mixture (0.6 mL) contained 150 mM Tris-HCl (pH 8.7), 5 mM $MgCl_2$, 150 mM KCl (buffer A), 1 mM DTT, 60 units of LDH (rabbit muscle; Sigma-Aldrich), 100 μM NADH, 500 nM EI, and different volumes of the HPr(His∼P) preparation. The reaction mixture was preincubated for 15 min at 15 °C before addition of 10 mM PEP to initiate the reaction, which reached completion in less than 1 min. The endogenous HPr concentration was then calculated from the ΔA_{340} resulting from the NADH-to-NAD^+ conversion (ε_{340} = 6300 M^{-1} cm^{-1}). Next, the total amount of HPr in the HPr(His∼P) preparation [i.e., HPr(His∼P) with endogenous HPr] was determined using the same assay after the HPr(His∼P) preparation had been heated for 3 min at 100 °C. The concentration of HPr(His∼P) was then deduced by subtracting the amount of endogenous HPr from the amount of total HPr.

Electrophoretic and Densitometric Analyses. Electrophoretic separations were performed at room temperature or 4 °C using a Bio-Rad Mini-Protean II or Mini-Protean Tetra Cell apparatus under either native or denaturing conditions (*31*). Densitometric analyses were performed as described previously (*27*), with the exception that Coomassie Blue staining was performed for 20 min. We experimentally determined, using proteins of known concentrations, that the densitometric

analyses gave values with an error ranging from 1 to 10% depending on the intensity of the protein spot (results not shown).

HPr(His∼P) Phosphohydrolysis. The rate of spontaneous phosphohydrolysis of HPr(His∼P) at pH 8.7 and 15 °C (condition A) and pH 7.5 and 37 °C (condition B), in the presence and absence of HprK/P (900 nM at pH 8.7 and 500 nM at pH 7.5), was determined by spectrophotometry using the EI-LDH-coupled assay described above, with the following modifications. For condition A, the HPr concentration was 25 μM. For condition B, the buffer was HEPES containing 20 units of LDH and the HPr concentration was 33 μM. After complete phosphorylation of HPr, the rate of phosphohydrolysis was measured by the decrease in the absorbance at 340 nm. The rate of NADH oxidation in the absence of PEP was subtracted from the rate obtained in the presence of PEP. The rate of HPr(His∼P) phosphohydrolysis in the presence of HprK/P was also determined by PAGE under the two sets of conditions mentioned above. For condition A, experiments were conducted in 150 μL of buffer A containing 25 μM HPr(His∼P). HprK/P (900 nM) was added after a 5 min preincubation period at 15 °C. Samples were withdrawn at intervals and mixed with 3× PAGE loading buffer containing 50 mM EDTA and were kept on ice until being analyzed by PAGE via 15% acrylamide gels. Scanned gels were analyzed by densitometry as described above. For condition B, experiments were conducted as described above, except that the buffer was HEPES, the final volume was 110 μL, the preincubation period was 2 min, and the concentrations of HPr(His∼P) and HprK/P were 33 μM and 500 nM, respectively.

HprK/P Kinase Activity Determined by Spectrophotometry. The kinetic constants of the ATP-dependent phosphorylation of HPr, HPr(H15D), and HPr(H15E) were measured spectrophotometrically by coupling the HprK/P-catalyzed reaction with those of pyruvate kinase (PK) and lactate dehydrogenase (LDH). A similar procedure was used to quantify adenylate kinase activity by determining the amount of ADP produced (*32*). The reaction was conducted in a total volume of 0.6 mL containing 150 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP (buffer B), 20 units of LDH (rabbit muscle; Sigma-Aldrich), 10 units of PK (rabbit muscle; Sigma-Aldrich), 2 mM PEP, 100 μM NADH (ε_{340} = 6300 M^{-1} cm^{-1}), and various amounts of HPr (0−300 μM), HPr(H15D) (0−425 μM), or HPr(H15E) (0−425 μM). The solution was preincubated for 15 min at 37 °C, and the reaction was initiated via addition of 150 nM HprK/P for HPr phosphorylation or 500 nM HprK/P for HPr(H15D) and HPr(H15E) phosphorylation. The reaction rates were measured by recording the absorbance at 340 nm. The rate of NADH oxidation in the absence of HprK/P was subtracted from the rate obtained in the presence of HprK/P. The initial rates were measured when less than 20% of the substrate had been consumed at ≤20 μM substrate or when less than 10% of the substrate had been consumed at >20 μM substrate. The linear regression coefficients (R^2) were ≥0.97.

The spectrophotometric HprK/P-PK-LDH-coupled assay was also used to determine the effect of FBP at pH 7.4 on the rates of HPr, HPr(H15D), and HPr(H15E) phosphorylation. Briefly, 50 μM HPr, HPr(H15D), or HPr(H15E) was phosphorylated at 37 °C in the presence of 5 mM ATP, 0 or 25 mM FBP, and 100 nM HprK/P for HPr or 500 nM HprK/P for the mutated proteins. The effect of KCl (10−200 mM) was also measured in the presence of 50 μM HPr and 100 nM HprK/P at 37 °C and pH 7.4. Under all conditions, initial rates were measured when less than 10% of the substrate had been consumed. The linear regression coefficients (R^2) were ≥0.95.

HprK/P Activity at pH 7.4 and 37 °C Determined by PAGE. Kinetic constants for HPr phosphorylation, and the effect of P_i on HprK/P kinase activity, were determined after the reaction products had been separated by PAGE. For the determination of the kinetic constants, phosphorylation experiments were conducted in 125−200 μL of buffer B containing increasing concentrations of HPr (0−171 μM) and 50 nM HprK/P to initiate the reaction after 10 min preincubation periods at 37 °C. Samples were withdrawn at intervals and mixed with 3× PAGE loading buffer containing 50 mM EDTA. They were heated at 100 °C for 3 min and were kept on ice until being deposited on 15% acrylamide gels and separated by PAGE. Gels were scanned and analyzed by densitometry. The effect of P_i (0−1 mM) on HPr phosphorylation rates was measured in the presence of 20 μM HPr and 25 nM HprK/P at 37 °C and pH 7.5. Under all conditions, the initial rates were linear and were measured when less than 20% of the substrate had been consumed. The correlation coefficients (R^2) were ≥0.95.

The effect of pH (pH 5.6−8.3) on the phosphorylation rates of HPr and HPr(H15D) was also determined by densitometric analyses of PAGE gels. The reaction mixtures (120−150 μL) contained the appropriate buffer at 150 mM (MES for pH 5.6, 6.0, and 6.5; HEPES for pH 7.0 and 7.5; Tris-HCl for pH 8.3), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, and 40 μM HPr or HPr(H15D). The mixtures were preincubated for 5 min at 37 °C before addition of 4−100 nM HprK/P for the phosphorylation of HPr or 20−200 nM HprK/P for the phosphorylation of HPr(H15D) to initiate the reaction. Under all conditions, the initial rates were linear and were measured when less than 20% of the substrate had been consumed. The correlation coefficients (R^2) were ≥0.90.

Phosphorylation of HPr(His∼P) by HprK/P at pH 7.5 and 37 °C. Phosphorylation experiments were conducted in 110 μL of buffer B, at pH 7.5, containing 150 mM KCl and 33 μM HPr or HPr(His∼P). The mixtures were incubated for 2 min at 37 °C. Then, 20 nM HprK/P for the phosphorylation of HPr or 500 nM HprK/P for the phosphorylation of HPr(His∼P) was added to initiate the reactions. Aliquots were taken at intervals and mixed with 3× PAGE loading buffer containing 50 mM EDTA. Samples from experiments conducted with HPr were heated for 3 min at 100 °C and then conserved on ice. Since the phosphoramidate bond of HPr(His∼P) is heat-labile (*33*), samples from experiments conducted with HPr(His∼P) were not heated but immediately placed on ice. Samples were deposited on 15% acrylamide gels and separated by PAGE. Gels were scanned and analyzed by densitometry. Experiments with HPr(His∼P) were analyzed as follows. Since the HPr(His∼P) preparations contained endogenous HPr, phosphorylation of HPr(His∼P) by HprK/P gave rise to HPr(Ser-P) and HPr-P2. The final solution also contained nonphosphorylated HPr and HPr(His∼P). HPr(Ser-P) and HPr(His∼P) migrated at the same position on PAGE gels, whereas HPr migrated more slowly and HPr-P2 more rapidly. The amount of HPr(Ser-P) was estimated by calculating the difference between the amounts of free HPr at time zero and x min. The amount of HPr(His∼P) was estimated by calculating the difference between the amount of HPr(His∼P) and HPr(Ser-P) and the amount of HPr(Ser-P) estimated as described above. Under all conditions, the initial rates were linear and were measured when less than 20% of the substrate had been consumed. The correlation coefficients (R^2) were ≥0.95.

Table 2: Kinetic Constants of *S. salivarius* HprK/P at pH 7.4 and 37 °C[a]

substrate	K_m (μM)	V_{max} (units/mg of HprK/P)[b]	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
HPr (spectro)	109 ± 7[c]	3.0 ± 0.1	1.9 ± 0.1	17×10^3
HPr (PAGE)	120 ± 30[d]	2.8 ± 0.4	1.7 ± 0.2	14×10^3
HPr(H15D) (spectro)	ND[e]	ND[e]	ND[e]	1.4×10^{3c}
HPr(H15E) (spectro)	ND[e]	ND[e]	ND[e]	1.2×10^{3c}

[a]All the constants were determined using the same enzyme preparation. [b]One unit is the amount of HprK/P that phosphorylated 1 μmol of HPr per minute at pH 7.4 and 37 °C. [c]The values (±standard error) were calculated from the curves shown in Figure 1 (panel A, curve with ○, and panel B). For each substrate concentration, the reaction rates were measured at least three times with the spectrophotometric HprK/P-PK-LDH-coupled assay (spectro). The constants for HPr were obtained by nonlinear regression using the Michaelis−Menten equation, whereas those for HPr(H15D) and HPr(H15E) were obtained using linear regressions. [d]The values (±standard error) were calculated from the best fit shown in Figure 1 (panel A, curve with ▽). For each substrate concentration, the reaction rates were measured at least three times. The rates were determined after separation of the reaction products by PAGE followed by densitometric analyses. The constants were obtained by nonlinear regression using the Michaelis−Menten equation. [e]Not determined.

Table 3: Kinetic Constants of *S. salivarius* HprK/P at pH 8.6 and 15 °C[a]

substrate	K_m (μM)	V_{max} (units/mg of HprK/P)[b]	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
HPr	183 ± 30	2.4 ± 0.2	1.5 ± 0.1	82×10^2
HPr-(H15D)	ND[c]	ND[c]	ND[c]	8.6×10^2
HPr-(His∼P)	ND[c]	ND[c]	ND[c]	3.1×10^2

[a]All the constants were determined using the same enzyme preparation, which possessed, however, a specific activity 2.5-fold higher than that of the enzyme preparation used to obtain the data listed in Table 2. The values (±standard error) were calculated from the curves shown in Figure 6. For each substrate concentration, the reaction rates were measured at least three times. The rates were determined after separating the reaction products by PAGE followed by densitometric analyses. The constants for HPr were obtained by nonlinear regression using the Michaelis−Menten equation, whereas those for HPr(H15D) and HPr(His∼P) were obtained using linear regressions. [b]One unit is the amount of HprK/P that phosphorylated 1 μmol of HPr per minute at pH 8.6 and 15 °C. [c]Not determined.

HprK/P Activity at Alkaline pH. Phosphorylation experiments were conducted in 150 μL of buffer A supplemented with 5 mM ATP, 0 or 25 mM FBP, and 25 μM HPr, HPr(H15D), or HPr(His∼P). After 5 min preincubation periods at 15 °C, 200 nM HprK/P for the phosphorylation of HPr and HPr(H15D) or 900 nM HprK/P for the phosphorylation of HPr(His∼P) was added to initiate the reactions. Aliquots were taken at intervals and analyzed by PAGE as described in the previous section. The effect of P_i (0−100 μM) on HPr phosphorylation rates was also measured at pH 8.7 in the presence of 25 μM HPr and 50 nM HprK/P for experiments conducted at 37 °C or 200 nM HprK/P for experiments conducted at 15 °C. Under all conditions, the initial rates were linear and were measured when less than 20% of the substrate had been consumed. The correlation coefficients (R^2) were ≥0.95.

Kinetic constants for HPr, HPr(H15D), and HPr(His∼P) phosphorylation were determined in buffer A at pH 8.6 and 15 °C using the protocol described above, with the following modifications. Reactions with HPr (0−250 μM) and HPr(H15D) (0−600 μM) were conducted in 200 μL of buffer A, containing 100 mM instead of 150 mM KCl and supplemented with 5 mM ATP, whereas those with HPr(His∼P) (0−175 μM) were conducted in 120 μL of buffer A supplemented with 5 mM ATP. Since the HPr(His∼P) preparations contained different proportions of endogenous HPr, the HPr(His∼P):HPr ratio was kept constant at 1.5:1 for the kinetic studies via addition of the appropriate amount of HPr to the reaction mixtures. Reactions were initiated by the addition of 75 nM HprK/P for HPr phosphorylation, 200 nM HprK/P for HPr(H15D) phosphorylation, or 900 nM HprK/P for HPr(His∼P) phosphorylation. Under all conditions, the initial rates were linear and were measured when less than 20% of the substrate had been consumed. The correlation coefficients (R^2) were ≥0.90.

Kinetic Data Analysis and pH Determination. The kinetics of HPr phosphorylation were analyzed with Kaleidagraph 3.51 (Synergy Software) using the Michaelis−Menten nonlinear regression curve function (*15*, *34*). The kinetics of HPr(H15D), HPr(H15E), and HPr(His∼P) phosphorylation were analyzed by linear regression using Microsoft Excel 2000 (Microsoft Corp.) (*35*). In all cases, the pHs of the reaction mixtures (±0.1) were determined in the test tubes using an InLab Micro microprobe (Mettler Toledo) after all the components were mixed.

RESULTS

Purification of HprK/P. The protocol used to purify *S. salivarius* recombinant HprK/P was optimized to preserve the activity of the enzyme during purification and storage. HprK/P was purified as previously described (*14*, *27*), with an additional anionic exchange chromatography purification step. Dialyzed enzyme preparations can be kept at −80 °C for more than 3 years without a significant loss of activity (data not shown). The specific kinase activity of HprK/P varied from preparation to preparation. The data presented in Table 2 were thus obtained from experiments conducted using a single enzyme preparation. The data presented in Table 3 were also obtained using a single enzyme preparation that, however, possessed 2.5-fold higher specific activity.

Measurement of HprK/P Kinase Activity by Spectrophotometry. As pointed out by Poncet et al. (*5*), a method that would enable the initial reaction rates of HprK/P to be more accurately measured would facilitate the study of the kinetic properties of this enzyme. For this purpose, we developed a coupled spectrophotometric assay using pyruvate kinase (PK) and lactate dehydrogenase (LDH). The ATP-dependent phosphorylation of HPr by HprK/P produces ADP and HPr(Ser-P). In the presence of PEP and PK, the newly formed ADP is converted to pyruvate and ATP, which is a substrate of HprK/P. Via addition of LDH and NADH, pyruvate is converted to lactate, and the resulting NADH-to-NAD^+ conversion is followed spectrophotometrically by the decrease in absorbance at 340 nm. Since one molecule of NADH is consumed for each molecule of HPr(Ser-P) produced, the initial reaction rates can be easily and accurately determined. To confirm that the assay specifically measured HprK/P kinase activity, we conducted a set of tests that showed that (i) the two coupling enzymes were not limiting, (ii) the Mg^{2+}, ATP, PEP, and NADH concentrations were in excess, (iii) PEP, NADH, PK, and LDH did not interfere with HprK/P activity, (iv) the initial rates were similar to those determined by PAGE between pH 5.6 and 7.7, and (v) the reaction rates were proportional to the HprK/P concentration within a relatively wide range (50−600 nM) (data not shown). However, the rates of

HPr phosphorylation by HprK/P determined using the spectrophotometric assay at pH >7.7 were lower than the rates determined by measuring, by PAGE, the amounts of HPr(Ser-P) produced over time (data not shown). The problem was not overcome by merely increasing the amount of PK and LDH in the reaction medium. Thus, the HprK/P kinase spectrophotometric assay described in this paper could only be used at pH ≤7.7.

Determination of S. salivarius HprK/P Kinetic Constants at pH 7.4 and 37 °C. We determined, using the spectrophotometric assay, the kinetic constants associated with the phosphorylation of HPr, and HPr(H15D) and HPr(H15E), two structural analogues that are believed to mimic HPr(His∼P). The experiments were conducted at pH 7.4 and 37 °C. The results for HPr (Figure 1A) were obtained by analyzing the data with the Michaelis−Menten equation. The results for HPr(H15D) and HPr(H15E) (Figure 1B) were analyzed by linear regression at low substrate concentrations ([S] ≪ K_m), which allows the specificity constants (k_{cat}/K_m) to be precisely determined (*35*). Values for the affinity constant (K_m), maximal velocity (V_{max}), turnover number (k_{cat}), and specificity constant (k_{cat}/K_m) are listed in Table 2. Under these conditions, the specificity constants for the mutated HPrs were ∼12−14 times lower than for HPr. For the kinetics of HPr phosphorylation, we also analyzed the data presented in Figure 1A using the Hill equation. A Hill coefficient (h) of 0.9 was found (data not shown), indicating that, as opposed to *B. subtilis* HprK/P (*17*), *S. salivarius* HprK/P did not bind HPr cooperatively. This result is consistent with previously published data (*15*).

To validate the HprK/P-PK-LDH-coupled assay, we determined the kinetic constants for HPr at pH 7.4 and 37 °C by separating the reaction products by PAGE as illustrated in Figure 2, followed by densitometric analyses. The Michaelis−Menten curve fit of the results is shown in Figure 1A, and the K_m, V_{max}, k_{cat}, and k_{cat}/K_m values are given in Table 2 (row 2). Although variations between measurements conducted with identical substrate concentrations were much higher than those observed using the spectrophotometric assay, the values of the kinetic constants were close to those obtained by spectrophotometry (Table 2, row 1).

HprK/P Activity in the Presence of Fructose 1,6-Bisphosphate, P_i, and KCl. To confirm previous results obtained with nonrecombinant *S. salivarius* HPr and HprK/P, and to determine whether FBP affects HprK/P kinase activity when HPr has a negative charge at position 15, we assessed the ATP-dependent phosphorylation of HPr, HPr(H15D), and HPr(H15E), at pH 7.4 and 37 °C in the presence of FBP (25 mM) and ATP (5 mM). The concentrations of the two metabolites were similar to those reported for glucose-grown cells of *Lactococcus lactis* (*36*). Consistent with previous results (*15*), no stimulation by FBP was observed with HPr as the substrate (data not shown). Also, no significant stimulation (≤18%) was observed with HPr(H15D) and HPr(H15E) (data not shown). As previously reported (*15*), 1 mM P_i inhibited the kinase activity of HprK/P by more than 80% but by less than 10% in the presence of 0.1 mM P_i at pH 7.5 and 37 °C (data not shown). However, at alkaline pH, the HprK/P kinase activity was strongly inhibited by low concentrations of P_i. At pH 8.7 and 37 °C, the kinase activity was reduced by 65% in the presence of 0.1 mM P_i and by more than 90% at 15 °C (data not shown). Lastly, unlike HprK/P from *Streptococcus pyogenes* (*34*), *S. salivarius* HprK/P kinase activity was not inhibited by KCl concentrations as high as 200 mM (data not shown).

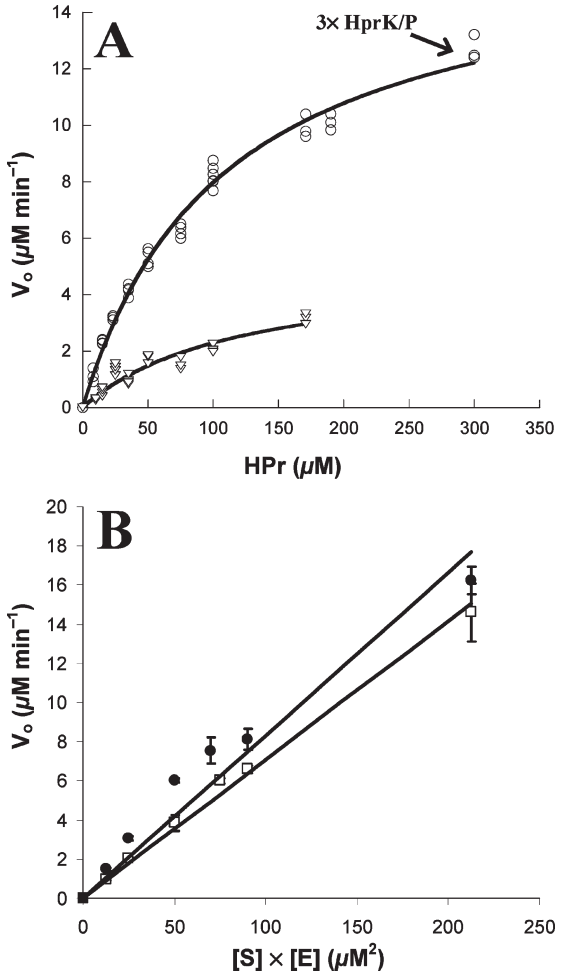

FIGURE 1: Kinetics of phosphorylation of HPr, HPr(H15D), and HPr(H15E) by HprK/P at pH 7.4 and 37 °C. Reactions with HPr were initiated with 150 nM HprK/P for the kinetics determined spectrophotometrically [A (○)] or 50 nM HprK/P for the kinetics determined by PAGE [A (▽)]. Reactions with HPr(H15D) (●) and HPr(H15E) (□) (B) were followed spectrophotometrically and initiated with 500 nM HprK/P. The rate of NADH oxidation in the coupled spectrophotometric assay was followed at 340 nm, whereas results obtained by PAGE were analyzed by densitometry. Data were collected at least in triplicate and were analyzed by nonlinear regression using the Michaelis−Menten equation for HPr (A) and by linear regressions for HPr(H15D) and HPr(H15E) (B). Correlation coefficients (R^2) for the fits were ≥0.94 (coupled assay) or ≥0.88 (PAGE). The concentrations of HprK/P were adjusted for each method to determine as accurately as possible the initial rates of reaction.

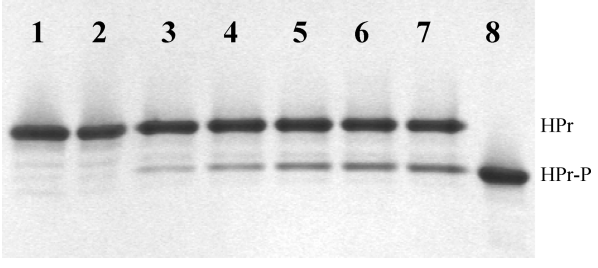

FIGURE 2: Kinetics of phosphorylation of HPr by HprK/P at pH 7.4 and 37 °C. The phosphorylation of 25 μM HPr as a function of time was followed by PAGE. Reaction was initiated with 50 nM HprK/P: lane 1, purified HPr; lane 2, 0 min; lane 3, 1 min; lane 4, 2 min; lane 5, 3 min; lane 6, 4 min; lane 7, 5 min; and lane 8, purified HPr(Ser-P) (indicated as HPr-P on the figure). Each lane contained 3 μg of protein.

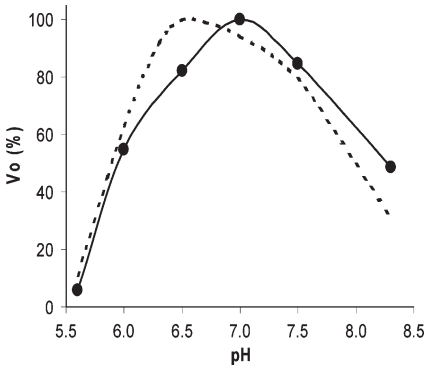

FIGURE 3: Phosphorylation of HPr and HPr(H15D) by HprK/P at 37 °C as a function of pH. The phosphorylation of 40 μM HPr and HPr(H15D) was assessed by densitometric analyses of PAGE gels. Reactions were initiated with 4−100 nM HprK/P for the experiments conducted with HPr (●) and 20−200 nM HprK/P for the experiments conducted with HPr(H15D) (···). Data were collected at least in triplicate. Maximal reaction rates were found at pH 7.0 for HPr (2.4 μmol min^{-1} mg^{-1}) and pH 6.5 for HPr(H15D) (0.3 μmol min^{-1} mg^{-1}). Since the amount of HprK/P used differed substantially with respect to the substrate, the results are expressed as relative reaction rates. The errors were ±21% or less.

HprK/P Activity as a Function of pH. The intracellular pH of streptococci decreases when the pH of the extracellular milieu drops (*37*). We previously showed that HPr-P2 synthesis, via EI-catalyzed PEP-dependent phosphorylation of HPr(Ser-P), is stimulated under acidic conditions (*27*). This prompted us to study the effect of pH (5.6−8.3) on the phosphorylation of HPr and HPr(H15D) by HprK/P. Since the spectrophotometric assay could not be used over the entire pH range, we assessed the effect of pH using densitometric analyses of PAGE gels. The results showed that (i) the rate of phosphorylation of HPr was not enhanced at acidic pH and (ii) the rate of phosphorylation of HPr(H15D) was only slightly higher (~1.3-fold) at pH 6.5 than at pH 7.5 (Figure 3).

HPr(His~P) Purification. The results obtained with HPr-(H15D) and HPr(H15E) suggested that HprK/P may be involved to some extent in HPr-P2 synthesis in streptococci. However, even though HPr(H15D) and HPr(H15E) are believed to mimic HPr(His~P) because of the negative charge at position 15 (*16*, *29*), the structural analogy with HPr(His~P) is obviously merely partial. Indeed, a side chain with two (D) or three (E) carbon atoms, including a carboxyl group at the end, does not have the same spatial orientation as a phosphorylated imidazole ring, nor is it as sterically encumbering and negatively charged. We thus attempted to determine the kinetic constants of HprK/P for HPr(His~P). To do so, HPr(His~P) had first to be synthesized and purified. The phosphorylation of HPr by EI is reversible and reaches an equilibrium even in the presence of large amounts of enzyme (*1*), which means that free HPr is not totally transformed into HPr(His~P). Moreover, the phosphoramidate bond of HPr(His~P) is subject to spontaneous phosphohydrolysis, the level of which is reduced at alkaline pH and low temperature (*33*). For this reason, the synthesis and purification of HPr(His~P) were conducted at pH 8.7 and 15 °C and at pH 8.8 and 4 °C, respectively. Using these conditions, we obtained HPr(His~P) preparations that were devoid of EI, PEP, pyruvate, and P_i but that nonetheless contained free endogenous HPr [approximately 25% of total HPr (results not shown)]. We confirmed that the purification and storage of HPr(His~P) at pH 8.8 did not affect the capacity of the protein to be phosphorylated by HprK/P (results not shown).

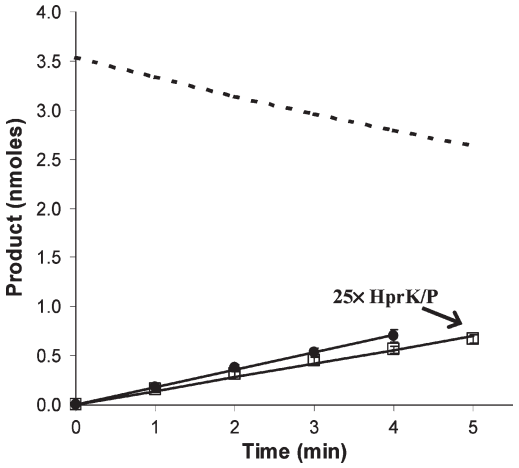

FIGURE 4: Phosphorylation of HPr and HPr(His~P) by HprK/P and phosphohydrolysis of HPr(His~P) at pH 7.5 and 37 °C. The phosphorylation of 33 μM HPr and HPr(His~P) was assessed by densitometric analyses of PAGE gels. Reactions were initiated with 20 nM HprK/P for the experiments conducted with HPr (●) and 500 nM HprK/P for the experiments conducted with HPr(His~P) (□). Data were collected at least in triplicate and were analyzed by linear regression. Correlation coefficients (R^2) for the fits were ≥0.99. The dotted line represents the rate of spontaneous phosphohydrolysis of HPr(His~P) determined spectrophotometrically in the presence of HprK/P as described in Experimental Procedures.

Phosphorylation of HPr(His~P) by HprK/P at pH 7.5 and 37 °C. The proportion of doubly phosphorylated HPr can account for more than 70% of the total HPr in streptococci (*20*, *21*). To determine whether streptococcal HprK/P might be involved in the synthesis of HPr-P2, we first attempted to determine whether HprK/P could phosphorylate HPr(His~P) under conditions near neutrality, namely pH 7.5, and at 37 °C. Given the presence of endogenous HPr in HPr(His~P) preparations, the spectrophotometric assay could not be used to measure initial rates. Reaction products were thus separated by PAGE and quantified by densitometry. The results indicated that HPr-(His~P) was slowly phosphorylated by HprK/P (Figure 4). However, as the spontaneous phosphohydrolysis of HPr(His~P) caused a decrease in the substrate concentration during the course of the reaction [Figure 4 (···)], it is likely that the reaction rate of HPr(His~P) phosphorylation was underestimated. Moreover, as the phosphoramidate bond in HPr-P2 should also be subject to spontaneous phosphohydrolysis, a significant amount of HPr-P2 should have been lost during the reaction. Thus, even though the results obtained at pH 7.5 clearly indicated that HPr(His~P) could be phosphorylated by HprK/P, the kinetic constants for HPr(His~P) could not be determined accurately under these conditions.

HPr(His~P) Phosphorylation by HprK/P at Alkaline pH and Low Temperature. Because the stability of the phosphoramidate bond is known to increase at alkaline pH and low temperature (*33*), we studied the phosphorylation of HPr(His~P) by HprK/P under these conditions. We first verified the stability of HPr(His~P) at pH 8.7 and 15 °C by spectrophotometry. For this purpose, we measured the rate of spontaneous phosphohydrolysis using the EI-LDH-coupled assay in the presence and absence of HprK/P. Under these conditions, phosphohydrolysis was undetectable for at least 20 min (data not shown). We also tested the stability of HPr(His~P) in the presence of HprK/P by PAGE. The results showed that HPr(His~P) was stable for at least 30 min [Figure 5 (···)].

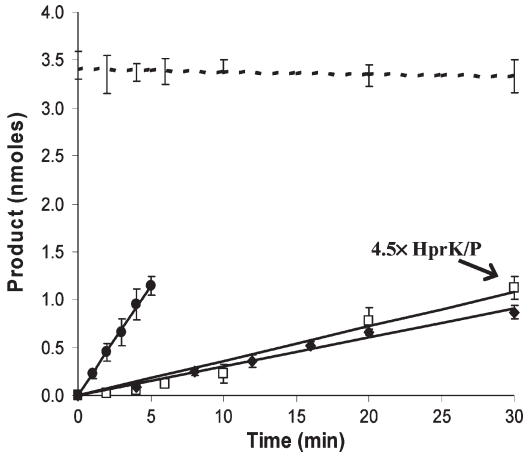

FIGURE 5: Phosphorylation of HPr, HPr(H15D), and HPr(His∼P) by HprK/P and phosphohydrolysis of HPr(His∼P) at pH 8.7 and 15 °C. The phosphorylation of 25 μM HPr, HPr(H15D), and HPr-(His∼P) was assessed by densitometric analyses of PAGE gels. Reactions were initiated with 200 nM HprK/P for the experiments conducted with HPr (●) and HPr(H15D) (◆) and 900 nM HprK/P for the experiments conducted with HPr(His∼P) (□). Data were collected at least in triplicate and were analyzed by linear regression. Correlation coefficients (R^2) for the fits were ≥0.96. The dotted line represents the rate of spontaneous phosphohydrolysis of HPr-(His∼P) determined by PAGE in the presence of HprK/P as described in Experimental Procedures.

Phosphorylation experiments conducted at pH 8.7 and 15 °C showed that HPr(His∼P) was phosphorylated by HprK/P ∼30 times more slowly than HPr (Figure 5). The inhibition factor was reduced to 7.5-fold when HPr(H15D) was used as the substrate (Figure 5). Like at pH 7.4 for HPr(H15D) and HPr(H15E), FBP did not affect the phosphorylation rate of HPr(His∼P) at pH 8.7 (data not shown).

The kinetic constants of HprK/P for HPr, HPr(H15D), and HPr(His∼P) were determined at pH 8.6 and 15 °C after densitometric analyses of PAGE gels. The Michaelis−Menten curve fit associated with HPr phosphorylation and the linear regressions of the data from HPr(H15D) and HPr(His∼P) phosphorylation are presented in Figure 6. The calculated kinetic constants are listed in Table 3. The results indicate that the specificity constant (k_{cat}/K_m) of HprK/P for HPr(H15D) was ∼9 times lower than that for HPr, whereas that for HPr(His∼P) was approximately 26 times lower.

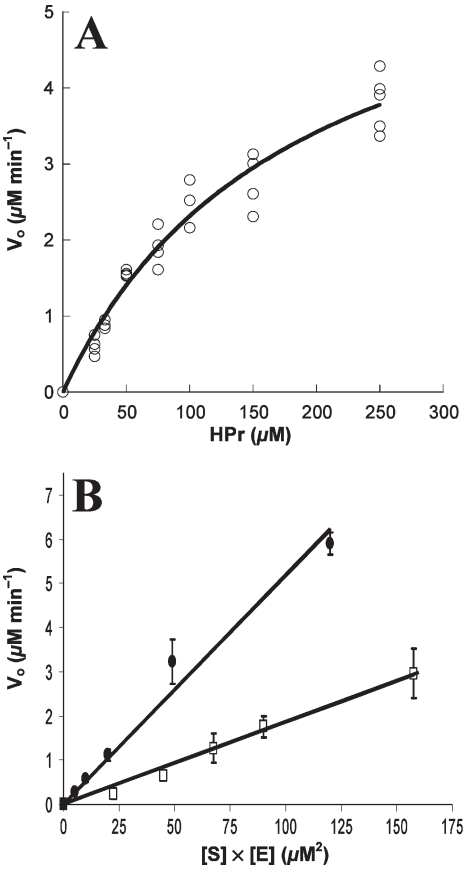

FIGURE 6: Kinetics of phosphorylation of HPr, HPr(H15D), and HPr(His∼P) by HprK/P at pH 8.6 and 15 °C. The phosphorylation of HPr, HPr(H15D), and HPr(His∼P) was assessed by densitometric analyses of PAGE gels. Reactions were initiated with 75 nM HprK/P for the experiments conducted with HPr (A), 200 nM HprK/P for the experiments conducted with HPr(H15D) [B (●)], and 900 nM HprK/P for the experiments conducted with HPr(His∼P) [B (□)]. Data were collected at least in triplicate and were analyzed by nonlinear regression with the Michaelis−Menten equation for HPr (A) and linear regressions for HPr(H15D) and HPr(His∼P) (B). The correlation coefficients (R^2) for the fits were ≥0.97.

DISCUSSION

The aim of this study was to determine whether streptococcal HprK/P participates in HPr-P2 synthesis via phosphorylation of HPr(His∼P). To do so, we first developed a quantitative spectrophotometric assay that allowed us to accurately measure HprK/P kinase activity. The advantages of this assay are (i) the short time required to accumulate a large number of data, (ii) the capacity to accurately measure initial reaction rates in real time, (iii) the low cost, and (iv) the fact that radioactive isotopes are not needed. We used the assay to measure the kinetic constants for recombinant HPr at pH 7.4 and 37 °C. The affinity constant was ∼4-fold higher than that reported previously for native HPr (110 μM versus 31 μM) (*15*). However, in the study by Brochu et al. (*15*), the range of concentrations of HPr used to determine the K_m was very narrow (0−30 μM) and the results were analyzed by linear regression, which prevents an accurate determination of this constant (*35*). In our study, we used a wider range of concentrations (0−300 μM) and a nonlinear regression approach, which is more accurate (*35*).

The specificity constants of HprK/P for HPr(H15D) and HPr(H15E) at pH 7.4 were ∼13 times lower than for HPr. These results suggested that the phosphorylation of HPr(His∼P) by streptococcal HprK/P may occur, albeit at a slow rate. Phosphorylation of HPr(His∼P) could not be studied with accuracy at 37 °C and near neutral pH because of the instability of the phosphoramidate bond. We therefore studied the phosphorylation of HPr(His∼P) under conditions where the spontaneous phosphohydrolysis of the phosphoramidate bond is negligible, namely, pH 8.6 and 15 °C. We found that the k_{cat}/K_m of HprK/P for HPr(His∼P) was ∼26 times lower than that for HPr and 3 times lower than that for HPr(H15D). However, since heat is generated during electrophoresis, and because the phosphoramidate bond is unstable at elevated temperature (*33*), it is likely that the proportions of HPr-P2 determined by densitometric analyses of PAGE gels were underestimated. To estimate the amount of HPr-P2 lost during electrophoresis, we compared the amounts of HPr(His∼P) measured spectrophotometrically before electrophoresis with those estimated by densitometry after electrophoresis. We found that ∼10% of HPr(His∼P) was transformed into HPr during electrophoresis, even when the electrophoresis was

conducted at 4 °C (results not shown). As the phosphoramidate bond of HPr-P2 is most likely as unstable as that of HPr(His∼P), the rate of synthesis of HPr-P2 determined by densitometry after electrophoresis was likely slightly underestimated. Nonetheless, it was obvious that HPr(H15D) was a better substrate for HprK/P than HPr(His∼P), which suggested that the mere presence of a negative charge at position 15 is not the only element that makes HPr(His∼P) a poorer substrate for HprK/P than HPr.

Previous NMR structural studies on HPr and HPr(His∼P) (*38*−*41*), as well as crystallographic studies (*42*, *43*), support this hypothesis. Indeed, it has been shown that the His_{15} active site of HPr exists in two different conformations, closed and open (*38*−*43*). Molecular dynamics (MD) simulations performed on several HPr structures have shown that the closed conformation is relatively rigid and is the predominant active site conformation in HPr, whereas the open conformation is more flexible (*44*). While the active site of unphosphorylated HPr can adopt both conformations, the active site of HPr(His∼P) exists only in the closed conformation, primarily because of favorable interactions between the phosphoryl group and the backbone amide protons of residues 16 and 17 (*39*, *44*). Since HprK/P recognizes the tertiary structure of its substrates (*13*), the rigidity of the His_{15} active site in HPr(His∼P), which is part of the HPr binding site (*11*, *41*), is likely an additional factor that interferes with the phosphorylation of HPr(His∼P).

The structure of the HPr(His∼P)−HprK/P complex is not yet available. However, the capping motif and the C-terminal α-helix (helix α4) of the enzyme have contacts with the cluster around the phosphorylatable histidyl residue of HPr in the HPr−HprK/P complex (*11*). Since helix α4 contains negatively charged residues (*11*, *12*), it might be involved in interactions that lower the affinity of the enzyme for HPr(His∼P) and HPr(H15D). However, as the aspartyl residue of HPr(H15D) is not as negatively charged as the phosphoryl group of HPr(His∼P), formation of a complex between HPr(H15D) and HprK/P should be less affected by these electrostatic repulsions. The structural information from NMR, crystallographic, and MD studies provides support for the hypothesis that the inhibition of the phosphorylation of HPr(His∼P) by HprK/P is caused by a combination of factors, including the spatial orientation of the imidazole ring, the presence of the negatively charged bulky phosphoryl group, and the structural rigidity of the active His_{15} site of HPr(His∼P).

Our results showed that streptococcal HprK/P, which has a very low catalytic efficiency, can synthesize HPr-P2 in vitro at a low rate when the concentrations of HPr(His∼P) and HprK/P are elevated. However, growing streptococcal cells do not appear to contain large amounts of HprK/P (*15*). Moreover, in many cases, when cells are grown in the presence of an abundant source of PTS sugar(s), cellular levels of HPr(His∼P) are low or undetectable (*20*, *21*). Under these conditions, it is unlikely that HprK/P synthesizes HPr-P2. The high levels of HPr-P2 found in streptococci and lactococci most likely come from the EI-catalyzed phosphorylation of HPr(Ser-P) (*27*), as both the substrate and the enzyme are present in large amounts (*20*, *45*). On the other hand, when the concentration of sugar(s) is low, cells accumulate large amounts of P_i and HPr(His∼P) (*21*, *36*). As HprK/P kinase activity is strongly inhibited by P_i (*14*, *15*), it is unlikely that HPr-P2 is synthesized via the phosphorylation of HPr(His∼P) by HprK/P. Given the results presented here, we suggest that the contribution of streptococcal HprK/P to the synthesis of HPr-P2 in vivo is marginal.

ACKNOWLEDGMENT

We thank Denis Brochu for his work on the pH15E construct, Dr. Jacques Lapointe for his help with the kinetic analyses and for valuable discussions, Eric Lebrun for his contribution to the purification of HPr, and Gene Bourgeau for editorial assistance.

REFERENCES

1. Deutscher, J., Francke, C., and Postma, P. W. (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev. 70*, 939–1031.
2. Lengeler, J. W., Jahreis, K., and Wehmeier, U. F. (1994) Enzymes II of the phosphoenolpyruvate-dependent phosphotransferase systems: Their structure and function in carbohydrate transport. *Biochim. Biophys. Acta 1188*, 1–28.
3. Saier, M. H. Jr., and Reizer, J. (1992) Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate: Sugar phosphotransferase system. *J. Bacteriol. 174*, 1433–1438.
4. Zeng, L., and Burne, R. A. (2009) Transcriptional regulation of the cellobiose operon of *Streptococcus mutans*. *J. Bacteriol. 191*, 2153–2162.
5. Poncet, S., Mijakovic, I., Nessler, S., Gueguen-Chaignon, V., Chaptal V., Galinier, A., Boël, G., Mazé, A., and Deutscher, J. (2004) HPr kinase/phosphorylase, a Walker motif A-containing bifunctional sensor enzyme controlling catabolite repression in Gram-positive bacteria. *Biochim. Biophys. Acta 1697*, 123–135.
6. Robinson, V. L., and Stock, A. M. (1999) High energy exchange: Proteins that make or break phosphoramidate bonds. *Structure 7*, R47–R53.
7. Monedero, V., Yebra, M. J., Poncet, S., and Deutscher, J. (2008) Maltose transport in *Lactobacillus casei* and its regulation by inducer exclusion. *Res. Microbiol. 159*, 94–102.
8. Warner, J. B., and Lolkema, J. S. (2003) CcpA-dependent carbon catabolite repression in bacteria. *Microbiol. Mol. Biol. Rev. 67*, 475–490.
9. Müller, W., Horstmann, N., Hillen, W., and Sticht, H. (2006) The transcription regulator RbsR represents a novel interaction partner of the phosphoprotein HPr-Ser46-P in *Bacillus subtilis*. *FEBS J. 273*, 1251–1261.
10. Pompeo, F., Luciano, J., and Galinier, A. (2007) Interaction of GapA with HPr and its homologue, Crh: Novel levels of regulation of a key step of glycolysis in *Bacillus subtilis*? *J. Bacteriol. 189*, 1154–1157.
11. Fieulaine, S., Morera, S., Poncet, S., Mijakovic, I., Galinier, A., Janin, J., Deutscher, J., and Nessler, S. (2002) X-ray structure of a bifunctional protein kinase in complex with its protein substrate HPr. *Proc. Natl. Acad. Sci. U.S.A. 99*, 13437–13441.
12. Fieulaine, S., Morera, S., Poncet, S., Monedero, V., Gueguen-Chaignon, V., Galinier, A., Janin, J., Deutscher, J., and Nessler, S. (2001) X-ray structure of HPr kinase: A bacterial protein kinase with a P-loop nucleotide-binding domain. *EMBO J. 20*, 3917–3927.
13. Saier, M. H. Jr. (1994) Bacterial protein kinases that recognize tertiary rather than primary structure? *Res. Microbiol. 145*, 647–650.
14. Frey, N., Nessler, S., Fieulaine, S., Vaillancourt, K., Frenette, M., and Vadeboncoeur, C. (2003) The HPr(Ser) kinase of *Streptococcus salivarius*: A hexameric bifunctional enzyme controlled by glycolytic intermediates and inorganic phosphate. *FEMS Microbiol. Lett. 224*, 67–72.
15. Brochu, D., and Vadeboncoeur, C. (1999) The HPr(Ser) kinase of *Streptococcus salivarius*: Purification, properties, and cloning of the *hprK* gene. *J. Bacteriol. 181*, 709–717.
16. Reizer, J., Hoischen, C., Titgemeyer, F., Rivolta, C., Rabus, R., Stülke, J., Karamata, D., Saier, M. H. Jr., and Hillen, W. (1998) A novel protein kinase that controls carbon catabolite repression in bacteria. *Mol. Microbiol. 27*, 1157–1169.
17. Ramström, H., Sanglier, S., Leize-Wagner, E., Philippe, C., Van Dorsselaer, A., and Haiech, J. (2003) Properties and regulation of the bifunctional enzyme HPr kinase/phosphatase in *Bacillus subtilis*. *J. Biol. Chem. 278*, 1174–1185.
18. Kravanja, M., Engelmann, R., Dossonnet, V., Blüggel, M., Meyer, H. E., Frank, R., Galinier, A., Deutscher, J., Schnell, N., and Hengstenberg, W. (1999) The *hprK* gene of *Enterococcus faecalis* encodes a novel bifunctional enzyme: The HPr kinase/phosphatase. *Mol. Microbiol. 31*, 59–66.

19. Steinhauer, K., Jepp, T., Hillen, W., and Stülke, J. (2002) A novel mode of control of *Mycoplasma pneumoniae* HPr kinase/phosphatase activity reflects its parasitic lifestyle. *Microbiology 148*, 3277–3284.
20. Roy, D. J., Casabon, I., Vaillancourt, K., Huot, J. L., and Vadeboncoeur, C. (2008) Streptococci and lactococci synthesize large amounts of HPr(Ser-P)(His∼P). *Can. J. Microbiol. 54*, 941–949.
21. Vadeboncoeur, C., Brochu, D., and Reizer, J. (1991) Quantitative determination of the intracellular concentration of the various forms of HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system in growing cells of oral streptococci. *Anal. Biochem. 196*, 24–30.
22. Halbedel, S., Hames, C., and Stülke, J. (2004) In vivo activity of enzymatic and regulatory components of the phosphoenolpyruvate: sugar phosphotransferase system in *Mycoplasma pneumoniae*. *J. Bacteriol. 186*, 7936–7943.
23. Singh, K. D., Schmalisch, M. H., Stülke, J., and Görke, B. (2008) Carbon catabolite repression in *Bacillus subtilis*: Quantitative analysis of repression exerted by different carbon sources. *J. Bacteriol. 190*, 7275–7284.
24. Joseph, B., Mertins, S., Stoll, R., Schär, J., Umesha, K. R., Luo, Q., Müller-Altrock, S., and Goebel, W. (2008) Glycerol metabolism and PrfA activity in *Listeria monocytogenes*. *J. Bacteriol. 190*, 5412–5430.
25. Cochu, A., Roy, D., Vaillancourt, K., Lemay, J. D., Casabon, I., Frenette, M., Moineau, S., and Vadeboncoeur, C. (2005) The doubly phosphorylated form of HPr, HPr(Ser-P)(His∼P), is abundant in exponentially growing cells of *Streptococcus thermophilus* and phosphorylates the lactose transporter LacS as efficiently as HPr(His∼P). *Appl. Environ. Microbiol. 71*, 1364–1372.
26. Lessard, C., Cochu, A., Lemay, J. D., Roy, D., Vaillancourt, K., Frenette, M., Moineau, S., and Vadeboncoeur, C. (2003) Phosphorylation of *Streptococcus salivarius* lactose permease (LacS) by HPr-(His∼P) and HPr(Ser-P)(His∼P) and effects on growth. *J. Bacteriol. 185*, 6764–6772.
27. Casabon, I., Couture, M., Vaillancourt, K., and Vadeboncoeur, C. (2006) Synthesis of HPr(Ser-P)(His∼P) by enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus salivarius*. *Biochemistry 45*, 6692–6702.
28. Halbedel, S., and Stülke, J. (2005) Dual phosphorylation of *Mycoplasma pneumoniae* HPr by Enzyme I and HPr kinase suggests an extended phosphoryl group susceptibility of HPr. *FEMS Microbiol. Lett. 247*, 193–198.
29. Christensen, D. P., Benson, A. K., and Hutkins, R. W. (1999) Mutational analysis of the role of HPr in *Listeria monocytogenes*. *Appl. Environ. Microbiol. 65*, 2112–2115.
30. Vadeboncoeur, C., Proulx, M., and Trahan, L. (1983) Purification of proteins similar to HPr and enzyme I from the oral bacterium *Streptococcus salivarius*. Biochemical and immunochemical properties. *Can. J. Microbiol. 29*, 1694–1705.
31. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
32. Colli, W., and Pullman, M. E. (1969) Adenosine diphosphate as the primary phosphoryl acceptor in oxidative phosphorylation. *J. Biol. Chem. 244*, 135–141.
33. Waygood, E. B., Erickson, E., El-Kabbani, O. A., and Delbaere, L. T. (1985) Characterization of phosphorylated histidine-containing protein (HPr) of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. *Biochemistry 24*, 6938–6945.
34. Reizer, J., Novotny, M. J., Hengstenberg, W., and Saier, M. H. Jr. (1984) Properties of ATP-dependent protein kinase from *Streptococcus pyogenes* that phosphorylates a seryl residue in HPr, a phosphocarrier protein of the phosphotransferase system. *J. Bacteriol. 160*, 333–340.
35. Copeland, R. A. (2000) Kinetics of single-substrate enzyme reactions. In Enzymes: A practical introduction to structure, mechanism, and data analysis , 2nd ed., pp 109−145, John Wiley & Sons, Inc., New York.
36. Neves, A. R., Pool, W. A., Kok, J., Kuipers, O. P., and Santos, H. (2005) Overview on sugar metabolism and its control in *Lactococcus lactis*: The input from in vivo NMR. *FEMS Microbiol. Rev. 29*, 531–554.
37. Kashket, E. R. (1987) Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance. *FEMS Microbiol. Rev. 46*, 233–244.
38. Kalbitzer, H. R., and Hengstenberg, W. (1993) The solution structure of the histidine-containing protein (HPr) from *Staphylococcus aureus* as determined by two-dimensional ^{1}H-NMR spectroscopy. *Eur. J. Biochem. 216*, 205–214.
39. Jones, B. E., Rajagopal, P., and Klevit, R. E. (1997) Phosphorylation on histidine is accompanied by localized structural changes in the phosphocarrier protein, HPr from *Bacillus subtilis*. *Protein Sci. 6*, 2107–2119.
40. Maurer, T., Döker, R., Görler, A., Hengstenberg, W., and Kalbitzer, H. R. (2001) Three-dimensional structure of the histidine-containing phosphocarrier protein (HPr) from *Enterococcus faecalis* in solution. *Eur. J. Biochem. 268*, 635–644.
41. Maurer, T., Meier, S., Kachel, N., Munte, C. E., Hasenbein, S., Koch, B., Hengstenberg, W., and Kalbitzer, H. R. (2004) High-resolution structure of the histidine-containing phosphocarrier protein (HPr) from *Staphylococcus aureus* and characterization of its interaction with the bifunctional HPr kinase/phosphorylase. *J. Bacteriol. 186*, 5906–5918.
42. Herzberg, O., Reddy, P., Sutrina, S., Saier, M. H. Jr., Reizer, J., and Kapadia, G. (1992) Structure of the histidine-containing phosphocarrier protein HPr from *Bacillus subtilis* at 2.0-Å resolution. *Proc. Natl. Acad. Sci. U.S.A. 89*, 2499–2503.
43. Jia, Z., Vandonselaar, M., Hengstenberg, W., Quail, J. W., and Delbaere, L. T. (1994) The 1.6 Å structure of histidine-containing phosphotransfer protein HPr from *Streptococcus faecalis*. *J. Mol. Biol. 236*, 1341–1355.
44. Homeyer, N., Essigke, T., Ullmann, G. M., and Sticht, H. (2007) Effects of histidine protonation and phosphorylation on histidine-containing phosphocarrier protein structure, dynamics, and physicochemical properties. *Biochemistry 46*, 12314–12326.
45. Thibault, L., and Vadeboncoeur, C. (1985) Phosphoenolpyruvate-sugar phosphotransferase transport system of *Streptococcus mutans*: Purification of HPr and enzyme I and determination of their intracellular concentrations by rocket immunoelectrophoresis. *Infect. Immun. 50*, 817–825.