Regulation and Mutational Analysis of the HPr Kinase/Phosphorylase from *Bacillus subtilis*†

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Received March 12, 2003; Revised Manuscript Received April 17, 2003

ABSTRACT: In most Gram-positive bacteria, catabolite repression is mediated by a bifunctional enzyme, the HPr kinase/phosphorylase (HprK/P). It has recently been shown that HprK/P could catalyze the phosphorylation of the protein HPr by using pyrophosphate (PP_i) as a phosphate donor instead of ATP. Here we showed that, as for ATP, PP_i binds to the enzyme with strong positive cooperativity. However, in contrast to ATP, PP_i binding does not modify the fluorescence properties of the unique Trp residue of *Bacillus subtilis* HprK/P. In addition, to understand how two conserved motifs, namely, the P-loop and the specific signature of this family, participate in the three enzymatic activities of HprK/Ps (ATP-kinase, PP_i-kinase, and phosphorylase), several site-directed mutants were generated. Whereas the three activities are mediated by the P-loop which is directly involved in the binding of ATP, PP_i, or Pi, the signature motif seems to be involved preferentially in the dephosphorylation reaction. On the basis of these results, we propose a model in which the binding of the allosteric activator FBP induces a conformational change of a central loop located above the active site of HprK/P, thereby allowing the ATP binding. However, this conformational change is not required for the binding of PP_i.

Carbon catabolite repression (CCR)[1] allows bacteria to alter catabolic gene expression in response to the availability of rapidly metabolizable carbon sources. In the low-GC Gram-positive bacteria, *Bacillus subtilis*, regulation of CCR involves the HprK/P, a bifunctional enzyme catalyzing both the phosphorylation and the dephosphorylation of HPr (histidine-containing protein) and its homologue Crh (catabolite repression HPr) at Ser46 (*1*−*3*). HprK/P appeared to be unrelated to the classical eukaryotic protein kinases, but the catalytic C-terminal domain contains a P-loop (or Walker A motif) commonly found in other nucleotide-binding proteins (*4*, *5*) and a signature motif found in all HprK/P proteins from different organisms (*6*). The sequence and structure of HprK/P are restrictedly but significantly homologous with those of another phosphotransferase, the phosphoenolpyruvate carboxykinase (PEPCK) (*7*, *8*). The X-ray structures of HprK/Ps from different species have been determined [PDB entries 1JB1 (*9*), 1KO7 (*10*), 1KKL, 1KKM (*11*), and 1KNX (*12*)], showing that they are homohexamers. The catalytic core of the protein forms dimers of trimers surrounded by three dimers of N-terminal domains. The function of the latter remains unknown.

The ATP-dependent phosphorylation of HPr or Crh is stimulated by glycolytic intermediates such as fructose 1,6-bisphosphate (FBP). It has been demonstrated that *B. subtilis* HprK/P is an allosteric enzyme which displays strong positive cooperativity for the binding of its allosteric activator, FBP, as well as the binding of the nucleotide ATP (*13*). Furthermore, inorganic phosphate (P_i) has been found to be another effector of HprK/P which inhibits the kinase activity and stimulates the dephosphorylation reaction. Indeed, *in vivo*, the kinase activity is predominant when high concentrations of ATP and FBP are present in the cell, whereas the dephosphorylation becomes prevalent when the concentration of P_i increases (*3*). The inhibitory effect of P_i was explained by demonstrating the competition between P_i and ATP for the same binding site (*14*) and by the crystal structure of HprK/P from *Lactobacillus casei* (*9*) and from *Staphylococcus xylosus* (*10*) that revealed the presence of one or two P_i molecules interacting with the P-loop. Recent experiments showed that P_i is not an activator of P-Ser-HPr dephosphorylation but the substrate of the reaction, which produces HPr and pyrophosphate (PP_i) (*15*). The phosphorylase

† This research was supported by the CNRS, the Université d'Aix-Marseille II, the Fondation pour la Recherche Médicale, and the ministère de la recherche "ACI-jeunes-chercheurs" (to A.G.).

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[1] Abbreviations: CCR, carbon catabolite repression; Crh, catabolite repression HPr; EDTA, ethylenediaminetetraacetic acid; FBP, fructose 1,6-bisphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; FRET, fluorescence resonance energy transfer; HPr, histidine-containing protein; HprK/P, HPr kinase/phosphorylase; Mant, 2′(3′)-*N*-methylanthraniloyl; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; P_i, inorganic phosphate; PP_i, pyrophosphate.

10.1021/bi034405i CCC: $25.00
Published on Web 05/16/2003

reaction seems to be reversible, but the physiological significance of the PP_i-dependent HPr phosphorylation remains unknown. This PP_i-dependent kinase activity of HprK/P is not stimulated by the effector FBP in contrast to the ATP-dependent kinase activity.

Analysis of HprK/P mutants of different Gram-positive bacteria revealed that both ATP-dependent phosphorylation and P_i-dependent dephosphorylation reactions are associated and linked to the Walker A motif, corresponding to the phosphate binding site or P-loop (*7*, *16*−*18*). The comparison of the crystal structure of the free HprK/P with the crystal structures of P-Ser-HPr−HprK/P and HPr−HprK/P complexes inferred the possible mechanisms of phosphorylation and dephosphorylation and gave some clues about some important residues forming the active site (*11*). In particular, Asp177 (*B. subtilis* amino acid numbering) was proposed to be the catalytic residue which deprotonates Ser46 in the phosphorylation reaction and acts as an acid catalyst in the dephosphorylation reaction (*7*, *11*).

Finally, divalent cations are required for both activities of HprK/P as confirmed by several biochemical studies (*14*, *19*). Indeed, investigation of the *L. casei* HprK/P crystal structure in complex with HPr or P-Ser-HPr demonstrated that it contains one Ca^{2+} in the active site at the position usually occupied by Mg^{2+} in other P-loop-containing proteins (*11*). However, up to now, all the HprK/P structures were determined with no bound nucleotide or FBP, and no data are available to help us understand, at the molecular level, their structural influence on HprK/P activities. Recently, new findings showed that the hexamer of HprK/P dissociates at high pH (pH 9.0), and the authors suggested that this dissociation could be related to a switch between the two activities; the dephosphorylation activity would be mainly supported by the hexameric form, whereas the kinase activity would reside in the trimeric form of HprK/P (*19*).

In this paper, using the recent structural data and the evidence that HprK/P uses a new phosphate donor, namely, PP_i (*11*, *15*), we describe new information concerning the functioning of this atypical bifunctional enzyme. We showed first that HprK/P displays a strong positive cooperativity for the binding of PP_i to the nucleotide binding site. Furthermore, we demonstrated that proteins with a mutation in the P-loop have lost simultaneously both types of kinases (ATP-dependent and PP_i-dependent) and phosphorolysis activities. With regard to the signature motif, we determined that it is mostly involved in the PP_i-dependent kinase reaction and in the phosphorylase reaction since single-point mutations in this signature of HprK/P do not significantly affect the ATP-dependent kinase activity. Then, characterizing HprK/P H138A and H171R mutations, we showed that His138 is not a catalytic residue but is rather involved in interactions with the magnesium. The role of His171 seems to be the stabilization of the active conformation(s) of HprK/P. Finally, we investigated the influence of FBP on HprK/P activity, and we propose a mechanism of phosphorylation stimulated by this compound.

EXPERIMENTAL PROCEDURES

Reagents. Mant-GTP was purchased from Molecular Probes. ATP and FBP were purchased from Sigma.

Site-Directed Mutagenesis. Plasmid pAG4 (*2*) was used as a template for introduction of point mutations into the *hprK* gene by PCR (*20*), and the presence of the correct mutations was checked by DNA sequencing.

Protein Purification. $HPr(His)_6$ and $HprK/P(His)_6$ wild-type and mutant proteins were purified on Ni−NTA agarose columns as previously described (*1*, *2*).

Limited Trypsin Digestion. Wild-type or mutant HprK/P proteins (6 μg) were digested by trypsin as previously described (*7*) before they were applied to a 15% SDS−PAGE gel.

Gel Filtration. Wild-type and mutant HprK/P proteins (200 μg) were loaded on a Superose 12 HR 10/30 column from Pharmacia and eluted at a rate of 0.3 mL/min with a 50 mM Tris-HCl (pH 7.4), 150 mM NaCl buffer. The column was previously calibrated with molecular mass markers.

Protein Activity Assays. A 10 μL ATP-dependent phosphorylation mixture contained 30 μM HPr, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 0.5 mM ATP, with or without 5 mM FBP and HprK/P (0.5−4 μM). A 10 μL PP_i-dependent phosphorylation reaction mixture contained 30 μM HPr, 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 4 mM PP_i (potassium salt, K_2PO_4), and HprK/P (0.5−4 μM). A 10 μL dephosphorylation reaction mixture contained 30 μM P-Ser-HPr, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM P_i (potassium salt, $K_4P_2O_7$), and HprK/P (1 μM). The mixtures were incubated at 37 °C for 10 min for the kinase reactions and for 60 min for the dephosphorylation reaction. All experiments were stopped by addition of 100 mM EDTA, and the samples were loaded onto a nondenaturing 12.5% polyacrylamide gel as described previously (*13*). After electrophoresis, the gel was stained with Coomassie Blue and scanned in a GS 800 densitometer from Bio-Rad. The results were analyzed with Quantity One software (Bio-Rad).

Fluorescence Measurements. All experiments were performed at 30 °C using a SAFAS flx-Xenius 5117 spectrofluorimeter. All spectra were corrected for buffer fluorescence. Fluorescence measurements were carried out as previously described (*13*).

RESULTS

ATP or PP_i as a Substrate of the Phosphorylation Reaction. It was recently shown that PP_i was the product of HPr dephosphorylation and can be used as a phosphate donor for HPr phosphorylation, whether FBP was present (*15*). The kinetic parameters were already reported (*15*), but no cooperativity was mentioned. To determine if the binding of PP_i to HprK/P follows a cooperativity mechanism, we analyzed HPr phosphorylation in the presence of PP_i (Figure 1A,B). The sigmoidal curve in Figure 1B reveals that the binding of PP_i to HprK/P follows a strong positive cooperativity mechanism. Since the binding of ATP induces a change in conformation around the unique Trp residue present in the sequence of *B. subtilis* HprK/P (*13*), we tried to use the same technique to assess the direct binding of PP_i or P_i to the enzyme. However, no change in intrinsic fluorescence due to the addition of PP_i or P_i was detected. This suggests that the conformational change is induced by the binding of the base−sugar part of the nucleotide outside the P-loop, close to the central loop. Therefore, experiments were carried out to analyze the possible competition between PP_i and phosphates of the Mant-GTP for the P-loop (*7*). We chose to use Mant-GTP as the affinity of HprK/P for this

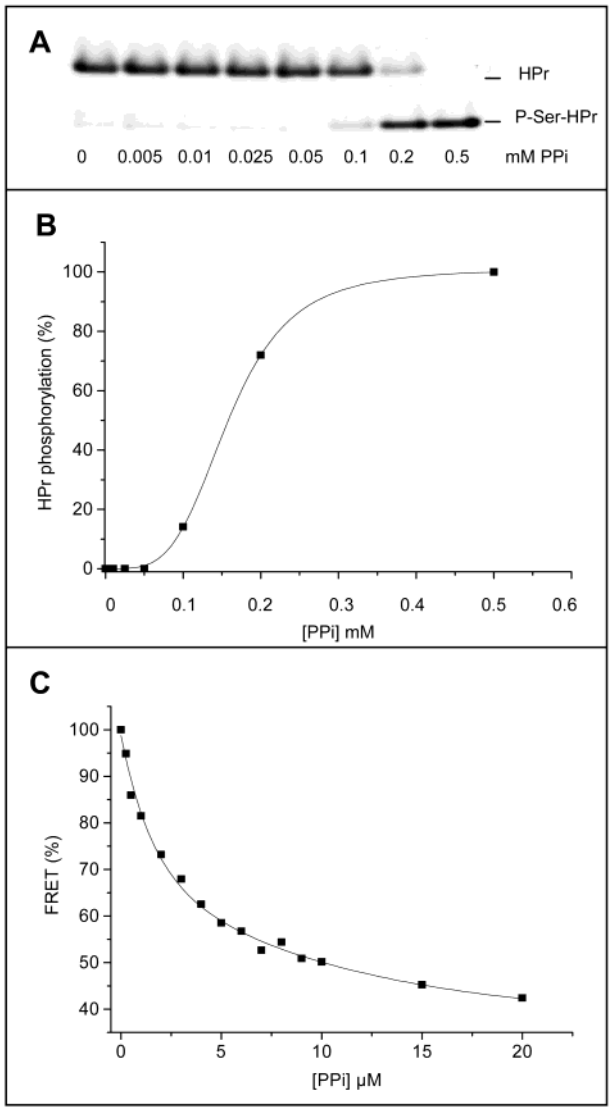

FIGURE 1: Interaction of HprK/P with PP_i. (A) Increasing concentrations of PP_i were added in a PP_i-dependent phosphorylation mixture [30 μM HPr, 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, and 3 μM HprK/P], and the enzymatic assay was incubated for 10 min at 37 °C. The phosphorylation reaction was stopped by adding 100 mM EDTA to the assay mixtures. (B) The amounts of phosphorylated and nonphosphorylated HPr separated on a 12.5% nondenaturing gel were quantified. The percentages of phosphorylated HPr were plotted as a function of PP_i concentration. (C) HprK/P (0.6 μM) was incubated in a 2 mL cuvette containing 25 mM Hepes/KOH (pH 8.0), 1 mM $MgCl_2$, and 10 μM Mant-GTP for 2 min at 30 °C, and increasing concentrations of PP_i were added. After excitation of the unique tryptophan of HprK/P at 295 nm, the fluorescence emission was recorded between 310 and 510 nm, 2 min after each addition of PP_i. The extent of fluorescence resonance energy transfer (FRET) obtained by integrating the emission peak around 430 nm characteristic of the fluorescence of Mant-GTP bound to HprK/P was plotted as a function of PP_i concentration.

compound is very high (*7*, *13*). The binding of 10 μM Mant-GTP produced a drastic quenching of the fluorescence emission spectrum of the Trp residue of HprK/P. Simultaneously, a new peak of fluorescence developed, centered at approximately 430 nm, which is indicative of the fluorescence resonance energy transfer (FRET) between the Trp residue and the Mant derivative (data not shown) (*7*). Addition of increasing concentrations of PP_i progressively relieved the quenching of the Trp fluorescence and concomitantly reduced the extent of fluorescence resonance energy transfer (Figure 1C). This shows that the addition of PP_i chased off the Mant-GTP previously bound to HprK/P. These results confirm that there is a direct competition between PP_i and Mant-GTP for the binding to the P-loop of HprK/P.

Construction of HprK/P Mutants. The crystal data obtained with the complex of *L. casei* HprK/P with HPr or P-Ser-HPr from *B. subtilis* (*11*) provided some information about the residues likely to be involved in the catalytic mechanism of phosphorylation and dephosphorylation. For instance, the authors proposed an essential role in both activities of HprK/P for residues Asp177, Lys159, His138, and Arg243 and a possible role of Glu202 and Ser160 in the interactions with the divalent cation (*B. subtilis* amino acid numbering). The importance of Asp177 and its neighbor Asp176 has already been analyzed by site-directed mutagenesis (*7*). To analyze how some of these key residues participate in the different HprK/P activities, site-directed mutagenesis was carried out in two highly conserved regions of the protein, and two other strictly conserved residues, His138 and His171, were also mutated (Figure 2). Six residues of the P-loop motif (Gly153, Ser155, Gly156, Gly158, Lys159, and Ser160) were mutated to Ala, but Lys159 was substituted with an Arg. The Ser residues were also replaced with a Cys at Ser155 or with a Thr at Ser160. Residues G153, G158, K159, and S160 are the canonical residues of the Walker A motif [GxxxxGK(S/T)], while S155 and G156 are specifically conserved in HprK/P. Three residues of the signature motif defined by Reizer et al. (*6*) (Glu202, Arg204, and Gly205) were also mutated to Ala. Site-directed mutagenesis was also carried out for conserved residues with the homologous protein PEPCK (*7*). Hence, His138, proposed to be essential based on the three-dimensional (3D) structures of HprK/P in complex with HPr or P-Ser-HPr (*11*), was mutated to Ala. Finally, the conserved His171, situated near the interface between the two trimers of HprK/P and close to a large spot of uninterpreted density in the crystals of *L. casei* HprK/P (*9*), was replaced with an Arg which is found to be conserved in the homologous protein PEPCK (*7*). After overexpression and purification of the mutant proteins, their stability was checked by limited trypsin digestion (*7*). All mutants were found to be properly folded and as stable as the wild-type protein since they had the same digestion profile as the wild-type HprK/P protein on SDS−PAGE gels (data not shown). Furthermore, all mutants exhibited the same elution profile as the wild-type protein when analyzed by size exclusion chromatography, suggesting a conserved hexameric state (data not shown). All these mutants were tested in several enzymatic assays: ATP-kinase activity in the presence and in the absence of 10 mM FBP, PP_i-kinase activity, and phosphorylase activity as described in Experimental Procedures. The direct binding of the nucleotides or FBP was monitored by the intrinsic fluorescence of the unique Trp235 of HprK/P (*13*). The binding of PP_i or P_i was indirectly detected by measuring the decrease in the extent of FRET between the Trp and the Mant moiety of the Mant-GTP (*7*).

The P-Loop Is Involved in the Two Kinase and Phosphorylase Activities. After determination of the residual activities of the mutants, four amino acids of the P-loop (Gly153, Gly156, Gly158, and Ser160) were found to be essential for all three activities of HprK/P (Table 1). For none of these

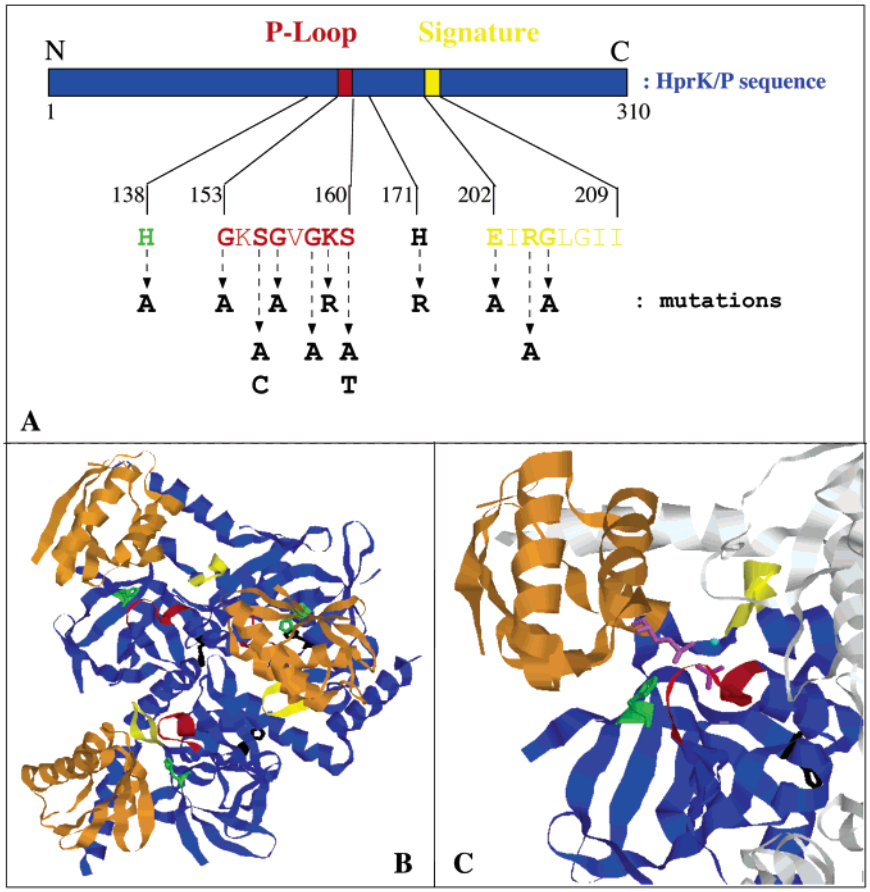

FIGURE 2: Location of the 11 mutated amino acids of HprK/P. (A) Schematic view of the *B. subtilis* HprK/P sequence. The Walker A motif, or P-loop, is shown in red and the signature motif in yellow. The designated substitutions are shown in black for each amino acid. (B) 3D structure of a trimer of HprK/P from *L. casei* (blue) in interaction with its substrate P-Ser-HPr (orange) (PDB entry 1kkm). Residues of the P-loop are colored red. Residues of the signature are colored yellow. His140 (His138 in *B. subtilis* HprK/P) is colored green and His173 (His171 in *B. subtilis* HprK/P) black. (C) Close-up view of the active site of one monomer of *L. casei* HprK/P in interaction with P-Ser-HPr. The phosphorylated Ser46 of HPr is colored purple. The P_i molecule is colored pink and the Ca^{2+} ion cyan.

Table 1: Activities and Kinetic Parameters of the Wild-Type and P-Loop Mutants of HprK/P[a]

	ATP-kinase (%)	ATP-kinase with FBP[b] (%)	PP_i-kinase (%)	phosphorylase (%)	FBP (increase), K_D,[c] F_{max}/F_0	ATP (quenching), K_D, F_{max}/F_0	ATP with FBP[b] (quenching), K_D, F_{max}/F_0	Mant-GTP (FRET), K_D, F_{max}	Mant-GTP with FBP[b] (FRET), K_D, F_{max}
wild type	100	100	100	100	4.2 ± 0.2 mM 1.7 ± 0.02	108 ± 12 μM 0.515 ± 0.01	74 ± 2 μM 0.316 ± 0.003	0.5 ± 0.03 μM 17308 ± 513	1.8 ± 0.1 μM 17070 ± 420
G153A	0	0	0	0	ND	ND	ND	ND	
S155A	81	100	100	81	4.6 ± 0.4 mM 1.45 ± 0.02	129 ± 39 μM 0.469 ± 0.02		0.65 ± 0.03 μM 6439 ± 155	0.66 ± 0.06 μM 12375 ± 402
S155C	0	0	30	0	ND	ND	ND	1 ± 0.1 μM 2174 ± 129	2.5 ± 0.4 μM 6211 ± 419
G156A	0	0	0	0	ND	ND	ND	ND	
G158A	0	0	0	0	ND	ND	ND	ND	
K159R	0	74	59	62	5.8 ± 4 mM 1.17 ± 0.05	ND	59 ± 30 μM 0.953 ± 0.004	0.37 ± 0.07 μM 1959 ± 121	
S160A	0	0	0	0	ND	ND		ND	
S160T	100	100	30	87	2.8 ± 0.2 mM 1.1 ± 0.004	99 ± 25 μM 0.885 ± 0.005		0.7 ± 0.04 μM 6000 ± 152	

[a] Relative enzymatic activities of the mutant proteins, compared to those of wild-type HprK/P, are indicated. The values for FBP and ATP were determined by intrinsic fluorescence and for Mant-GTP by FRET. ND indicates that no variation in the fluorescence signal was detected. [b] Concentrations used in the tests in the presence of FBP: 10 mM for fluorescence experiments and 5 mM for activity measurements. [c] K_D is the affinity constant, and F_{max}/F_0 is the maximum ratio of fluorescence detected.

four mutants were we able to measure the affinity constants of the enzymes for FBP, ATP, or Mant-GTP (Table 1). Ser160 appeared to be essential, but this residue can be replaced efficiently with a Thr residue which restored nearly completely the activity of wild-type HprK/P (Table 1), in agreement with the consensus sequence GxxxxGK(S/T). On the other hand, the mutation of another amino acid of the P-loop, namely Ser155, to Ala had no effect on the activities of the protein, but substitution with a Cys had the opposite effect. Indeed, the mutant HprK/P S155C has lost all

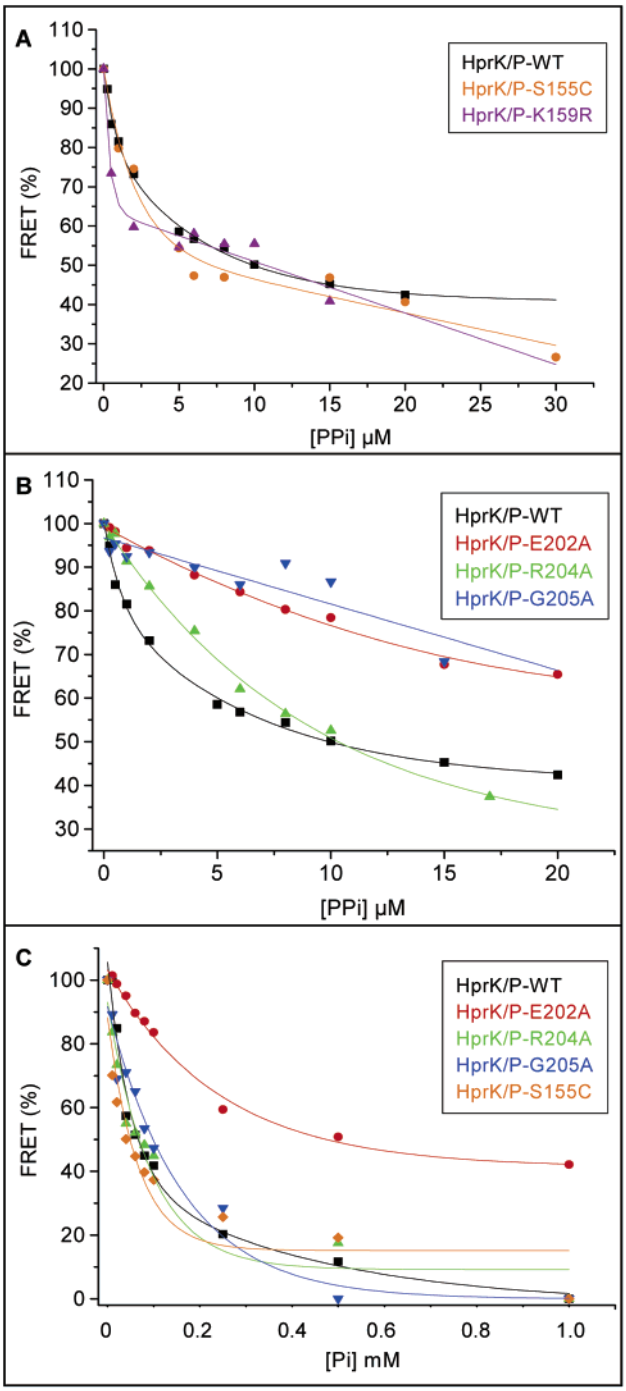

FIGURE 3: Competition of P_i or PP_i for the binding site of Mant-GTP on wild-type or mutant HprK/P. (A) Wild-type or P-loop mutants of HprK/P (0.6 μM) were incubated in a 2 mL cuvette containing 25 mM Hepes/KOH (pH 8.0), 1 mM $MgCl_2$, and 10 μM Mant-GTP for 2 min at 30 °C, and increasing concentrations of PP_i were added. After excitation of the unique tryptophan of HprK/P at 295 nm, the fluorescence emission was recorded between 310 and 510 nm, 2 min after each addition of PP_i. The extent of fluorescence resonance energy transfer (FRET) obtained by integrating the emission peak around 430 nm characteristic of the fluorescence of Mant-GTP bound to HprK/P was plotted as a function of PP_i concentration. (B) The same experiments that are described for panel A were carried out for the signature mutants of HprK/P. (C) Wild-type and mutants of HprK/P (0.6 μM) were incubated in a 2 mL cuvette containing 25 mM Hepes/KOH (pH 8.0), 1 mM $MgCl_2$, and 5 μM Mant-GTP for 2 min at 30 °C, and increasing concentrations of P_i were added. After excitation of the tryptophan of HprK/P at 295 nm, the fluorescence emission was recorded between 310 and 510 nm, 2 min after each addition of P_i. The extent of FRET obtained by integrating the emission peak around 430 nm was plotted as a function of P_i concentration.

activities but conserved a small residual PP_i-kinase activity. We observed that all the proteins mutated in the P-loop are not only affected in their ATP-dependent activity of HprK/P but also affected in their PP_i-dependent phosphorylation reaction and in their phosphorylase reaction, with the only exception being the HprK/P S155C mutant (Table 1).

To investigate HprK/P S155C behavior, we determined the binding parameters of this mutant, as previously described, by measuring the intrinsic fluorescence or the extent of FRET. The effect of PP_i or P_i on Mant-GTP binding is the same for the mutant HprK/P S155C as for the wild-type protein (Figure 3A,C). Surprisingly, we observed by FRET that Mant-GTP was able to bind to HprK/P S155C with an unchanged apparent affinity, while the quenching of intrinsic fluorescence for the binding of ATP or FBP was not detected (Table 1). We also observed that FBP was still able to bind to the enzyme by comparing the binding parameters of Mant-GTP in the absence and presence of 10 mM FBP (Table 1). This mutant might therefore be able to bind NTP or FBP but not to induce the conformational change observed for their binding to the wild-type protein. The substitution of Lys159 with an Arg only slightly modified the activities of HprK/P. Although no activity was detected under the conditions used in the test (Table 1), the ATP-kinase activity was not abolished since it was still detectable with more enzyme (data not shown) or in the presence of 5 mM FBP. This Lys residue is described as being essential in the P-loop motif (*21*) with a catalytic but also structural role (*22*). Nevertheless, the Arg residue can be replaced more or less efficiently with a Lys. We were unable to detect the binding of ATP to the mutant HprK/P K159R, but in the presence of 10 mM FBP, ATP binding was still detectable (Table 1). With regard to the two other mutants, HprK/P S155A and S160T, with normal activity, we were able to determine the kinetic parameters for the binding of FBP, ATP, and Mant-GTP. All the affinity constants that were measured were identical to those of the wild-type protein (Table 1).

Analysis of Mutants of the HprK/P Signature. The residual activities and the kinetic parameters were also measured for the mutants of the signature motif $_{202}$E[LIVM]RG[LIVM]G-[LIVM]$_2$[NDEQ][LIVMF]$_{211}$ defined by Reizer et al. (*6*) and forming the structural capping motif described in the *L. casei* structure (*9*). This study revealed that two of these mutants (HprK/P E202A and G205A) have lost not only the phosphorylase activity but also the PP_i-kinase activity (Table 2). However, they both conserved a normal ATP-dependent kinase activity. We suggest that the PP_i-dependent phosphorylation is the reversible reaction of phosphorolysis or is at least intimately linked with this P_i-dependent dephosphorylation. The respective role of these two residues seems, however, to be different. Indeed, the affinity constant of HprK/P E202A for ATP was increased by at least 6 times compared to that of the wild-type protein but was back to normal in the presence of FBP (Table 2), and the effects of PP_i and P_i on Mant-GTP binding were highly reduced (Figure 3B,C). The affinity of the other mutant, HprK/P G205A, for ATP was 5-fold better than for the wild-type enzyme, but the maximum of fluorescence that was detected was highly reduced (Table 2), reflecting that a modified conformational change took place around the Trp. Gly205 plays an important structural role, allowing a sharp turn in the capping motif. Trp235 is the first residue of a flexible loop close to the

Table 2: Activities and Kinetic Parameters of the Wild-Type and Signature Mutants of HprK/P[a]

	ATP-kinase (%)	ATP-kinase with FBP[b] (%)	PP_i-kinase (%)	phosphorylase (%)	FBP (increase), K_D,[c] F_{max}/F_0	ATP (quenching), K_D, F_{max}/F_0	ATP with FBP[b] (quenching), K_D, F_{max}/F_0
wild type	100	100	100	100	4.2 ± 0.2 mM 1.7 ± 0.02	108 ± 12 μM 0.515 ± 0.009	74 ± 2 μM 0.316 ± 0.03
E202A	80	100	0	0	4.5 ± 0.2 mM 1.76 ± 0.09	668 ± 48 μM 0.736 ± 0.06	77 ± 4 μM 0.463 ± 0.005
R204A	0	29	52	0	5.1 ± 0.6 mM 1.16 ± 0.01	115 ± 7 μM 0.856 ± 0.01	417 ± 81 μM 0.670 ± 0.02
G205A	79	100	0	0	ND	20 ± 6 μM 0.906 ± 0.04	32 ± 9 μM 0.819 ± 0.008

[a] Relative enzymatic activities of the mutant proteins, compared to those of wild-type HprK/P, are indicated. The values for FBP and ATP were determined by intrinsic fluorescence and for Mant-GTP by FRET. ND indicates that no variation in the fluorescence signal was detected. [b] Concentrations used in the tests in the presence of FBP: 10 mM for fluorescence experiments and 5 mM for activity measurements. [c] K_D is the affinity constant, and F_{max}/F_0 is the maximum ratio of fluorescence detected.

capping motif, and mutation G205A may modify the environment of the Trp and its fluorescence. Furthermore, we observed that only the effect of PP_i, and not P_i, on Mant-GTP binding was reduced (Figure 3B,C). These data are in agreement with the loss of the PP_i-kinase activity. Finally, the mutant HprK/P R204A behaves in a manner different from that of the two other mutants of the signature. This mutant has lost, like the two others, the phosphorylase activity but exhibited a reduced ATP-kinase activity and a normal PP_i-kinase activity. The apparent affinity constants determined for ATP and FBP were identical to those for the wild-type protein HprK/P (Table 2), and the competition curves of Mant-GTP binding with P_i or PP_i could be superimposed onto the competition curves for the wild-type enzyme (Figure 3B,C). Surprisingly, the apparent affinity constant for ATP in the presence of 10 mM FBP was increased by 6 times (Table 2). The mutation lowered the stabilization effect of FBP for the ATP conformation. A possible hypothesis may be that Arg204 belongs to the still unknown FBP binding site. Hence, the maximum of fluorescence due to FBP binding is highly reduced, and the binding of ATP is affected by the presence of FBP. Arg204 might be important for the conformational change induced by FBP binding. In light of these results, it seems obvious that the capping motif is involved in the conformational change of HprK/P shifting from one activity to the other.

His138 Is Not a Catalytic Residue. Taking advantage of the *L. casei* HprK/P 3D structures and because the sequence and structure HprK/P are significantly homologous with those of the PEPCK (*7*, *9*, *11*), we carried out the HprK/P H138A substitution to determine its possible involvement in HprK/P activities. In the structure of *L. casei* HprK/P bound to HPr (*11*) and in *Mycoplasma pneumoniae* HprK/P (*12*), His138 interacts with Asp177 which was shown to play a role in the catalysis of phosphate transfer. In the structure of *Staphylococcus xylosus* HprK/P (*10*), His138 adopts an alternative conformation. In the free *L. casei* HprK/P, it is disordered (*9*). We found that this mutant is only slightly affected in the PP_i-kinase activity of HprK/P and not at all in the two others under the conditions that were tested (data not shown). The K_D values for FBP, ATP, and Mant-GTP were effectively identical to the K_D of the wild-type protein [K_D(FBP) = 3.6 mM, K_D(ATP) = 57 μM, and K_D(Mant-GTP) = 0.6 μM]. Therefore, His138 is not a catalytic residue. However, we observed that the ATP-kinase activity was affected when the reaction was not carried out in the presence of saturating concentrations of $MgCl_2$. To compare the wild-type and mutant proteins, 1 μg of protein was incubated at 37 °C (1.5 min for wild-type HprK/P and 10 min for H138A) in the ATP reaction mixture, with increasing $MgCl_2$ concentrations (Figure 4). The HPr-phosphorylated form was separated from the nonphosphorylated form by nondenaturing gels which were then scanned to allow quantification of the protein bands (Figure 4A,B). We observed a distinct difference between the curves (Figure 4C), suggesting that His138 is involved in the interactions with divalent cation in the active site. Ser160 and Glu202 were also described as being involved in the interactions with the divalent cation in the 3D structure of *L. casei* HprK/P. The position of Glu202 is identical to the metal binding site usually observed in P-loop-containing proteins (*5*). However, the apparent affinity of HprK/P E202A protein affected in the reversible phospho-

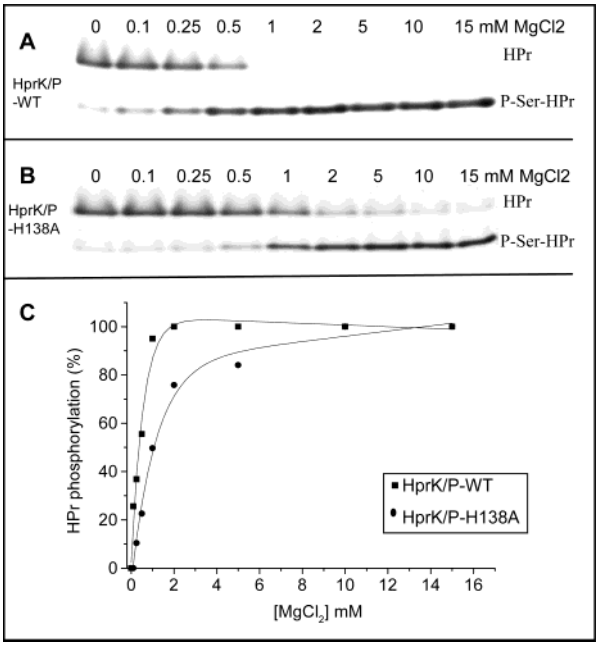

FIGURE 4: Mg^{2+} binding on wild-type HprK/P and HprK/P H138A. Increasing concentrations of $MgCl_2$ were added to ATP-dependent phosphorylation mixtures containing 30 μM HPr, 50 mM Tris-HCl (pH 8.0), 0.5 mM ATP, 5 mM FBP, and 1 μM wild-type HprK/P or H138A. The assays were incubated for 1.5 min for wild-type HprK/P and 10 min for HprK/P H138A at 37 °C, and then stopped by adding 100 mM EDTA. The substrate HPr and the product P-Ser-HPr were separated by nondenaturing gel electrophoresis. The experiments were carried out in parallel for (A) the wild-type protein and for (B) the mutant HprK/P H138A. (C) The percentages of phosphorylated HPr were plotted as a function of $MgCl_2$ concentration for wild-type HprK/P (■) and HprK/P H138A (●).

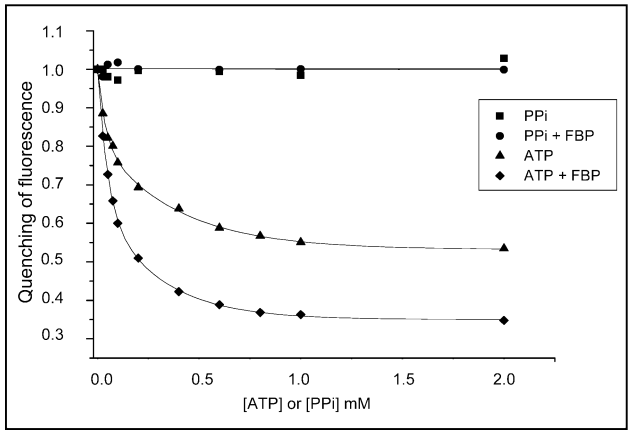

FIGURE 5: Effect of phosphate donors on the HprK/P fluorescence. Increasing amounts of either ATP (▲ and ◆) or PP_i (■ and ●) were added to a 2 mL mixture [25 mM Hepes/KOH (pH 8.0) and 1 mM $MgCl_2$] with (◆ and ●) or without (▲ and ■) 10 mM FBP and 0.6 μM HprK/P preincubated for 2 min at 30 °C, before each addition of phosphate donors. The emission of fluorescence was scanned in the range of 310−380 nm upon excitation at 295 nm of the unique tryptophan of HprK/P. The variations in the fluorescence emission peak due to ligand binding were monitored and plotted as a function of phosphate donor concentration.

rolysis reaction, determined for a range of Mg^{2+} concentrations, was identical to that of the wild-type protein (data not shown). We were unable to determine the apparent affinity of the mutant HprK/P S160A for Mg^{2+} as this enzyme is totally inactive.

His171 Is Implicated in the Stabilization of the Active Conformation(s) of HprK/P. We have also mutated another His of *B. subtilis* HprK/P, His171, which, in the HprK/P hexamer, is located near the interface between two trimers (Figure 2B). This His171 is highly conserved in the sequences of HprK/P from different organisms, and the X-ray structures show that it interacts with the similarly conserved Glu276. The His171 position is also conserved in the sequence of the homologous PEPCK proteins, but an Arg is found instead of a His. We decided to replace His171 with an Arg. Phosphorylation and dephosphorylation tests showed that this residue is essential for the activities of the protein as the mutant HprK/P H171R has lost all activities (data not shown). Since His171 is buried in the core of the hexamer, far from the active site, its role should be at a structural level. Furthermore, the mutant protein was found to be oligomeric by gel-filtration analysis as is the wild-type protein (data not shown), confirming that it is not directly involved in the oligomerization.

Conformational Changes Due to FBP Binding. We studied the effect of FBP on the ATP or PP_i binding to HprK/P by intrinsic fluorescence in the presence or absence of FBP. As previously observed (*13*), we found that FBP modifies the conformational change due to the binding of ATP (Figure 5) without altering the affinity of the enzyme for ATP. This result suggests that FBP stimulates the ATP-dependent phosphorylation of HPr by inducing a highly efficient and specific conformational change in the 3D structure of HprK/P that we were able to observe by the modification of the environment of the unique Trp of the enzyme. In contrast, the binding of PP_i does not induce conformational changes that can be detected by intrinsic fluorescence either in the presence or in the absence of FBP (Figure 5). We propose that the change in conformation induced by the binding of the FBP improves the binding of the base part of ATP and is not needed for the binding of PP_i.

DISCUSSION

The aim of this paper was to provide insights into the functioning of *B. subtilis* HprK/P. We showed that HprK/P displays a strong positive cooperativity for the binding of PP_i to the nucleotide binding site. Furthermore, proteins with a mutation in the P-loop have lost simultaneously all HprK/P activities, whereas residues in the signature sequence are mostly involved in the reversible phosphorolysis. Characterization of the mutant HprK/P H138A revealed that this residue is not a catalytic residue but is involved in interactions with divalent cation. Finally, we propose a mechanism for the stimulation of kinase activity by the allosteric activator, FBP.

Characteristics of PP_i Binding to HprK/P. We first investigated the PP_i-dependent phosphorylation to determine which is the primordial phosphate donor in the HPr phosphorylation. Here, we observed that PP_i, like ATP, binds to the P-loop of HprK/P with a strong positive cooperativity. Furthermore, the apparent affinities for the binding of ATP or PP_i to the enzyme, described in a previous paper (*15*), are compatible with the intracellular concentrations of PP_i, FBP, and NTP in the presence or absence of glucose (*15*). Thus, the enzymatic properties of HprK/P and the physiological conditions in the *B. subtilis* cells are favorable for both kinds of phosphorylation, even if ATP and PP_i bind to the same site. The question of the preference between the two primordial high-energy compounds, ATP and PP_i, has already been discussed for other enzymes such as the PP_i-dependent phosphofructokinase (PFK) (*23*). This enzyme catalyzes the transfer of phosphate from PP_i to fructose 6-phosphate to yield FBP and P_i. The authors showed that this enzyme displays a strong preference for PP_i over ATP. The PFK was proposed to originally evolve from an enzyme that used ATP as the phosphoryl donor, and the utilization of PP_i happened as an evolving process that occurred later during evolution. In the case of HprK/P, Mijakovic and al. (*15*) proposed that PP_i-dependent phosphorylation of HPr and dephosphorylation by phosphorolysis could be a relic of early life and the use of ATP as phosphoryl donor in the presence of FBP was gained during evolution. Furthermore, we tried to construct mutant proteins of HprK/P which carry only one kinase activity and with a normal phosphorylase activity. We have performed a mutational analysis of the conserved regions of HprK/P: the P-loop and the signature. We did not obtain proteins inactivated in only one kinase activity. Indeed, numbers of the P-loop residues are essential for all HprK/P activities, whereas the residues of the signature seem to be mostly implicated in the phosphorolysis and the PP_i-kinase activity, suggesting that either the PP_i phosphorylation is the reverse reaction of the phosphorolysis or these two reactions are highly connected.

P-Loop Mutants of HprK/P. We observed that several residues of the P-loop are essential for both kinase and phosphorylase activities of HprK/P: Gly153, Gly156, Gly158, and Ser160. Furthermore, the P-loop appeared to be the binding site of substrates ATP, PP_i, and P_i, explaining why the three activities are highly connected. The role of Gly156

was studied in the PEPCK. In this enzyme, the main chain NH group of Gly253 (corresponding to Gly156 of HprK/P) was proposed to stabilize the transition state for P−O bond cleavage in phosphoryl transfer (*24*). Since HprK/P and PEPCK belong to the same family of phosphotransferases (*7*, *8*), it is relevant that this Gly residue, conserved in all PEPCKs, is also conserved in this position in the P-loop sequence GXX**G**XGKSE in all HprK/Ps described so far. Surprisingly, the substitution of Lys159 with an Arg only slightly modified the activities of HprK/P. In several other studies, the substitution of the Lys of the P-loop with the conservative residue Arg has been found to be insufficient to conserve a residual activity (*18*, *25*). This Lys residue is therefore described as being essential in the P-loop motif (*25*, *26*). In the PEPCK, a crucial role of this equivalent Lys was proposed in the phosphoryl transfer (*21*, *24*). The lysine interacts with both β- and γ-phosphoryl groups of ATP in the 3D structures of the PEPCK (*27*) and of the HprK/P, and the side chains of this conserved Lys and the following Ser of the P-loop interact with the P_i molecule (*9*). In the mutant protein HprK/P K159R, the Arg residue seems to replace more or less efficiently this Lys. This result could be explained by the fact that the positive charge, which is a major requirement for NTP binding (*24*, *28*), is conserved by the amino acid substitutions. Furthermore, we observed that this mutant HprK/P K159R is still able to bind NTP but not to induce the conformational change due to ATP binding on the wild-type protein. The Ser residue in the P-loop following the conserved Lys has been described as being important for Mg^{2+} binding (*5*, *29*). The substitution of Ser160 with an Ala completely abolishes all activities of HprK/P. It was already shown that the Mg^{2+} was required for HprK/P activities and for nucleotide and HPr or Crh binding (*14*). If this modified protein is no longer able to bind Mg^{2+}, it can bind neither nucleotides nor protein substrates (Table 1). The substitution with a Thr restores a HprK/P with normal activities. In the PEPCK and many other P-loop-containing proteins, the residue following the conserved Lys in the P-loop is a Thr which was shown to contribute an oxygen atom to the Mg^{2+} coordination sphere (*27*). Gly153, Gly158, Ser155, and Ser160 of HprK/P were mutated previously (*16*, *17*), and similar results were found in terms of residual activities; all amino acids were found to be essential for both activities except Ser155. Here we also showed that substitution of Ser155 with an Ala had no effect on the activities, while the substitution with a Cys residue nearly abolished all activities. The only residual activity was the PP_i-kinase activity. The X-ray structures of HprK/Ps (*10*, *11*) show that Ser155 H-bonds with the transferred phosphate. In the free *L. casei* structure (*9*), it H-bonds to the catalytic Asp177 that adopts an alternative conformation compared to the other structures. The substitution with an Ala abolishes these interactions, while they would be maintained with the Cys mutation. The results of these mutations therefore suggest that these interactions are not essential for the activity of the enzyme. Besides, the position is not strictly conserved among HprK/Ps, an Ala being observed in *Xylella fastidiosa*.

Mutants of the HprK/P Signature. Whereas we were unable to find mutants only inactivated for one of the two kinase activities, we were able to identify mutants only inactivated for the phosphorylase activity. All the mutants of the signature motif, HprK/P E202A, R204A, and G205A, were indeed inactivated for the phosphorylase activity. The mutants HprK/P E202A and G205A conserved a normal ATP-kinase activity but have lost the PP_i-kinase activity. The phosphorolysis conformation is conserved in the PP_i-dependent phosphorylation, but the ATP-dependent kinase activity needs a conformational change. The mutants HprK/P E202A and G205A destabilize the P_i conformation but not the ATP conformation. The P_i conformation needs the integrity of the signature sequence forming the structural capping motif (*9*), while the ATP conformation is less sensitive to mutations in this region. We also found that the apparent affinity of the mutant HprK/P E202A for ATP was 6-fold higher than the affinity of the wild-type protein but was back to the normal value in the presence of FBP. This residue is situated at the surface of the protein, close to the active site. In the structure of HprK/P in complex with P-Ser-HPr (*11*), it interacts with the bound cation and with the side chain of the conserved Arg from the flexible loop. Glu202 seems, therefore, to be implicated in the stabilization of the flexible loop in the P_i conformation. The mutated protein has a higher affinity for nucleotides than for P_i or PP_i, which is in agreement with the hypothesis that the capping motif is important for the stabilization of the P_i conformation. The other signature mutant, HprK/P R204A, conserved a normal PP_i-kinase activity but was strongly affected in the ATP-kinase activity. In one subunit of the *S. xylosus* HprK/P structure (*10*), the side chain of Arg204 interacts with both phosphates present in the active site. In the other structures, it is either disordered or without interactions. These observations suggest that Arg204 could be implicated in the stabilization of the ATP conformation. Arg204 has previously been mutated (*18*) to Lys instead of Ala, and the residual activities detected in this case were a normal ATP-kinase activity and a complete loss of the phosphorylase activity. The Lys residue seems to efficiently replace the Arg for its potential role in the stabilization of the ATP conformation but not for its role in the dephosphorylation reaction.

H138 Is Not a Catalytic Residue but Interacts with the Divalent Cation. In several phosphorylation reactions, the nucleotide binds and donates a phosphoryl group to the enzyme, usually a His residue, generating a phosphoenzyme intermediate. The enzyme-bound phosphoryl group is in turn donated to a second substrate, yielding the final product (*21*). The His138 residue preceding the P-loop, conserved in HprK/P and PEPCK, occupies the same spatial position in the two structures. In PEPCK, this residue is involved in direct and water-mediated interactions with the γ-phosphate of ATP and appears to be functionally important (*30*, *31*). However, the substitution of H138 with an Ala does not affect the kinetic parameters which are identical to those of the wild-type enzyme for FBP, ATP, and Mant-GTP; however, the kinase activities are slightly affected when not tested at saturating concentrations of Mg^{2+}. Therefore, this residue is not an essential residue as predicted by the structural data (*9*, *11*, *32*). Nevertheless, we found that this His is involved in the interaction with the Mg^{2+}. Because His138 does not interact with the cation observed in the active site of *L. casei* HprK/P, this may indicate that a second cation could be involved in the HprK/P mechanism, as suggested previously (*5*, *14*). The comparable His in the *Escherichia coli* PEPCK (His233) has also been shown to

be important for Mn^{2+} binding (*31*), the second divalent cation found in the 3D structure of the PEPCK.

Role of FBP in the ATP-Kinase Activity Stimulation. FBP is an activator of the ATP-kinase activity, but no effect on the PP_i-kinase activity was detected (*15*). On one hand, we previously observed that the binding of ATP induces a change in the intrinsic fluorescence which corresponds to a change in conformation around the unique Trp residue present in the sequence of HprK/P (*13*). On the other hand, we did not detect any change in intrinsic fluorescence due to the addition of PP_i. Furthermore, the mutant HprK/P S155C was found to be inactive for all activities except for the PP_i-kinase activity, and conformational changes around the unique Trp of this mutant protein due to the binding of ATP or FBP were not detected. We suggest that HprK/P can bind PP_i without inducing the conformational change near Trp235, whereas for the larger molecule like ATP, this conformational change is needed to open the active site. We propose that the change in conformation induced by the binding of the FBP allows the binding of ATP. This hypothesis is supported by the structural data despite the fact that, up to now, all the HprK/P structures that have been obtained do not contain either ATP or FBP. However, the modeling of an ATP molecule in the active site of HprK/P is impossible without a conformational change in the loop, called the central loop (residues 262−267 in *B. subtilis*), from a second subunit and situated above the active site (*32*). Several mutations affecting the central loop specifically lowered the phosphorylase but not the kinase activity of HprK/P from *L. casei* and *B. subtilis* (*17*). Therefore, FBP would stimulate the ATP-kinase activity by inducing a conformational change in the protein that will move away the central loop and open the active site. The binding of ATP would initiate the same change but less efficiently as shown by the quenching of fluorescence for ATP with or without FBP. This hypothesis is in agreement with the results obtained for the mutant HprK/P R204A. Thus, the conformational change due to FBP binding is reduced for this mutant, and the affinity for ATP is reduced in the presence of FBP. Arg204 seems to be important for an efficient conformational change induced by FBP and might be located in the still unknown FBP binding site. It has previously been proposed that the positive cooperativity mechanism of FBP binding was more consistent with a concerted symmetry model between two populations of oligomers than a change in the affinity for FBP binding sites of the same oligomer (*13*). Several enzymes involved in sugar utilization are allosterically regulated by the FBP. The structures of enzymes allosterically regulated by FBP and crystallized in the presence of FBP show that it is difficult to predict the FBP binding site and the mechanism of FBP regulation. In the case of pyruvate kinase, the 3D structures obtained in the presence and absence of FBP clearly demonstrate that the allosteric activation is limited to small movements of a limited number of side chains involved in the binding of substrate and of metal ions. The consequence is an increase in the affinity for the PEP substrate. The 3D structures of *E. coli* glycerol kinase (GK) in the presence and absence of FBP revealed a most unusual type of FBP binding site formed between two Gly-Arg loops where one half of the binding site is donated by each monomer at the regulatory interface (*33*). FBP is an inhibitor of GK and was proposed to promote dimer−tetramer assembly and to inactivate the tetramers.

Role of HprK/P Oligomerization. In a recent paper (*19*), the authors proposed that the switch between the kinase and the phosphorylase activity was regulated by pH and the oligomeric state of the enzyme. The kinase activity would be predominant when the pH of the cell is ~8.0 and would be carried out by the dimer or trimer of HprK/P. However, under starvation conditions, the pH of the cell would be ~6.0, which favors the phosphorylase activity carried out by the hexamer. We analyzed all mutants by gel filtration and found that all of them were hexameric. The experiments were carried out in a Tris buffer at neutral pH (7.4), which would favor the dimeric form of the enzyme and the kinase activity. All the mutants, even those with only a kinase activity which were expected to be dimeric or trimeric, were also oligomeric like wild-type HprK/P. It seems therefore that the regulation of the switch between the kinase and the phosphorylase activity is more complex that the pH-dependent association or dissociation of the hexamer. Furthermore, we observed that the PP_i substrate binds to the enzyme HprK/P with a positive cooperativity. This positive cooperativity has also been observed for the other substrates (ATP, P_i, and HPr) and the effector FBP of HprK/P (*13*, *19*). Therefore, it would be tempting to speculate that the mechanism of positive cooperativity for the binding of the substrates and effector could be explained by a change in the oligomerization state of HprK/P. However, for the moment, only the conformational change induced by the binding of FBP and ATP has been characterized. It has been linked to a change near the flexible loop containing the unique Trp235 of HprK/P but has not yet been linked to a change in the oligomerization state.

ACKNOWLEDGMENT

We are grateful to Cécile Lecampion for technical assistance and to Josef Deutscher for helpful discussions. We thank the reviewers for improving the English version of the manuscript.

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BI034405I