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The HPr(Ser) kinase of *Streptococcus salivarius*: a hexameric bifunctional enzyme controlled by glycolytic intermediates and inorganic phosphate

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Abstract

Phosphorylation of HPr, the small phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system, on Ser46 by the HPr(Ser) kinase (HPrK/P) is a vital step in catabolite repression in Gram-positive bacteria. *Streptococcus salivarius* HPrK/P is reported to be a multimeric protein not regulated by metabolic intermediates. We re-evaluated the molecular mass of *S. salivarius* HPrK/P using sedimentation equilibrium ultracentrifugation, demonstrated that *S. salivarius* HPrK/P dephosphorylated HPr(Ser-P) and further characterised the effect of fructose 1,6-bisphosphate and other metabolic intermediates on enzyme activities. The molecular mass of *S. salivarius* HPrK/P was 201 305 Da, suggesting that streptococcal HPrK/P was a hexameric protein. Fructose 1,6-bisphosphate poorly activated streptococcal HPrK/P but protected kinase activity against inhibition by inorganic phosphate and inhibited dephosphorylation of HPr(Ser-P). Phosphoenolpyruvate and 2-phosphoglycerate, but not fructose 1-P, fructose 6-P, and ribulose 1,5-bisphosphate, also protected kinase activity against inhibition by inorganic phosphate. Thus, unlike previous reports, we show that fructose 1,6-bisphosphate and other key glycolytic intermediates played a pivotal role as a modulator of streptococcal HPrK/P activities.

Keywords: Protein kinase/phosphatase; HPr(Ser) kinase; Catabolite repression; Protein phosphorylation

1. Introduction

Carbon catabolite repression and inducer exclusion allow bacteria to selectively metabolise carbon and energy sources. In Gram-positive bacteria, a small protein called HPr, a component of the phosphoenolpyruvate (PEP):sugar phosphotransferase transport and signal transduction system (PTS), is involved in carbon catabolite repression and inducer exclusion [1–3]. HPr can be phosphorylated on the histidine at position 15 by enzyme I of the PTS at the expense of PEP and on the serine at position 46 by a protein kinase at the expense of adenosine triphosphate (ATP). When phosphorylated on His15, HPr participates in sugar transport and phosphorylation by transferring its phosphate group to other PTS components and controls the activity of transcriptional regulatory proteins by phosphorylation. The principal currently recognised functions of HPr(Ser-P) are control of sugar permease activity by protein–protein interaction (inducer exclusion) and regulation of gene transcription by association with the protein CcpA and binding of the resulting complex to a DNA sequence called *cre* located in the vicinity of promoters controlling the expression of genes sensitive to carbon catabolite repression.

The protein kinase that phosphorylates HPr on Ser46 has been purified and characterised from *Bacillus subtilis* [4–6], *Enterococcus faecalis* [7], *Staphylococcus xylosus* [8], *Lactobacillus casei* [9], *Mycoplasma pneumoniae* [10] and *Streptococcus salivarius* [11]. Size exclusion chromatography experiments showed that HPr kinases are oligomers

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with a number of subunits that varies from two in *E. faecalis* [7] to 10 in *S. salivarius* [11]. The crystal structures of the HPr kinases from *L. casei* (Protein Data Base (PDB) code 1JB1) [12], *S. xylosus* (PDB code 1KO7) [13] and *M. pneumoniae* (PDB code 1KNX) [14] indicate that they are hexamers. For two of them, equilibrium ultracentrifugation experiments showed that the hexamer is the major conformation in solution [12,13]. Data obtained using similar experimental approaches suggest that the *B. subtilis* enzyme is an octamer [6]. Nevertheless, re-interpretation of a new set of data confirmed that *B. subtilis* HPr kinase is also a hexamer (data not shown). Because the amino acid sequences of the C-terminal catalytic domain, which is involved in the oligomerisation, are highly conserved among all HPrK/Ps [12], the molecular masses of the different proteins need to be re-evaluated to verify whether they all are hexamers.

The enzyme also catalyses the transformation of HPr(Ser-P) to HPr [7] and is thus designated HPr kinase/phosphorylase (HPrK/P). In *B. subtilis* [6,7], *L. casei* [9] and *M. pneumoniae* [10], the dephosphorylation of HPr(Ser-P) is dependent on the presence of inorganic phosphate (Pi). In *E. faecalis*, the dephosphorylation of HPr(Ser-P) is not strictly dependent on but is activated by Pi [7]. The *S. xylosus* enzyme seems to be able to dephosphorylate HPr(Ser-P) in a Pi-independent manner [8]. Since Mijakovic et al. [15] demonstrated that Pi is not an activator of the dephosphorylation reaction but rather a substrate that is transformed into pyrophosphate during a phosphorolysis reaction, it is necessary to determine whether HPrK/Ps from various organisms use the same catalytic mechanism.

Whether HPrK/P kinase activity is activated by metabolic intermediates, particularly fructose 1,6-biphosphate (FBP), is uncertain. The HprK/P kinase activity of *B. subtilis* and *L. casei* is stimulated by FBP at low ATP concentrations [6] while FBP has no effect on HPrK/P kinase activity of *E. faecalis* [7] and *M. pneumoniae* [10]. The kinase activity of *S. xylosus* HPrK/P is activated by FBP even at high ATP concentrations [8,13]. The effect of FBP on the kinase activity of *S. salivarius* HPrK/P was tested with cellular extracts at various ATP concentrations [16]. Kinase activity is stimulated two-fold by 5 mM FBP in the presence of 0.05 mM ATP. The stimulation is less than 20% with 0.1 mM ATP while FBP has no effect in the presence of 1 mM ATP [16]. The activity of the purified enzyme is also not stimulated by FBP in the presence of 1 mM ATP [11]. However, the effect of FBP at lower ATP concentrations has not been tested with purified streptococcal HPrK/P.

In this paper, the molecular mass of *S. salivarius* HPrK/P was re-evaluated using sedimentation equilibrium ultracentrifugation, the dephosphorylation of HPr(Ser-P) by *S. salivarius* HPrK/P was studied for the first time and the effects of FBP and other metabolic intermediates on enzyme activities were measured.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *S. salivarius* was cultured as described previously [11]. *Escherichia coli* was grown with aeration at 37°C in Luria Bertani (LB) medium for strain TOP10, in RM medium (20 g casamino acids, 6 g Na_2HPO_4 and 3 g KH_2PO_4 per l, 8.5 mM NaCl, 28 mM NH_4Cl, 1 mM $MgCl_2$ and 11 mM glucose) for strain LMG194, and in SOC medium (20 g tryptone and 5 g yeast extract per l, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, and 20 mM glucose) for strain BL21(DE3). When necessary, 50 μg of ampicillin per ml and 30 or 50 μg of kanamycin per ml were added.

2.2. DNA manipulation

Chromosomal DNA was isolated from *S. salivarius* as described previously [17]. Unless otherwise mentioned, DNA manipulations were performed using standard procedures [18].

2.3. Overproduction of S. salivarius (His$_6$)HPr, (His$_6$)HPrK/P and HPrK/P in E. coli and purification of the recombinant proteins

The cloning of *S. salivarius ptsH*, the gene coding for the protein HPr, was reported to be toxic in *E. coli* [19]. To overexpress HPr in *E. coli*, *S. salivarius ptsH* was cloned into pBAD (Invitrogen), a vector designed for the expression of toxic proteins that adds a small peptide containing six histidines (His_6) at the N-terminus of the protein encoded by the cloned gene. The resulting plasmid (pHPW18) was transformed into *E. coli* LMG194 and expression of (His_6)HPr was induced with 0.02% arabinose. Cells were harvested by centrifugation and suspended in 100 mM Tris–HCl (pH 7.0) containing 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM benzamidine-HCl and lysozyme (0.2 mg ml^{-1}). After 30 min, the cells were ruptured by sonication with a Heat System ultrasonic model W 350 sonicator. The cellular extract was centrifuged at $10\,000 \times g$ for 25 min at 4°C. The supernatant fluid was centrifuged at $120\,000 \times g$ for 30 min at 4°C. The final supernatant was loaded onto a 2 ml Ni-NTA Superflow column equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.4 M NaCl, 10 mM imidazole, and 10% glycerol. The column was washed with 10 ml of the same buffer containing 20 mM imidazole, and the recombinant *S. salivarius* (His_6)HPr was eluted with 10 ml of the equilibration buffer containing 250 mM imidazole. The fractions containing (His_6)HPr were pooled and the protein was further purified on a Superdex 75-HR column (Pharmacia). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed that the

protein was over 99% pure. We previously reported the cloning of *S. salivarius hprK*, the gene coding for the HPr(Ser) kinase [11]. The following procedure was used to obtain a clone overproducing (His_6)HPrK/P. *Nde*I and *Bam*HI sites were created by polymerase chain reaction (PCR) at the 5′ and 3′ ends respectively of *S. salivarius hprK* using the oligonucleotide primers HPRK1837F (5′-CGGAGGAAATCATATGACAGTAACCGTTAAA-A-3′) and HPRK2775R (5′GAGCAATAGGATCCA-TAGTAGCAAACATTAGT-3′). After digestion with *Nde*I and *Bam*HI, the DNA fragment was cloned into the overexpression plasmid pET-28a(+) (Novagen), adding a His_6-tag at the N-terminus of HPrK/P. *E. coli* BL21(DE3) was transformed with the resulting plasmid (pHPKHis) and expression was induced with isopropyl thiogalactose (IPTG) according to the manufacturer's instructions (Novagen). The recombinant protein was purified on a Ni-NTA column as described above. Purification of *S. salivarius* HPrK/P without a His-tag from *E. coli* TOP10 bearing pHPK229 was previously reported [11].

2.4. Synthesis and purification of (His_6)HPr(Ser-P)

The synthesis of (His_6)HPr(Ser-P) was carried out with purified *S. salivarius* HPrK/P (5 μg) and (His_6)HPr (500 μg), which were incubated for 60 min at 37°C in 50 mM Tris–HCl (pH 7.5) containing 2 mM ATP and 5 mM $MgCl_2$. The reaction was stopped by heating the solution at 80°C for 5 min. The reaction product (His_6)HPr(Ser-P) was purified by chromatography on a Ni-NTA column as described above.

2.5. Determination of HPrK/P activities

Phosphorylation of (His_6)HPr by *S. salivarius* (His_6)-HPrK/P was performed at 37°C using 350 nM (His_6)-HPrK/P and 30 μM (His_6)HPr, in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$, 0.05, 0.5 or 1 mM ATP, and 0–50 mM FBP. Dephosphorylation of (His_6)HPr(Ser-P) by *S. salivarius* (His_6)HPrK/P was carried out in the same buffer containing 0–18 mM Pi, 0–4 mM $MgCl_2$, and 0–10 mM FBP but without ATP. The effect of other metabolic intermediates was tested at concentrations indicated in the text. The reaction products, (His_6)HPr(Ser-P) or (His_6)HPr, were separated by PAGE under native conditions [19]. After electrophoresis, the proteins were stained with Coomassie blue R-250. The image of the gel was digitalised (RS-170/CCIR digital camera, Bio-Rad) and analysed using the Quantity One quantification software package (Bio-Rad). The kinetic experiments were repeated at least twice and were reproducible.

2.6. Analytical ultracentrifugation

Analytical ultracentrifugation was carried out on a Beckman Optima XL-A equipped with an An60Ti four-hole rotor and a cell with two-channel 12-mm path length centrepieces. The molecular mass of *S. salivarius* HPrK/P was determined as described by Jault et al. [6]. Equilibrium sedimentation centrifugations were carried out at 5000, 8000 and 12 000 rpm at 4°C. Data were analysed for average molecular mass in terms of a single homogeneous species. Calculations used a specific volume of 0.7328 ml g^{-1} estimated from the sequence and a solvent density of 1.01616 g ml^{-1} calculated from the buffer content (20 mM bis-Tris propane (pH 7.3), 375 mM NaCl, 1 mM EDTA and 0.1 mM dithiothreitol (DTT)).

3. Results

3.1. Molecular mass of S. salivarius HPrK/P

To obtain accurate information on the quaternary structure of *S. salivarius* HPrK/P, equilibrium analytical ultracentrifugation was performed. The experiment was performed at three different ultracentrifugation speeds with a protein concentration of 0.3 mg ml^{-1}. Each data set, which was analysed separately, gave a single homogeneous species with an average molecular mass of 193–207 kDa. A simultaneous fit was then performed that yielded an average mass of 201305 Da (Fig. 1).

Table 1
Strains and plasmids

Strain/plasmid	Relevant genotype/characteristics	Source/reference
Strains		
S. salivarius ATCC 25975	wild-type	I.R. Hamilton, University of Manitoba
E. coli LMG194	F^- Δ*lacX74 galE thi rpsL* Δ*phoA* (*pvu* II) Δ*ara714 leu*::Tn*10*	Invitrogen
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Novagen
Plasmids		
pET-28a(+)	expression vector	Novagen
pBAD/His	expression vector	Invitrogen
pCR-Blunt	cloning vector	Invitrogen
pHPK229	contains *S. salivarius hprK* and its promoter cloned into pCR-Blunt	[11]
pHPW18	contains the *ptsH* gene of *S. salivarius* cloned into pBAD	this work
pHPKHis	contains the *hprK* gene of *S. salivarius* cloned into pET-28a(+)	this work

3.2. Effect of FBP on the kinase activity of HPrK/P

We measured the rate of phosphorylation of HPr using purified HPrK/P in the presence of 0.05 and 0.5 mM ATP and various concentrations of FBP. Up to 10 mM FBP had virtually no effect on the phosphorylation of HPr in the presence of 0.5 mM ATP, while 5 mM FBP in the presence of 0.05 mM ATP increased the phosphorylation rate by a factor of approximately two (Fig. 2). A two-fold increase in the concentration of FBP did not result in greater stimulation of HPrK/P kinase activity while lower concentrations of FBP, i.e., 0.5 and 1 mM, did not significantly stimulate the phosphorylation of HPr (data not shown). In the presence of 0.05 mM ATP without FBP, 40% of HPr was transformed into HPr(Ser-P) after 2 min, indicating that *S. salivarius* HPrK/P was able to phosphorylate HPr on Ser46 in the absence of FBP even at very low concentrations of ATP (Fig. 2).

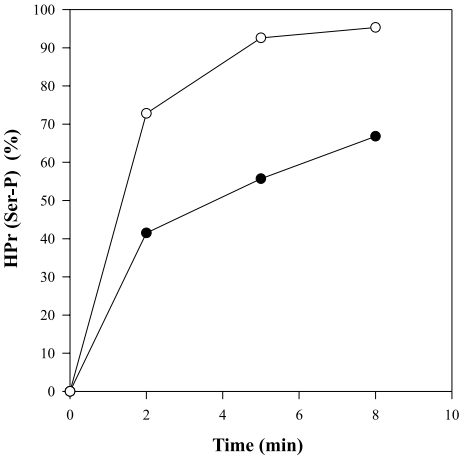

Fig. 2. HPr phosphorylation by *S. salivarius* HPrK/P. The reaction medium contained 350 nM (His_6)HPrK/P and 30 μM (His_6)HPr in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$, 0.05 mM ATP and 0 mM FBP (●) or 5 mM (○) FBP.

3.3. FBP neutralises Pi inhibition of HPrK/P kinase activity

Pi strongly inhibits the kinase activity of streptococcal HPrK/P [11]. Concentrations as low as 1 mM Pi almost totally prevented phosphorylation of HPr by HPrK/P in the presence of 1 mM ATP (Fig. 3). Addition of 10 (data not shown) to 15 mM (Fig. 3A) FBP restored HPrK/P kinase activity to optimal levels. In the presence of higher concentrations of Pi (5 and 10 mM), FBP only partially restored kinase activity, even at concentrations as high as 50 mM (data not shown). We determined whether the following molecules at concentrations from 1 to 10 mM could overcome the inhibition caused by 1 mM Pi: fructose 1-phosphate, fructose 6-phosphate, ribulose 1,5-bisphosphate, PEP and 2-phosphoglycerate. The hexoses and the pentose were unable to overcome Pi inhibition while PEP (Fig. 3B), and to a lesser extent 2-phosphoglycerate (data not shown), interfered with Pi inhibition.

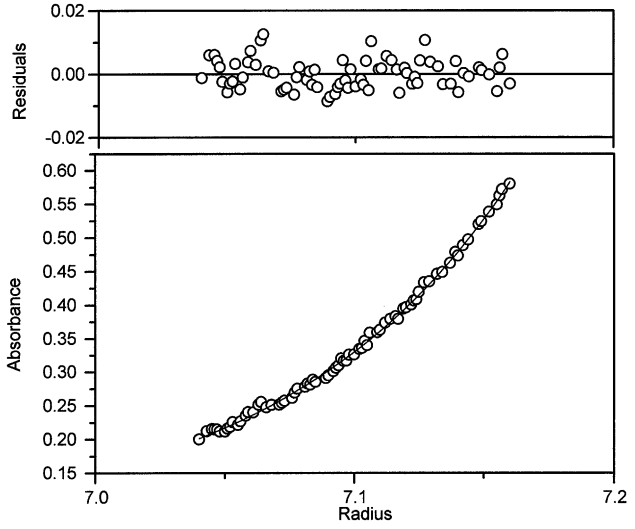

Fig. 1. Equilibrium sedimentation of *S. salivarius* HPrK/P. The absorbance at 280 nm is plotted against the radial position expressed in cm. The upper part of the figure shows the residual difference between experimental and fitted values by standard deviation. The average molecular mass calculated in terms of a single homogeneous species was 201305 Da. For more clarity, only the result of the simultaneous fit obtained with the data collected at 8000 rpm is shown. The other fits were of the same quality.

3.4. S. salivarius HPrK/P dephosphorylates HPr(Ser-P) in the presence of Pi

A 20-min incubation of HPrK/P with HPr(Ser-P) without Pi did not result in the formation of HPr. Addition of Pi, however, resulted in the dephosphorylation of HPr(Ser-P), confirming that the HPr(Ser) kinase of *S. salivarius* was a bifunctional enzyme. The maximal rate of HPr(Ser-P) dephosphorylation was observed at 8 mM Pi (not shown). At this Pi concentration and under the conditions described in Section 2, more than 95% of HPr(Ser-P) was transformed into HPr after 9 min. The reaction

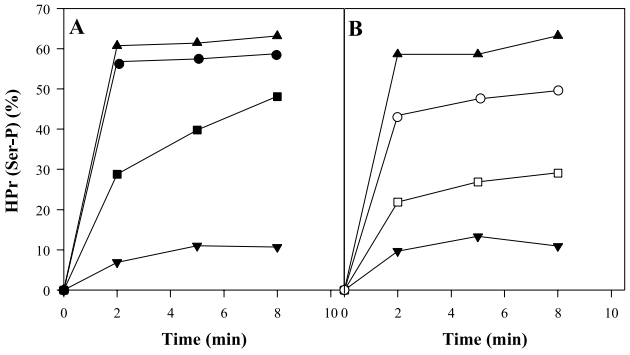

Fig. 3. HPr phosphorylation by HPrK/P in the presence of Pi and (A) FBP or (B) PEP. The reaction medium was as in Fig. 2 with 1 mM ATP. The reaction medium contained also: no addition (▲); 1 mM Pi (▼); 1 mM Pi and 5 mM FBP (■); 1 mM Pi and 15 mM FBP (●); 1 mM Pi and 1 mM PEP (□); 1 mM Pi and 5 mM PEP (○).

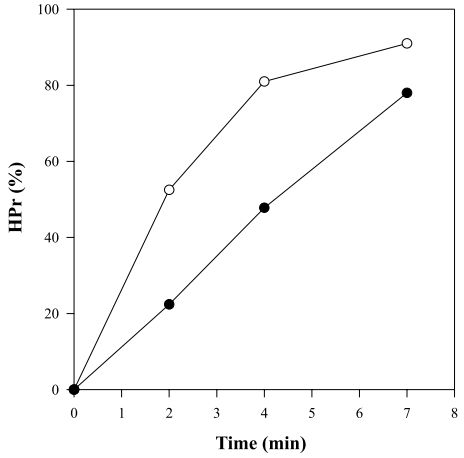

Fig. 4. HPr(Ser-P) dephosphorylation by *S. salivarius* HPrK/P. The reaction medium contained 350 nM (His_6)HPrK/P and 30 μM (His_6)HPr(Ser-P) in 50 mM Tris–HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM Pi and 0 mM FBP (○) or 5 mM (●) FBP.

required $MgCl_2$, which gave a maximal rate at a concentration of 2 mM in the presence of 10 mM Pi. To determine whether FBP could interfere with the dephosphorylation of HPr(Ser-P), we measured HPr(Ser-P) dephosphorylation with 10 mM Pi in the presence and absence of FBP. We observed a 2.5-fold decrease in HPr(Ser-P) dephosphorylation in the presence of 5 and 10 mM FBP (Fig. 4). Under the same conditions, the presence of 0.5 and 1 mM FBP did not interfere with HPr(Ser-P) dephosphorylation. The following intermediates, tested at concentrations found in actively metabolising streptococcal cells [20,21], had no effect: 0.5 mM PEP, 0.5 mM glucose-6-phosphate, 1 mM NAD^+ and 1 mM pyruvate. The K_m of *S. salivarius* HPrK/P for HPr(Ser-P) was measured by determining the rate of HPr(Ser-P) dephosphorylation in the presence of 10 mM Pi, and 4, 6, 8, 12, 20, and 30 μM HPr(Ser-P), and plotting the reciprocal of the velocity against the reciprocal of the substrate concentration. We found a K_m of approximately 5 μM.

4. Discussion

The molecular mass calculated for *S. salivarius* HPrK/P by equilibrium analytical ultracentrifugation was 201305 Da. This value corresponds to 5.9 times the calculated molecular mass of the monomer (34400 Da [11]), indicating that *S. salivarius* HPrK/P is also a hexameric protein. Previous reports suggesting that HPrK/P can adopt other quaternary structures are based on data obtained from experiments using size exclusion chromatography, which is a less accurate technique and dependent on the shape of the protein. The high levels of sequence identity between HPrK/Ps from various species [12] suggest that all are hexamers, a hypothesis strengthened by the results reported in this paper.

Some studies reported that phosphorylation of HPr by HPrK/P is stimulated by FBP whereas others observed only weak or no stimulation [6–11,13,16]. In the present work, the phosphorylation of HPr by purified *S. salivarius* HPrK/P using small amounts of enzyme (350 nM) in the presence of 0.05 and 0.5 mM ATP showed that FBP did not stimulate kinase activity at 0.5 mM ATP, unlike the *B. subtilis* enzyme, which is stimulated three- to four-fold under these conditions [6]. At 0.05 mM ATP, *S. salivarius* HPrK/P still exhibited substantial activity without FBP and the addition of 5 mM FBP resulted in only two-fold increase in the rate of HPr phosphorylation. These results demonstrate that the *Streptococcus* and the *Bacillus* HPrK/Ps respond differently to the presence of FBP.

From a physiological point of view, the slight activation of streptococcal HPrK/P kinase activity by FBP observed in the absence of Pi at low ATP concentrations has no obvious significance in a cellular context as very low levels of ATP, moderate to high levels of FBP and no Pi may not arise. Indeed, growth conditions that promote the synthesis of ATP also generate high cellular levels of FBP and, conversely, conditions that do not favour the synthesis of ATP result in a decrease in FBP levels [20–24]. However, while FBP was a poor activator of *S. salivarius* HPrK/P kinase activity, we found that its presence prevented inhibition of this activity by Pi. In fact, the enzyme was virtually unable to phosphorylate HPr in the presence of 1 mM Pi. Inhibition by Pi could be partially or totally overcome by FBP at concentrations found in growing cells [20–24], with the extent of relief dependent on the relative concentrations of the two metabolites. Thus, FBP, rather than being an activator of streptococcal HPrK/P, serves to protect kinase activity against Pi inhibition as it was found for *Mycoplasma* HPrK/P [10].

Dephosphorylation of HPr(Ser-P) by *S. salivarius* HPrK/P was strictly Pi dependent. This result is consistent with the finding that Pi is not a cofactor but a substrate of *B. subtilis* HPrK/P [15]. We also showed that FBP at concentrations of 5 mM or more reduced the rate of HPr(Ser-P) dephosphorylation two- to three-fold demonstrating the key role of FBP as a modulator of dual streptococcal HPrK/P activities. ATP and Pi compete for binding in the P-loop and trigger HPr phosphorylation or HPr(Ser-P) dephosphorylation respectively [12]. Our results suggest that FBP modulated *S. salivarius* HPrK/P activities by enhancing the affinity of the active site for ATP and, conversely, lowering the affinity for Pi. We assessed the specificity of the FBP regulatory site by testing the effect of other metabolic intermediates on HPrK/P activities. As neither fructose 6-phosphate nor fructose 1-phosphate could protect HPrK/P kinase activity from the inhibitory effect of 1 mM Pi, we suggest that stabilisation of FBP at the binding site involved the negative charges of the two phosphate groups. However, ribulose 1,5-biphosphate, which possesses only one carbon less than FBP, could not prevent Pi inhibition, suggesting that the spatial orientation of the two negative phosphate charges was crit-

ical for binding at the regulatory site. Intriguingly, 2-phosphoglycerate and PEP, which bear two negative charges and are smaller than ribulose 1,5-biphosphate, were able to regulate HPrK/P. These results emphasise the complexity of HPrK/P regulation. The determination of the structure of the enzyme with a regulator bound to it will provide a better understanding of the regulation mechanism.

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