*Articles*

# Insights into the Functioning of *Bacillus subtilis* HPr Kinase/Phosphatase: Affinity for Its Protein Substrates and Role of Cations and Phosphate†

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ABSTRACT: In *Bacillus subtilis*, carbon catabolite repression is mediated by the HPr kinase/phosphatase (HprK/P) which catalyzes both an ATP-dependent phosphorylation and a dephosphorylation on Ser-46 of either HPr (histidine-containing protein) or Crh (catabolite repression HPr). By using a surface plasmon resonance approach, it was shown here that the presence of magnesium is a prerequisite for the interaction of HprK/P with either HPr or Crh. HprK/P binds both protein substrates with a similar affinity ($K_D$ of about 40 nM), and addition of nucleotides increases by about 10-fold its affinity for each substrate. In addition, the specificity and the concentration of the cation required for the binding of protein substrates are different from that exhibited by the cation-binding site involved in the nucleotide binding, suggesting the presence of two cation-binding sites on HprK/P. The effects of phosphate on enzymatic activities of HprK/P were also investigated. Phosphate was able to unmask the phosphatase activity, especially in the presence of ATP or both ATP and fructose 1,6-bisphosphate whereas it was shown to inhibit the kinase activity of HprK/P. An apparent competition between phosphate and a fluorescent analogue of nucleotide led to the suggestion that phosphate mediates its effect by binding directly to the ATP-binding site of the enzyme.

Carbon catabolite repression (CCR)[1] is the paradigm of signal transduction. It allows bacteria to turn on and off catabolic gene expression in response to the availability of rapidly metabolizable carbon sources. In low GC Gram-positive bacteria, CCR is mediated by an atypical bifunctional enzyme, the HPr kinase/phosphatase (HprK/P) which catalyzes both an ATP-dependent phosphorylation and a dephosphorylation of HPr (histidine-containing protein) on Ser-46 (*1*−*3*). An HPr-like protein, Crh, is also found in *Bacillus subtilis* and is likewise reversibly phosphorylated by HprK/P on Ser-46 (*4*). The phosphorylation by HprK/P of either HPr or Crh was shown to be stimulated by fructose 1,6-bisphosphate (FBP) (*4*−*6*). This phosphorylation triggers a protein−protein interaction between HPr or Crh and the transcriptional regulator CcpA (catabolite control protein A) (*7*, *8*). The resulting protein complexes, CcpA/P-Ser-HPr or CcpA/P-Ser-Crh, specifically interact with an operator site called catabolite responsive element and regulate expression of many genes (*9*), since for instance about 10% of all genes are submitted to glucose regulation in *B. subtilis* (*10*).

In both Gram-positive and Gram-negative bacteria, HPr plays also a key role in the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (*11*). The PTS catalyzes the transport and concomitant phosphorylation of carbohydrates via a protein phosphorylation chain. In the presence of PEP, HPr is phosphorylated on His-15 by enzyme I (EI), and then P-His-HPr phosphorylates the sugar-specific IIA's (*12*). In Gram-positive bacteria, ATP-dependent phosphorylation at Ser-46 is a prerequisite for the interaction of HPr with CcpA, whereas PEP-dependent phosphorylation of HPr at His-15 prevents the formation of the HPr/CcpA complex, thus linking PTS-mediated sugar transport to CCR (*7*).

Sequence comparison revealed that HprK/P is found not only in most Gram-positive bacteria but also in some pathogenic Gram-negative bacteria (*5*, *13*). The HprK/Ps constitute a new family of protein kinase/phosphatase which does not contain the domain structure typical for eukaryotic protein kinases (*14*, *15*) but possesses an ATP-binding motif, called the P-loop (or A-motif) (*16*, *17*). Since disruption of the *hprK* gene, encoding HprK/P, leads to severe growth

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[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; CcpA, catabolite control protein A; CCR, carbon catabolite repression; Crh, catabolite repression HPr; EDTA, ethylenediaminetetraacetic acid; FBP, fructose 1,6-bisphosphate; FRET, fluorescence resonance energy transfer; HPr, histidine-containing protein; HprK/P, HPr kinase/phosphatase; Mant, 2′(3′)-*N*-methylanthraniloyl; PEP, phosphoenolpyruvate; $P_i$, inorganic phosphate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; SPR, surface plasmon resonance.

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defects of the *Staphylococcus xylosus* mutant strain (*2*), HprK/P might constitute a potential target for new antimicrobial agents in pathogenic bacteria (*5*).

The switch between the phosphatase and the kinase activities of HprK/P appears to be controlled by the presence of low molecular weight effectors (*1*). Hence, in the presence of the allosteric activator FBP, the kinase activity of HprK/P is predominant, but the stimulation of this activity by FBP essentially occurs at low concentrations of both ATP and HprK/P (*6*). On the other hand, inorganic phosphate was shown to inhibit kinase activity whereas it activates phosphatase activity in the absence of effectors (*1*, *18*).

In this paper, we described new information concerning the functioning of this atypical bifunctionnal enzyme. First, interactions of *B. subtilis* HprK/P with its protein substrates, HPr and Crh, were investigated by using a surface plasmon resonance (SPR) technique. It was found that (i) the presence of magnesium is necessary and sufficient for the interaction of either HPr or Crh with HprK/P, (ii) HprK/P binds HPr or Crh with a similar affinity, and (iii) the presence of nucleotides equally enhances the affinity of HprK/P for both substrates. In addition, the cation required for the binding of the protein substrates showed a different specificity than that involved in the binding of the nucleotide, suggesting that two cation-binding sites coexist on the enzyme. On the other hand, the effect of inorganic phosphate ($P_i$) on the molecular switch between the phosphatase and the kinase activities was analyzed in the presence of ATP and FBP. The results obtained from phosphorylation and dephosphorylation experiments performed in the presence of ATP alone or in the presence of both ATP and FBP showed that $P_i$ strongly inhibited the kinase activity whereas it unmasked the phosphatase activity. The use of a fluorescent ATP analogue, 2′(3′)-*N*-methylanthraniloyl-ATP (Mant-ATP), suggested that the effect of inorganic phosphate is most likely mediated by a direct binding of this effector to the ATP-binding site of the enzyme.

## EXPERIMENTAL PROCEDURES

*Reagents.* Mant-ATP was purchased from Molecular Probes, and 5′-adenylyl imidodiphosphate (AMPPNP) was purchased from Sigma.

*Protein Purification.* $HPr(His)_6$, $Crh(His)_6$, and $HprK/P(His)_6$ were purified on Ni-NTA−agarose columns as described previously (*4*, *5*).

*Protein Phosphorylation and Dephosphorylation.* A typical 20 $\mu$L phosphorylation mixture contained 30 $\mu$M purified $HPr(His)_6$ or $Crh(His)_6$, 0.5 $\mu$M purified $HprK/P(His)_6$, 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 500 $\mu$M ATP. A typical 20 $\mu$L dephosphorylation mixture contained 30 $\mu$M purified P-Ser-$HPr(His)_6$ or P-Ser-$Crh(His)_6$, 0.5 $\mu$M purified $HprK/P(His)_6$, 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. The phosphorylation and the dephosphorylation mixtures were incubated for 10 and 60 min, respectively. These experimental conditions were used unless otherwise stated in the figure legend. Both phosphorylation and dephosphorylation experiments were stopped by addition of 100 mM EDTA to the assay mixtures since both reactions were dependent on the presence of magnesium (data not shown and ref *13*). The samples were then loaded onto a nondenaturing 12.5% polyacrylamide gel as described previously (*19*). After electrophoresis, the gel was stained with Coomassie Blue and scanned in a Densitometer SI (Molecular Dynamics); results were analyzed with ImagQuant V1.2 software (Molecular Dynamics) (*6*).

*Surface Plasmon Resonance (SPR).* Real time binding experiments were performed on a BIAcore biosensor system (Biacore AB, Uppsala, Sweden). All experiments were performed at 25 °C. HPr or Crh in 20 mM potassium acetate, pH 4.5, were directly coupled through their amino groups to the surface of a CM5 sensor chip activated by *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide according to the manufacturer's instructions. The remaining reactive groups were then inactivated with 1 M ethanolamine. Each ligand was immobilized at concentrations described in the figure legends. For control experiments, the sensor surface was treated as above in the absence of protein (mocked derivatized sensor chip). Interaction experiments were done at a flow rate of 20 $\mu$L/min using a running buffer containing 20 mM Tris-HCl, pH 8.0, and 0.005% surfactant P-20 (buffer A) in the presence of additional effectors as indicated in the figure legends. At the end of each cycle, bound HprK/P was removed by injecting 10 $\mu$L of 25 mM EDTA. Sensorgrams were analyzed using BIAevaluation 2.0 software (Biacore AB, Uppsala, Sweden), and kinetic constants were obtained by nonlinear fitting of the sensorgrams to a single-site binding model. The equation $R = R_0 \exp[-k_{off}(t - t_0)]$ was used for the dissociation phase, where $t_0$ is the start time for dissociation and $R_0$ is the response at the start of the dissociation. $k_{on}$ was calculated using the model A + B = AB and by fitting the data to the equation $R = R_{eq}(1 - \exp[-k_{on}C + k_{off}(t - t_0)])$, where $C$ is the molar concentration of the analyte and $R_{eq}$ is the steady-state response level. The apparent $K_D$ value was determined from the ratio of the kinetic constants ($k_{off}/k_{on}$). The goodness of the fit was assessed by inspecting the $\chi^2$ values ($<5$) and the random distribution of the residuals. Sensorgrams were also analyzed using linear transformation of the primary data, as kinetic constants can be obtained by linear transformation of a set of sensorgrams using a plot of $\ln(dR/dt)$ versus time for each analyte concentration. These plots give a line of which the slope is $k_s$. A secondary plot of these slopes ($k_s$) versus $C$ (concentration of the analyte) is then used to determine $k_{on}$ and $k_{off}$ from the linear relationship $k_s = k_{on}C + k_{off}$.

*Fluorescence Measurements.* All experiments were performed at 25 ± 0.1 °C using a Photon Technology International Quanta Master I spectrofluorometer as described previously (*6*). The measurements were automatically corrected for intensity fluctuation in lamp emission. All spectra were corrected for buffer fluorescence. Fluorescence measurements were carried out after dilution of HprK/P (1 $\mu$M final concentration) and equilibration for 10 min in 2 mL of buffer containing 25 mM Hepes/KOH, pH 8, and 0.1 mM EDTA. After specific excitation of the tryptophan residue at 295 nm, the fluorescence emission was recorded between 310 and 540 nm before and after addition of increasing concentrations of $KP_i$ as indicated. The fluorescence resonance energy transfer (FRET) between the tryptophan residue of HprK/P and bound Mant-ATP was monitored by the appearance of a fluorescence emission peak (400−540 nm), characteristic of bound nucleotide analogues. Peak integration was carried out at each phosphate concentration with Felix

Table 1: Effect of Different Cations on the Binding of either Crh or Mant-ATP to HprK/P[a]

| | cation | | | | | |
|---|---|---|---|---|---|---|
| | $Mg^{2+}$ | $Ca^{2+}$ | $Mn^{2+}$ | $Co^{2+}$ | $Cu^{2+}$ | $Ni^{2+}$ |
| binding of Crh | +++ | +++ | − | − | − | − |
| binding of Mant-ATP | +++ | +++ | +++ | + | nd | + |

[a] The ability of each cation listed in the table, at a concentration of 5 mM, to allow the binding of either Crh (monitored by surface plasmon resonance as in Figure 1) or Mant-ATP (monitored by fluorescence resonance energy transfer; see Experimental Procedures) was determined. (+++) indicates that the cation maximally stimulated the binding; (+) indicates that the cation moderately stimulated the binding; (−) indicates that the cation had no effect on the binding. nd = not determined due to the precipitation of the Mant-ATP.

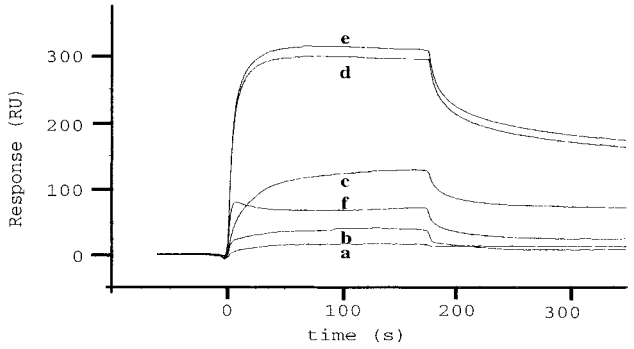


FIGURE 1: Influence of $Mg^{2+}$ and nucleotides on the interaction between HprK/P and HPr monitored by surface plasmon resonance. HPr was covalently immobilized (b−f; quantity corresponding to about 1000 resonance units, RU) or not (a) on sensor surfaces as described under Experimental Procedures. HprK/P (1 $\mu$M) in buffer A was then injected over the surface at a flow rate of 20 $\mu$L/min. Plots: (a) HprK/P in buffer A injected over a mock derivatized sensor chip; (b) HprK/P in buffer A; (c) HprK/P in buffer A containing 5 mM $MgCl_2$; (d) HprK/P in buffer A containing 5 mM $MgCl_2$ and 1 mM ADP; (e) HprK/P in buffer A containing 5 mM $MgCl_2$ and 1 mM AMPPNP; (f) HprK/P in buffer A containing 5 mM $MgCl_2$ and 1 mM ATP.

1.21 software (Photon Technology International), and the observed changes in fluorescence resonance energy transfer were used for the calculation of the $P_i$ concentration which produced a 50% release of the previously bound Mant-ATP ($K_{0.5}$). To analyze the requirement for cations in the binding experiments using Mant-ATP, HprK/P (1 $\mu$M final concentration) was incubated for 10 min in 2 mL of buffer containing 25 mM Hepes/KOH, pH 8, and 0.1 mM EDTA and in the presence of 5 $\mu$M Mant-ATP. This concentration of the analogue was used since it is too low to saturate the nucleotide-binding site in the absence of added cations. The fluorescence emission was then recorded between 310 and 540 nm before and after addition of 5 mM indicated cation (see Table 1).

## RESULTS

*Effect of Magnesium and Nucleotides on the Interactions of HprK/P with HPr or Crh.* The molecular interaction between HprK/P and HPr or Crh was analyzed using surface plasmon resonance detection. HPr or Crh (ligands) were coupled to the sensor chip through their amine functions. Figure 1 shows typical sensorgrams representing the real time interaction between bound HPr and HprK/P (analyte). The interaction is strictly dependent on the presence of $Mg^{2+}$ (compare sensorgrams b and c) and is enhanced by the presence of a nonhydrolyzable analogue of ATP, 5′-adenylyl imidodiphosphate (AMPPNP), or ADP (sensorgrams d and e, respectively). In the presence of ATP (sensorgram f) the kinetics of the interaction is greatly perturbed. After a rapid association that lasts only a few seconds following the injection of HprK/P, there is an apparent very slow dissociation during the remainder of the time course of the injection. Since this experimental condition reflects that used for the phosphorylation of the substrate, the dissociation might be interpreted as the result of both a very rapid association of the enzyme with its substrate and an even more rapid dissociation of the enzyme from its phosphorylated substrate. At the end of the injection, the dissociation of HprK/P from HPr occurs at a rate similar to those observed in the other sensorgrams. Interactions were completely reversed by addition of EDTA, allowing a recycling of the sensor chip with an EDTA-containing buffer.

*Is There a Unique Cation-Binding Site on HprK/P?* To analyze whether a unique cation-binding site is involved in the binding of both the protein substrate and the nucleotide, a comparison of the cation requirement was performed for the binding of these two substrates. Table 1 shows the ability of different cations to allow the binding of either Crh or Mant-ATP to HprK/P. Mant-ATP was chosen since it was shown previously to efficiently replace ATP in a phosphorylation assay of HPr (*6*) and also because, at the concentration used here, this analogue was unable to bind to the enzyme in the absence of added cation. Whereas $Ca^{2+}$ was the only cation tested in Table 1 able to replace $Mg^{2+}$ for Crh binding, both $Ca^{2+}$ and $Mn^{2+}$ substituted efficiently for $Mg^{2+}$ in the binding experiment with Mant-ATP. Furthermore, no interaction between HprK/P and Crh could be detected when the concentration of $Mg^{2+}$ was below 1 mM in the SPR experiment, whereas an equimolar concentration of $Mg^{2+}$ with Mant-ATP (i.e., 60 $\mu$M) was sufficient for the maximal binding of the ATP analogue in the fluorescence experiment (not shown). It should be noted that addition of 1 mM AMPPNP, ATP, or ADP in the SPR experiments did not modify the concentration of cation required to allow the interaction between HprK/P and its protein substrates, showing that the presence of nucleotides did not affect the affinity of this cation-binding site.

*Determination of the Kinetic Parameters for the Interactions between HprK/P and Its Protein Substrates.* Different concentrations of HprK/P were allowed to interact with a sensor chip covalently derivatized with HPr, and the binding of HprK/P was monitored by an increase in the SPR signal. Figure 2A shows the sensorgrams from a representative experiment performed with different concentrations of HprK/P. The kinetics of the interaction between HprK/P and HPr could be fitted to a single-site binding model with an apparent $K_D$ value of 45 nM. Almost the same value was obtained when a six His-tagged HprK/P was immobilized onto a surface of a NTA sensor chip coated with $Ni^{2+}$ and HPr used as the analyte (result not shown). The consistency of this result was also cross-checked by linear transformation of the data (Figure 2B). As can be seen, the plot of $k_s$ versus [HprK/P] is linear and supports a 1:1 interaction model. Moreover, the $k_{on}$ value obtained from the slope is nearly the same as that obtained by nonlinear fitting ($1.9 \times 10^5$ $M^{-1}$ $s^{-1}$ and $1.3 \times 10^5$ $M^{-1}$ $s^{-1}$, respectively). The $k_{off}$ value derived from the linear approach is approximately 2.5 times higher than

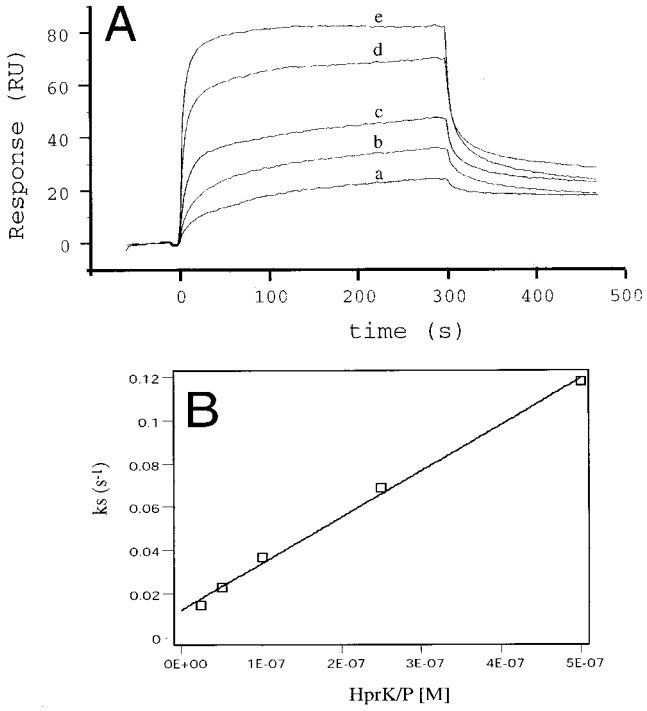


FIGURE 2: Kinetics of the interaction of HprK/P with HPr. (A) Increasing concentrations of HprK/P were injected in buffer A containing 5 mM $MgCl_2$ on a sensor surface coated with approximately 500 RU of HPr. The HprK/P concentrations were 25 nM (a), 50 nM (b), 100 nM (c), 250 nM (d), and 500 nM (e). (B) Plot of $k_s$ versus [HprK/P] resulting from the linear transformation of the sensorgrams obtained in (A) (see Experimental Procedures).

Table 2: Kinetic Constants for the Interaction of HprK/P with HPr or Crh[a]

| ligand | analyte | $k_{on} \times 10^{-4}$ ($M^{-1}$ $s^{-1}$) | $k_{off} \times 10^{-4}$ ($s^{-1}$) | $K_D$ (nM) |
|---|---|---|---|---|
| HPr | HprK/P | $13 \pm 2$ | $58 \pm 7$ | $45 \pm 8$ |
| Crh | HprK/P | $1.2 \pm 0.2$ | $4.7 \pm 0.7$ | $39 \pm 6$ |
| HPr | HprK/P + AMPPNP | $51 \pm 4$ | $16 \pm 3$ | $3.1 \pm 0.4$ |
| Crh | HprK/P + AMPPNP | $4.3 \pm 0.4$ | $2.2 \pm 0.3$ | $5.1 \pm 0.7$ |

[a] The kinetic constants were determined using nonlinear fitting of the sensorgrams as described under Experimental Procedures. The interaction was measured in a running buffer containing 5 mM $MgCl_2$ and, when indicated, 1 mM AMPPNP. The values represent the mean of triplicate measurements.

that determined using the nonlinear approach ($1.4 \times 10^{-2}$ $s^{-1}$ instead of $5.8 \times 10^{-3}$ $s^{-1}$). Such a small discrepancy is not unusual, and the better accuracy of the nonlinear method over the linear method to determine the $k_{off}$ value has been demonstrated previously (*20*, *21*).

Similar experiments were also performed with Crh, and the kinetic parameters analyzed by the sensorgrams for the different combinations are summarized in Table 2. It was found that HprK/P binds HPr or Crh with a similar affinity (apparent $K_D$ value of 45 nM for HPr and 39 nM for Crh) but with some differences in the $k_{on}$ and $k_{off}$ values. HprK/P associates more slowly with Crh than with HPr ($1.2 \times 10^4$ $M^{-1}$ $s^{-1}$ instead of $1.3 \times 10^5$ $M^{-1}$ $s^{-1}$), but it also dissociates more slowly ($4.7 \times 10^{-4}$ $s^{-1}$ instead of $5.8 \times 10^{-3}$ $s^{-1}$). In the presence of AMPPNP, both of the apparent $K_D$ values for HPr and Crh were decreased by about 10-fold (3.1 nM and 5.1 nM, respectively). In each case, this is due to both a 3−4-fold increase in the $k_{on}$ values and a 3−4-fold decrease in the $k_{off}$ values. Whatever the conditions used, addition of 1 mM phosphate did not change the kinetic parameters significantly (not shown). When the interactions between HprK/P and the phosphorylated substrates P-Ser-HPr or P-Ser-Crh were also investigated by using the same technique, we were unable to detect any interaction between these partners. This suggests that the affinities of HprK/P for the phosphorylated substrates drop to a level below the threshold limit of the BIAcore apparatus. This assumption is in agreement with the result shown in Figure 1 where a very rapid dissociation of HprK/P occurs in the presence of ATP, a condition leading to the phosphorylation of the protein substrate. It was also investigated whether the nature of the protein substrate, Crh or HPr, modulated the efficiencies of either the kinase or the phosphatase activities of HprK/P. However, no major differences were observed between the two protein substrates, regardless of the enzymatic reaction considered (not shown).

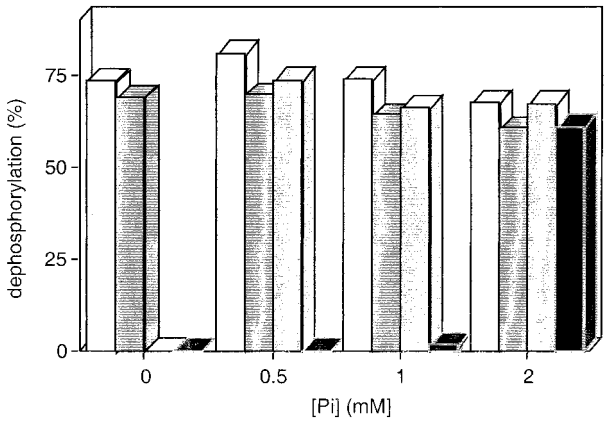


FIGURE 3: Effect of phosphate on P-Ser-Crh dephosphorylation catalyzed by HprK/P. A 20 $\mu$L dephosphorylation mixture containing 0.5 $\mu$M HprK/P, 30 $\mu$M P-Ser-Crh, 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$ (white bars), and also 5 mM FBP (dark gray bars) or 1.25 mM ATP (light gray bars) or both 5 mM FBP and 1.25 mM ATP (black bars) was incubated for 60 min at 37 °C in the presence of the $KP_i$ concentrations indicated in the figure. The dephosphorylation reactions were stopped by addition of 100 mM EDTA to the assay mixtures before loading them onto a nondenaturing 12.5% polyacrylamide gel. The gels were scanned in a personal Densitometer SI, and the data were analyzed with the software ImagQuant V1.2 (Molecular Dynamics).

*Effect of Phosphate on the Enzymatic Activities of HprK/P.* To investigate how inorganic phosphate ($P_i$) modulates each HprK/P activity in the presence of ATP, dephosphorylation and phosphorylation experiments were carried out in the presence of $P_i$. In the presence of a low concentration of *B. subtilis* HprK/P (30 nM) and 5 min of incubation time, $P_i$ was shown to be required for HPr dephosphorylation (*18*). In the experimental conditions used (i.e., 0.5 $\mu$M HprK/P and an incubation time of 60 min), to obtain a higher level of dephosphorylation (Figure 3), addition of phosphate barely affected the efficiency of dephosphorylation by HprK/P in the absence of other effectors. Experiments were also performed in the presence of other effectors, i.e., FBP, ATP, or both, and by adding increasing concentrations of $P_i$ (Figure 3). The presence of FBP alone did not significantly affect the dephosphorylation activity of HprK/P, regardless of the $P_i$ concentration added. By contrast, the presence of 1.25 mM ATP completely abolished the dephosphorylation of Crh, unless 0.5 mM $P_i$

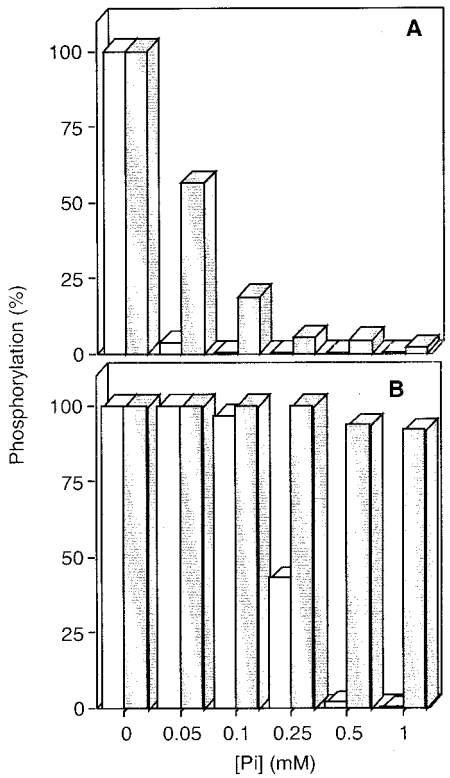


FIGURE 4: Effect of phosphate on Crh phosphorylation catalyzed by HprK/P. (A) A 20 $\mu$L phosphorylation mixture containing 1 $\mu$M HprK/P, 30 $\mu$M Crh, 50 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$ was incubated for 15 min at 37 °C in the presence of either 0.5 mM ATP (white bars) or 4 mM ATP (gray bars) and the concentrations of $KP_i$ indicated in the figure. (B) Same as in (A) except that the experiment was performed in the presence of 5 mM FBP. The phosphorylation reactions were stopped by addition of 100 mM EDTA to the assay mixtures before loading them onto a nondenaturing 12.5% polyacrylamide gel. The gels were scanned in a personal Densitometer SI, and the data were analyzed with the software ImagQuant V1.2 (Molecular Dynamics).

was present in the assay medium. Addition of both ATP and FBP also blocked the dephosphorylation of Crh, but in this case higher concentrations of $P_i$, at least 2 mM, were necessary to fully reverse the inhibitory effect of ATP plus FBP on the phosphatase activity. The same experiments were carried out with $NaP_i$ instead of $KP_i$ or AMPPNP instead of ATP, and similar results were obtained (data not shown).

The effect of $P_i$ was then analyzed on the phosphorylation of Crh by HprK/P. In the absence of FBP (Figure 4A) and at a low concentration of ATP (0.5 mM), a strong inhibition of Crh phosphorylation was observed for the lowest concentration of $P_i$ used, i.e., 0.05 mM. However, when a higher ATP concentration was used (4 mM), a much higher concentration of $P_i$ was necessary to inhibit the phosphorylation of Crh. Addition of the allosteric activator of the kinase activity, FBP, required an almost hundred times higher concentration of $P_i$ (0.5 mM) to inhibit the kinase activity obtained in the presence of 0.5 mM ATP (Figure 4B). In this case, increasing the concentration of ATP to 4 mM almost completely prevents the inhibitory effect of $P_i$.

*Does $P_i$ Exert Its Effects by Binding Directly to the ATP-Binding Site?* To identify the type of inhibition produced by $P_i$ on the kinase activity of HprK/P, an attempt was made to obtain a Dixon plot using different ATP concentrations. Unfortunately, it has been shown previously that the enzyme exhibited a positive cooperativity mechanism for the binding of ATP/Mg (*6*), and a similar mechanism most likely occurs for the binding of phosphate. Indeed, whereas in the presence of 2 mM ATP the phosphorylation of Crh was weakly inhibited by addition of 0.05 mM $P_i$, it was almost completely abolished when 0.1 mM $P_i$ was used (data not shown). Therefore, to determine if $P_i$ exerts its effect by binding directly to the ATP site, or to another specific site on the enzyme, e.g., the FBP binding site, experiments were carried out to analyze the effect of $P_i$ on the binding of Mant-ATP. The binding of 60 $\mu$M Mant-ATP produced a drastic quenching of the fluorescence emission spectrum of the unique tryptophan residue present in HprK/P. Simultaneously, a new peak of fluorescence developed, centered at approximately 430 nm, which is indicative of the fluorescence resonance energy transfer between the tryptophan residue and the Mant derivative (Figure 5). Addition of increasing concentrations of $P_i$ progressively relieved the quenching of the tryptophan fluorescence and concomitantly reduced the fluorescence resonance energy transfer, as shown by the disappearance of the fluorescence peak centered at

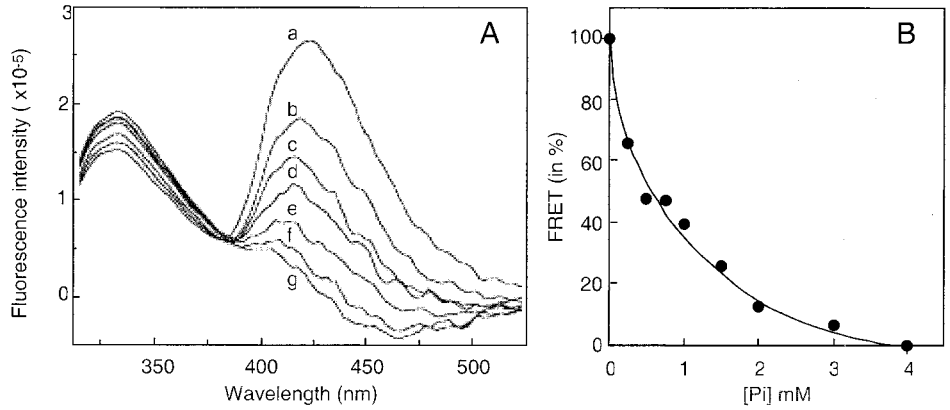


FIGURE 5: Effect of phosphate on Mant-ATP previously bound to HprK/P. (A) HprK/P (1 $\mu$M) was incubated in a 2 mL cuvette containing 25 mM Hepes/KOH, pH 8, 1 mM EDTA, and 60 $\mu$M Mant-ATP during 10 min at 23 °C. After specific excitation of the tryptophan residue at 295 nm, the fluorescence emission was recorded between 310 and 540 nm after each addition as follows: (a) no addition; (b) 0.25 mM, (c) 0.5 mM, (d) 1 mM,(e) 1.5 mM, (f) 2 mM, and (g) 3 mM $KP_i$ from a 1 M stock solution at pH 8. Additional concentrations of $KP_i$ were also used but are not shown for the sake of clarity. (B) The fluorescence resonance energy transfer (FRET) obtained by integrating the emission peak (400−540 nm) characteristic of the fluorescence of the Mant-ATP bound to HprK/P was plotted as a function of the $P_i$ concentration.

430 nm. At 4 mM $P_i$, there was virtually no more fluorescence resonance energy transfer between the tryptophan residue and the Mant derivative. This indicates that the addition of $P_i$ displaced the Mant-ATP previously bound to HprK/P. Conversely, prior incubation of HprK/P with 10 mM $P_i$ totally prevented the subsequent binding of Mant-ATP to the enzyme (data not shown). Altogether, these results suggest that there is a direct competition between $P_i$ and Mant-ATP for binding to the ATP-binding site of HprK/P.

## DISCUSSION

This paper describes the characterization of the mechanistic properties of the HprK/P bifunctional enzyme from *B. subtilis*. It is shown that the presence of magnesium is necessary, and sufficient, for the interaction of HprK/P with its protein substrates, HPr or Crh. In the absence of nucleotide, HprK/P binds HPr and Crh with a similar affinity, and the addition of nucleotides equally enhances the affinity of HprK/P for each protein substrate.

*A Second, Low-Affinity, Cation-Binding Site Specifically Involved in the Interaction with the Protein Substrates.* Comparison of the cation specificity for the binding of either the protein substrates or the nucleotide analogue suggests that the enzyme harbors two different cation-binding sites. The first site has a high affinity in the micromolar range (or even lower) for $Mg^{2+}$, $Ca^{2+}$, or $Mn^{2+}$, and the cation bound to this site interacts specifically with the nucleotide. This relative lack of specificity for the so-called metal nucleotide-binding site is not a unique property of HprK/P since it has been found in other ATP-utilizing enzymes harboring a P-loop motif, for instance, the $F_1$-ATPase (*22*) or the P-glycoprotein (*23*). In contrast, the second cation-binding site of HprK/P involved in the binding of the protein substrates appears more selective than the first site since it can accommodate only $Mg^{2+}$ or $Ca^{2+}$, although it exhibits a much lower affinity, in the millimolar range, for these two cations. Such a low affinity of the second cation-binding site of *B. subtilis* HprK/P is compatible with the concentration range of cation required by an HprK/P partially purified from *Streptococcus pyogenes* to efficiently phosphorylate HPr (*24*). In fact, the presence of two different cation-binding sites is a fairly common feature among enzymes (*25*), especially those involved in phosphoryl transfer and regardless of the substrate to be phosphorylated (*26*). Interestingly, we have recently shown that the nucleotide-binding site of HprK/P bears a significant structural resemblance to that of the phosphoenolpyruvate carboxykinase enzyme (*27*), the latter enzyme being known to harbor two metal-binding sites. Apart from the metal nucleotide-binding site classically found in many P-loop-containing enzymes, the second metal-binding site of the phosphoenolpyruvate carboxykinase involved a conserved histidine residue (*28*). Superimposition of the nucleotide-binding sites of HprK/P and phosphoenolpyruvate carboxykinase showed that an equivalent histidine is found in HprK/P (*27*), which could be tentatively assigned as a residue involved in the second cation-binding site of HprK/P.

*Binding Affinity of HprK/P for Its Protein Substrates, HPr and Crh.* Previously, it has been reported that *B. subtilis* HprK/P exhibited a $K_m$ for HPr of about 80 $\mu$M (*13*), consistent with that found, for instance, in *S. pyogenes* (61 $\mu$M) or *Streptococcus salivarius* (31 $\mu$M) (*24*, *29*). Here, by using the SPR technique, the affinity of HprK/P for HPr or Crh was directly measured for the first time and is in the nanomolar range. Although HprK/P is structurally and functionally unrelated to any known protein kinase, its affinity values for its two protein substrates are in the same range as those observed with several eukaryotic protein kinases for their respective substrate (*30*−*32*). More importantly, HPr plays a pivotal role in the PTS in both Gram-negative and Gram-positive bacteria and, as such, interacts with many cellular partners (*33*). In a few cases, the affinities controlling interactions between HPr and its physiological partners have been measured. Hence, the dissociation constant between HPr and enzyme I was reported to be about 10 $\mu$M (*34*), whereas the interaction between HPr and glycogen phosphorylase occurred with a much higher affinity (dissociation constant ~10 nM) (*35*). Remarkably, a similar surface on HPr is used to bind three different protein partners, namely, enzyme I, glucose-specific enzyme IIA, or glycogen phosphorylase (*36*, *37*), and the same HPr interface is also involved in binding HprK/P (*38*). In the latter case, the characteristics of the interface area on HPr suggested that the interaction with HprK/P involves mainly a hydrophobic region (*38*), and this would be compatible with the high affinity found here for this complex.

Intriguingly, the interaction between HprK/P and HPr could be fitted with a single binding site model whereas HprK/P was shown previously to display a positive cooperativity for the binding of either nucleotide, in the presence of $Mg^{2+}$, or FBP (*6*). Therefore, each homooligomer of HprK/P contains either a unique binding site for the protein substrate or, at least, two equivalent binding sites for the protein substrate. In fact, it is quite possible that HprK/P displays a mechanism of cooperativity for the binding of some, but not all, of its substrates or effectors. Cooperativity was observed with HprK/P for the binding of either nucleotide, in the presence of magnesium, or FBP. FBP is an allosteric effector of several bacterial enzymes involved in sugar metabolism (*39*), and its concentration is greatly influenced by the culture conditions (*40*−*42*). Likewise, the concentration of ATP is strongly influenced by the growth conditions (*42*, *43*), and many enzymes bind this substrate with a cooperative mechanism. By contrast, the concentration of HPr (or Crh) in the bacteria is always far in excess over the HprK/P concentration and mostly, given the pivotal role of HPr in the carbon catabolite repression mechanism (*44*, *45*), well above the affinity of the protein kinase for its substrate. For instance, the cellular concentration of HPr in streptococci has been estimated to be in the micromolar range (*46*), and furthermore, the cellular content of HPr was rather unaffected by variation in growth conditions (*46*, *47*). Therefore, a regulatory mechanism of HprK/P activity based on a variation in the concentration of its protein substrates, HPr or Crh, appears very unlikely.

*HPr and Crh Behave as Similar Substrates for HprK/P.* Although we were unable to detect significant differences between the ability of HprK/P to phosphorylate and dephosphorylate either HPr or Crh, previous studies have shown that these two "relay" proteins do not display the same efficiency in the CCR. Thus, a *ptsH1* mutant, in which Ser-46 of HPr is replaced with a nonphosphorylatable alanyl residue, exhibited a reduced glucose repression of several

operons (*48*), whereas a disruption of the *crh* gene barely affected the CCR (*4*). This result showed that P-Ser-Crh is not able to fully substitute for P-Ser-HPr in carbon regulation. Hence, to alleviate the carbon catabolite repression of many operons, a double mutant affected in both HPr and Crh had to be constructed (*4*). The feebler implication of Crh than that of HPr in the control of CCR, as opposed to their similar behavior as HprK/P substrates, therefore suggests that there is a difference in either their biosynthesis or their ability to interact with CcpA.

*Effect of $P_i$ on the Phosphatase and Kinase Activities of HprK/P and Location of Its Binding Site.* We have also investigated the effect of $P_i$ on the molecular switch between the phosphatase and the kinase activities. $P_i$ inhibits the kinase activity whereas it unmasks the phosphatase activity (*18*). At the intracellular level, one of the first consequences caused by the presence of glucose is an increase in the FBP concentration and a decrease in the $P_i$ concentration (*41*, *42*, *49*). The intracellular pool of these two compounds often acts as an energy sensor to properly balance the catabolism of glucose in the low G + C Gram-positive bacteria by modulating the activity of some key enzymes (*43*). For instance, FBP stimulates the activity of lactate dehydrogenases of some Gram-positive bacteria (*50*, *51*) while phosphate can act as an inhibitor of these enzymes (*50*, *52*). Moreover, phosphate has been shown to act as a competitive inhibitor of FBP binding to lactate dehydrogenase from *Streptococcus cremoris* (*53*), suggesting that these two effectors share the same binding site. In the case of glycerol kinase where both FBP and phosphate behave as inhibitors, crystal structures have been obtained in the presence of each effector showing that they do bind to the same site (*54*, *55*). The hypothesis that phosphate and FBP share the same binding site on HprK/P cannot be ruled out on the basis of the results reported here. On the other hand, the competition between the fluorescent ATP analogue and phosphate argues in favor that both effectors bind to the same site, although we cannot exclude that an additional binding site for phosphate is present on HprK/P and is responsible for activation of its phosphatase activity. Besides, the recent determination of the 3D structure of a truncated form of *Lactobacillus casei* HprK/P grown in the presence of 400 mM phosphate revealed the presence of only one binding site for this anion (*56*). On the basis of the structure of other nucleotide-binding proteins containing a P-loop, this phosphate would be located at the expected position for the $\beta$-phosphate of ATP (*56*), strengthening the idea that ATP and phosphate share the same binding site on HprK/P. We cannot exclude, however, the presence of an additional $P_i$-binding site on the missing part of the *L. casei* HprK/P truncated structure.

The effect of phosphate on the phosphatase activity of HprK/P seems to depend on the bacterial origin of the enzyme under consideration. Previously, phosphate alone was found to strongly stimulate the phosphatase activity of *L. casei* HprK/P (*3*, *18*) whereas it was not required to activate the phosphatase activity of HprK/P from *S. xylosus* (*2*). When phosphate was added to a mixture containing both FBP and ATP, the kinase activity of *B. subtilis* HprK/P was switched off and the phosphatase activity became prevalent (*1*), which corroborates the results reported here. Likewise, the stimulatory effect of FBP on the kinase activity of HprK/P was observed with enzymes purified from *B. subtilis* (*5*, *13*) or *L. casei* (*3*) but not with those purified from *S. salivarius* (*29*), *S. xylosus* (*2*), or *Enterococcus faecalis* (*1*). This different behavior, depending on the origin of the HprK/P, might again reflect the intracellular variation of the two metabolites, FBP and $P_i$, that occurs during CCR in each species.

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