The HPr kinase from Bacillus subtilis is a homo-oligomeric enzyme which exhibits strong positive cooperativity for nucleotide and fructose 1,6-bisphosphate binding

Jean-Michel Jault, Sonia Fieulaine, Sylvie Nessler, Philippe Gonzalo, Attilio Di Pietro, Josef J. Deutscher, Anne Galinier

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 3, Issue of January 21, pp. 1773–1780, 2000

Printed in U.S.A.

The HPr Kinase from *Bacillus subtilis* Is a Homo-oligomeric Enzyme Which Exhibits Strong Positive Cooperativity for Nucleotide and Fructose 1,6-Bisphosphate Binding*

(Received for publication, October 4, 1999)

Jean-Michel Jault‡, Sonia Fieulaine§, Sylvie Nessler§, Philippe Gonzalo‡, Attilio Di Pietro‡, Josef Deutscher¶, and Anne Galinier‡‖

From the ‡Institut de Biologie et Chimie des Protéines, UPR 412 CNRS, 69367 Lyon Cedex 07, France, the §Laboratoire d'Enzymologie et de Biochimie Structurales, UPR 9063 CNRS, bât. 34, 91198 Gif-sur-Yvette, France, and the ¶Laboratoire de Génétique des Microorganismes, INRA and CNRS ERS-567, 78850 Thiverval-Grignon, France

Carbon catabolite repression allows bacteria to rapidly alter the expression of catabolic genes in response to the availability of metabolizable carbon sources. In *Bacillus subtilis*, this phenomenon is controlled by the HPr kinase (HprK) that catalyzes ATP-dependent phosphorylation of either HPr (histidine containing protein) or Crh (catabolite repression HPr) on residue Ser-46. We report here that *B. subtilis* HprK forms homo-oligomers constituted most likely of eight subunits. Related to this complex structure, the enzyme displays strong positive cooperativity for the binding of its allosteric activator, fructose 1,6-bisphosphate, as evidenced by either kinetics of its phosphorylation activity or the intrinsic fluorescence properties of its unique tryptophan residue, Trp-235. It is further shown that activation of HPr phosphorylation by fructose 1,6-bisphosphate essentially occurs at low ATP and enzyme concentrations. A positive cooperativity was also detected for the binding of natural nucleotides or their 2′(3′)-*N*-methylanthraniloyl derivatives, in either phosphorylation or fluorescence experiments. Most interestingly, quenching of the HprK tryptophan fluorescence by using either iodide or acrylamide revealed a heterogeneity of tryptophan residues within the population of oligomers, suggesting that the enzyme exists in two different conformations. This result suggests a concerted-symmetry model for the catalytic mechanism of positive cooperativity displayed by HprK.

In bacteria, the histidine-containing protein (HPr)[1] participates in the phosphotransferase system-catalyzed transport and phosphorylation of carbohydrates (1). Being part of a protein phosphorylation chain, HPr is phosphorylated by enzyme I at His-15, in the presence of phosphoenolpyruvate (2), and transfers the phosphoryl group to a sugar-specific enzyme IIA. In Gram-positive bacteria, the phosphoryl-carrier protein HPr is also phosphorylated at a regulatory serine (Ser-46), by the HPr kinase (HprK) in the presence of ATP (3–8). This ATP-dependent phosphorylation of HPr mediated by HprK regulates expression of several catabolic genes and therefore plays a central role in carbon catabolite repression (9). A recently discovered HPr-like protein of *Bacillus subtilis*, catabolite repression HPr (Crh) (10), is likewise phosphorylated by HprK at Ser-46 and involved too in carbon catabolite repression (11). In *B. subtilis*, phosphorylation by HprK of either HPr or Crh was shown to be stimulated by fructose 1,6-bisphosphate (FBP) (5, 6, 10). Signal transduction in carbon catabolite repression continues with a phosphorylation-mediated protein-protein interaction between HPr or Crh and the transcriptional repressor/activator CcpA (catabolite control protein A) (12, 13). The ATP-dependent phosphorylation at Ser-46 is a prerequisite for the interaction of either HPr or Crh with CcpA. The resulting protein complexes, CcpA·Ser(P)-HPr or CcpA·Ser(P)-Crh, specifically interact with an operator site called catabolite responsive element (11, 14–16).

Although HPr kinase activity was detected 15 years ago in several Gram-positive bacteria (3, 4) *hprK*, the gene encoding HprK, was only recently identified and cloned during the *B. subtilis* sequencing program. Preliminary characterization of the purified recombinant *B. subtilis* HprK showed that it was indeed able to specifically phosphorylate HPr or Crh (5, 6). Sequence comparison revealed that this enzyme is found not only in most Gram-positive bacteria, but also in some pathogenic Gram-negative bacteria like *Bordetella pertussis, Treponema pallidum,* and *Neisseriae*. The HprKs constitute a new family of protein kinases which do not contain the domain structure typical for eukaryotic serine/threonine/tyrosine kinases (17, 18). Instead, an ATP-binding motif, called the P-loop (or A-motif) and typically found in some nucleotide-binding proteins such as F1-ATPase or $p21^{ras}$ (19, 20), has been detected in the primary structure of this new class of protein kinases. Since disruption of the *hprK* gene leads to severe growth defects of the *B. subtilis* mutant strain, HprK might constitute a potential target for new antimicrobial agents in pathogenic bacteria (5).

Recent experiments showed that HprK is a bifunctional enzyme which also possesses Ser(P)-HPr phosphatase activity (8). The molecular switch between the phosphatase and the kinase activities appears to be controlled by the presence of low molecular weight effectors. In the presence of FBP and ATP, the kinase activity of HprK is predominant, whereas the phosphatase activity becomes prevalent when the concentration of inorganic phosphate rises (8).

Tryptophan fluorescence provides a very sensitive and widely used tool to monitor the binding of effectors, especially

* This work was supported by the CNRS, the Université de Lyon, and the Université d'Orsay. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Institut de Biologie et Chimie des Protéines, UPR 412 CNRS, 7 Passage du Vercors, F-69367 Lyon Cedex 07, France. E-mail: a.galinier@ibcp.fr; Tel.: 33-472722679; Fax: 33-472722601.

[1] The abbreviations used are: HPr, histidine containing protein; FBP, fructose 1,6-bisphosphate; CcpA, catabolite control protein A; Crh, catabolite repression HPr; HprK, HPr kinase; PAGE, polyacrylamide gel electrophoresis; Mant, 2′(3′)-*N*-methylanthraniloyl; Bis-Tris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

nucleotides and their derivatives, to numerous nucleotide-binding proteins (21–25). Since the molecular mechanism underlying HPr phosphorylation by the recently discovered HprK was poorly understood, the binding properties of HprK were first investigated by following the variation of its intrinsic tryptophan fluorescence in the presence of different effectors. Our preliminary results suggested that HprK can bind nucleotides or FBP in a cooperative manner and a thorough characterization of its kinetic properties was therefore carried out. This report describes the results obtained from both biochemical and biophysical experiments, and it unambiguously shows that HprK is a homo-oligomeric enzyme which exists in two different conformations. This latter property leads most likely to the positive cooperativity mechanism reported here for the binding of either nucleotides or FBP. Furthermore, it is shown that the enhancement of HPr phosphorylation by FBP essentially occurs at low concentrations of both ATP and HprK.

EXPERIMENTAL PROCEDURES

Reagents—*N*-Methylanthraniloyl (Mant) derivatives of nucleotides were prepared by reaction of nucleotides with *N*-methylisatoic anhydride as described by Hiratsuka (26) except that the obtained derivatives were purified on DEAE-cellulose column (27) by elution with a linear gradient of 10 to 800 mM triethylammonium bicarbonate. The fluorescent nucleotides eluted far after the unreacted nucleotides and the excess of *N*-methylisatoic acid. The pooled fractions were dried under vacuum and remaining triethylamine was removed by three successive additions and evaporations of methanol. Purity and homogeneity of the products were tested by both thin-layer chromatography according to Ref. 26 and reverse-phase high performance liquid chromatography using a C-18 column and elution with a 0 to 60% gradient of acetonitrile in 50 mM potassium phosphate, pH 6.0. The concentration of fluorescent nucleotide was determined by UV absorbance at 255 nm for MantADP (ϵ = 23,300 M^{-1} cm^{-1}) and MantGDP (ϵ = 22,600 M^{-1} cm^{-1}).

Protein Purification—HPr$(His)_6$ and HprK$(His)_6$ were purified on Ni-NTA-agarose columns as described previously (5, 10). Proteins were stored at −80 °C in 20 mM ammonium bicarbonate.

Gel Filtration—Size exclusion chromatography experiments were performed on a Superdex 200 HR 10/30 FPLC column (Amersham Pharmacia Biotech). In all experiments, the equilibration buffer contained 20 mM Bis-Tris propane, pH 7.0, and 200 mM NaCl. The samples were centrifuged for 10 min at 15,000 rpm at 4 °C prior to loading 500-μl aliquots onto the column at a protein concentration of 170 μM. The system was calibrated under the same conditions using the Bio-Rad gel filtration standard kit. Proteins present in the eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis.

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 centerpieces. Radial scans of absorbance at 280 nm were performed with the sample buffer used as blank. Sedimentation-velocity experiments were performed at 30,000 rpm and 4 °C. Apparent sedimentation coefficients of the species present in the cell were obtained by the time derivative method of W. Stafford (28) and the SVEDBERG program of Philo (29). Equilibrium-sedimentation centrifugations were carried out at 5,600, 8,000, and 12,000 rpm at 4 °C. The high molecular weight aggregates that accumulated at the bottom of the cells were not taken into account. Determination of the molecular mass was performed with the software package provided by Beckman. Data were analyzed for average molecular mass in terms of a single homogeneous species according to the equation: $A_r = A_0 \exp [H M (x^2 - x_0^2)] + E$ with $H = (1 - v_{bar} \rho) \omega^2/2 RT$, where A_r is the absorbance at radius x, A_0 the absorbance at a reference radius x_0, v_{bar} the partial specific volume of HprK estimated from its sequence (0.735 ml g^{-1} at 4 °C), ρ the solvent density (1.009 g ml^{-1}), ω the angular velocity, R the gas constant, T the absolute temperature, M the molecular mass, and E the baseline offset.

Protein Phosphorylation—Phosphorylation of HPr$(His)_6$ by the HprK$(His)_6$ was carried out as described previously (5). A typical 20-μl phosphorylation mixture contained 30 μM purified HPr$(His)_6$, 350 nM purified HprK$(His)_6$, 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, and 50 μM ATP, unless otherwise stated in the figure legend. The reaction mixture was incubated for the time specified in the figure legends. In all cases, a kinetic experiment was performed under the same conditions to ensure that an initial rate of phosphorylation was maintained during the time interval chosen. The phosphorylation reaction was stopped by adding 100 mM EDTA to the assay mixtures before loading the samples onto a nondenaturing 12.5% polyacrylamide gel. On this type of gels, phosphorylated HPr is well separated from the unphosphorylated protein (30). After electrophoresis, the gel was stained with Coomassie Blue and scanned in a Densitometer SI (Molecular Dynamics); results were analyzed with the ImagQuant V1.2 software (Molecular Dynamics). For each sample, the percent of Ser(P)-HPr was automatically calculated by integrating the area of the lower band relatively to that of the upper band, corresponding to phosphorylated and unphosphorylated HPr, respectively.

Fluorescence Measurements—All experiments were performed at 25 ± 0.1 °C using a Photon Technology International Quanta Master I spectrofluorometer. The measurements were automatically corrected for intensity fluctuation in lamp emission. All spectra were corrected for buffer fluorescence. Fluorescence measurements were routinely carried out after dilution of HprK (1 μM final concentration) and equilibration for 10 min in 2 ml of buffer containing 25 mM Hepes/KOH, pH 8.2, and 0.1 mM EDTA (except where stated otherwise). Increasing concentrations of nucleotides, Mant-derivatives, or FBP were then added and the emission fluorescence was scanned in the range of either 310–380 nm for nucleotides or FBP or 310–530 nm for Mant-derivatives, upon excitation at 295 nm. Binding of ligands was monitored by the variation of tryptophan-intrinsic fluorescence of HprK (between 310 and 380 nm) produced after addition of increasing concentrations of effectors, and corrections for both the variation of volume and the inner-filter effect of the ligands were performed under the same conditions by using *N*-acetyltryptophanamide. Fluorescence resonance-energy transfer between tryptophan residues of HprK and bound Mant-nucleotide derivatives was monitored by the appearance of a fluorescence emission peak between 400 and 530 nm, characteristic of bound nucleotide analogues. Peak integration was carried out at each ligand concentration with the Felix 1.21 software (Photon Technology International) and the observed changes in fluorescence intensity or fluorescence resonance-energy transfer were used for the calculation of ligand affinity. Curve fitting of the data was performed using either the Graphit 2.11 software (Erithacus Software) for the monophasic binding of effectors as described in Ref. 31, or the MacCurvefit 1.0.8 software for the cooperative binding of effectors.

Quenching experiments in the presence of either potassium iodide or acrylamide were performed by successive additions of aliquots from concentrated stock solutions. The potassium iodide stock solution also contained 0.1 mM potassium thiosulfate in order to prevent I_3^- formation, which would quench tryptophan fluorescence emission (32). A control experiment conducted with similar concentrations of KCl indicated that ionic strength did not significantly modify the fluorescence emission of HprK. In the case of acrylamide, the inner-filter effect at the emission wavelength was corrected according to Calhoun *et al.* (33). The fluorescence quenching data in the presence of either acrylamide or iodide were analyzed according to the Stern-Volmer equation (34, 35) which, when all quenching is collisional (no static quenching), is: $F_o/F = 1 + K_{sv}[Q]$, where F_o and F are the fluorescence intensities in the absence or presence of quenchers, respectively, K_{sv} the collisional Stern-Volmer constant, and $[Q]$ the quencher concentration. The plot of F_o/F *versus* $[Q]$ is linear if the population of emitting fluorophores is homogenous. In contrast, a downward curvature is observed for a heterogenous population of fluorophores, and a linear plot can then be obtained by using the modified Stern-Volmer relationship introduced by Lehrer (36): $F_o/(F_o - F) = 1/([Q]f_aK_Q) + 1/f_a$ where f_a is the fractional number of accessible fluorophores and K_Q their collisional constant. The plot of $F_o/(F_o - F)$ *versus* $1/[Q]$ allows a graphical determination of f_a.

RESULTS

HprK from B. subtilis Is a Homo-oligomeric Enzyme—Analysis of the HprK oligomerization state was first undertaken by size exclusion chromatography. At the elution volume expected for the monomer (36 kDa), a very small peak was observed the composition of which has not been determined. The major peak containing HprK eluted at a volume corresponding to about 260 kDa, indicating that this enzyme is highly oligomeric (data not shown). Analysis of the chromatograms revealed a small shoulder at the leading edge of the peak, suggesting that some aggregation occurred during the experiments. No noticeable change in the protein elution volume was observed when HprK was incubated with ATP-Mg or FBP prior loading onto the column. In order to obtain additional information on the size of

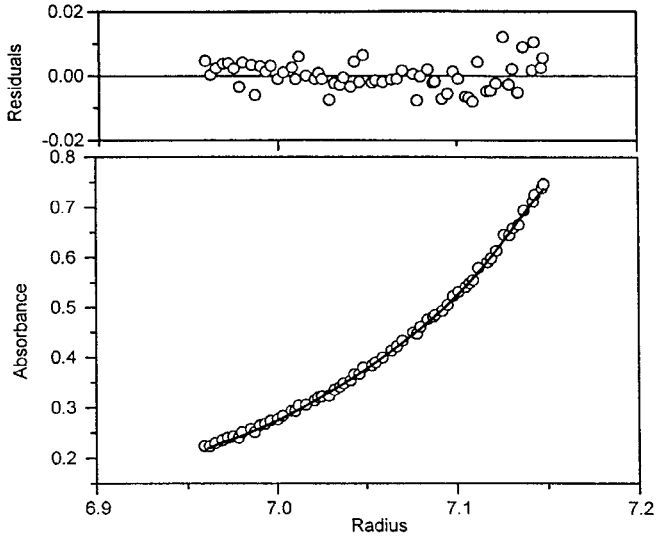

FIG. 1. **Equilibrium sedimentation of HprK.** The absorbance at 280 nm is plotted against the radial position expressed in centimeters. The *upper part* of the figure shows the residual difference between experimental and fitted values by its standard deviation. This experiment was carried out at three different centrifugation speeds, and repeated three times for each centrifugation speed. An identical fit was performed for all nine experiments, but only the result obtained with HprK at 5,600 rpm is shown here for more clarity. The other eight fits were of the same quality.

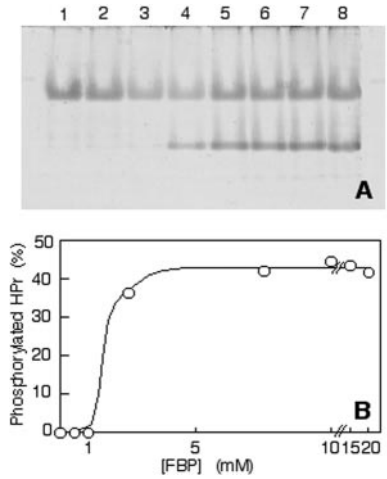

FIG. 2. **Effect of increasing FBP concentrations on HPr phosphorylation by HprK.** *A*, 20 μl of a phosphorylation mixture contained 30 μM HPr, 350 nM purified HprK, 50 mM Tris/HCl, pH 8, 10 mM $MgCl_2$, 25 μM ATP and the following concentrations of FBP: 0 mM (*lane 1*), 0.5 mM (*lane 2*), 1 mM (*lane 3*), 2.5 mM (*lane 4*), 7.5 mM (*lane 5*), 10 mM (*lane 6*), 15 mM (*lane 7*), 20 mM (*lane 8*). The phosphorylation mixture was incubated for 10 min at 37 °C and the reaction was stopped by adding 100 mM EDTA before loading samples onto nondenaturing 12.5% polyacrylamide gel (30). After electrophoresis, the gel was stained with Coomassie Blue. The *upper band* represents HPr, the *lower band* Ser(P)-HPr. *B*, the gel was scanned in a personal Densitometer SI and the data were analyzed with the software ImagQuant V1.2 (Molecular Dynamics). The figure represents the percent of HPr which is phosphorylated in response to increasing concentrations of FBP.

HprK, an analytical ultracentrifugation was conducted on the protein. First, homogeneity was tested by a sedimentation-velocity experiment. A major population (about 85%) was observed with an apparent sedimentation coefficient of $s_{20\,°C}$ = 9.2. Minor populations of higher molecular weight were also observed. The quaternary structure of HprK was studied by sedimentation-equilibrium analytical ultracentrifugation. The experiment was performed at three different ultracentrifugation speeds and repeated three times. Each of the 9 data sets analyzed separately gave a single homogeneous species of average molecular mass 240–320 kDa. A simultaneous fit was then performed yielding an average mass of 274,200 Da, corresponding to 7.6 times the calculated molecular mass of the monomer, *i.e.* 36,068 (Fig. 1). This result suggests that the native form of *B. subtilis* HprK is an octamer, although the possibility of a more rarely observed heptamer cannot be ruled out.

FBP Stimulates the Phosphorylation of HPr and Its Binding to HprK Follows a Positive Cooperativity Mechanism—In order to investigate the effect of FBP on HprK, the initial rate of HPr phosphorylation was analyzed at low concentrations of both HprK and ATP and increasing concentrations of FBP (Fig. 2). The results show that no stimulation of HPr phosphorylation was observed at FBP concentrations below 1 mM. However, when the FBP concentration was raised above 1 mM, HPr phosphorylation by HprK was strongly stimulated, and after a sharp increase, the activity reached a plateau at about 5 mM FBP. The sigmoidal curve in Fig. 2 suggests that binding of FBP to HprK, HPr, or to both proteins follows a positive cooperativity mechanism.

Evidence for direct binding of FBP to HprK was provided by the results presented in Fig. 3. We took advantage that HprK contains a single tryptophan (Trp-235) in its sequence (SWISS-PROT accession number 034483) to study FBP binding by intrinsic fluorescence measurements. In the absence of FBP and with the excitation wavelength set at 295 nm, HprK exhibited a fluorescence emission spectrum characteristic of a rather accessible tryptophan residue (λ_{max} around 340 nm) (Fig. 3*A, lower curve*). A progressive increase of the FBP concentration from 0.5 to 12 mM enhanced the fluorescence intensity of HprK (Fig. 3*A*, *upper curves*). When the ratio of the HprK fluorescence intensity measured in the presence and absence of FBP (F/F_o) was plotted as a function of the FBP concentration, a sigmoidal curve was obtained confirming that FBP binding to HprK follows a positive cooperativity mechanism (Fig. 3*B*). Curve fitting of the data allowed estimation of the parameters for binding of FBP to HprK (F_{max}/F_o = 1.46 ± 0.0062; n_H = 2.19 ± 0.067 and apparent K_D = 6.03 mM). The apparent discrepancy between the maximal concentrations of FBP required to stimulate the kinase activity in phosphorylation tests (Fig. 2) and to saturate the FBP-binding site of HprK in fluorescence experiments (Fig. 3*B*) might be due to different temperatures used (25 and 37 °C for fluorescence and kinetic experiments, respectively) but also to differences in buffer composition, for instance, the lack of magnesium in the fluorescence experiments. Magnesium was omitted from the fluorescence buffer since it produced a slow precipitation of the protein during the experiment. Addition of agents known to stabilize proteins, such as 20% glycerol or use of different buffers, did not solve this problem. As a consequence, the fluorescence experiments were carried out in the absence of magnesium. The effect of FBP on HprK fluorescence seems to be very specific, as neither fructose 1-phosphate nor fructose 6-phosphate had any significant effect on the fluorescence emission spectrum of the kinase (Fig. 3*B*). This indicates that either these two compounds do not bind to HprK or that they do bind to the enzyme but without inducing any conformational change. Accordingly, fructose 1-phosphate and fructose 6-phosphate were without effect on the phosphorylation activity of HprK (data not shown (6)).

Tryptophan Heterogeneity Reveals a Heterogenous Population of HprK Oligomers—The positive cooperativity displayed by HprK for binding of FBP implies that this enzyme contains at least two classes of FBP-binding sites. Whether the binding of FBP to (*a*) high affinity site(s) induced a change in the affinity of a second class of FBP-binding sites within the same

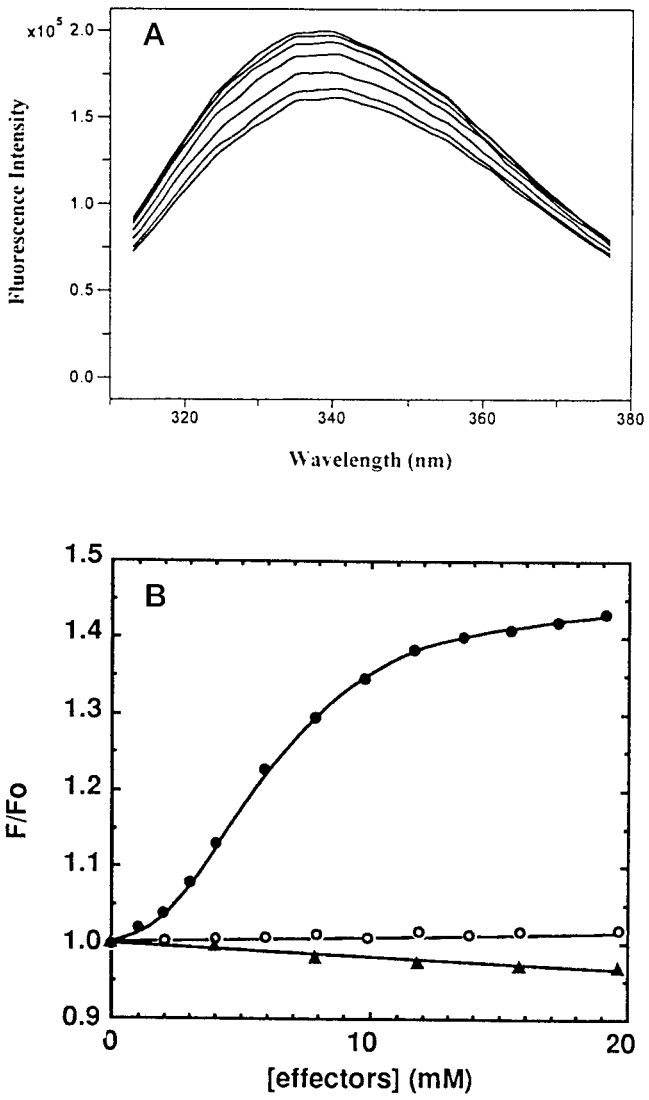

FIG. 3. **Effect of FBP on the HprK tryptophan fluorescence.** *A*, increasing concentrations of FBP were added to a 2-ml mixture containing 1 μM HprK, and the fluorescence intensity was recorded after each addition as described under "Experimental Procedures." From the lower to the upper curves, the concentration of FBP was 0, 2, 4, 5.9, 7.8, 9.8, and 11.7 mM, respectively. Each curve was corrected for the fluorescence of the buffer alone containing the same concentration of FBP. *B*, the increase in HprK fluorescence produced by FBP binding was plotted *versus* the concentration of FBP (●), after correction for the inner-filter effect of FBP on *N*-acetyltryptophanamide. Identical experiments were carried out using fructose 1-phosphate (○) or fructose 6-phosphate (▲) instead of FBP.

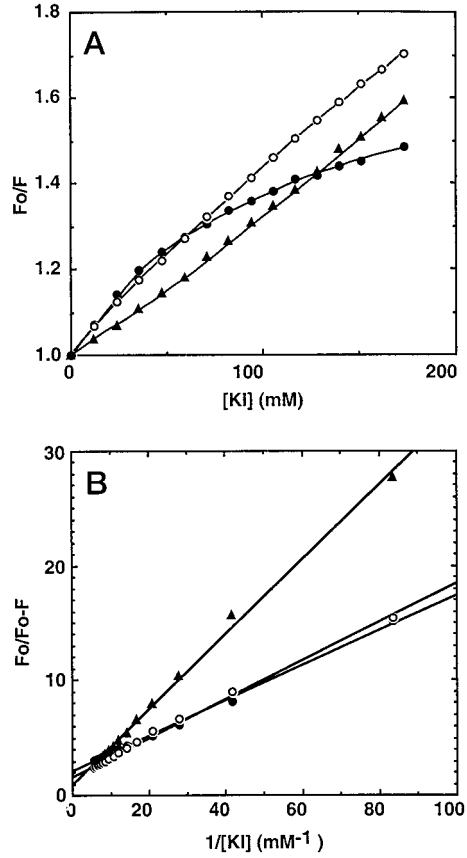

FIG. 4. **Quenching of HprK fluorescence by iodide.** Increasing concentrations of potassium iodide were added to a 2-ml mixture containing either 1 μM HprK (●), 1 μM HprK preincubated for 10 min with 20 mM FBP (○), or 1 μM HprK preincubated overnight with 6 M guanidine hydrochloride (▲), and the fluorescence intensity was recorded after each addition. F_o and F are integrated values of fluorescence intensities determined from the emission spectrum (between 310 and 380 nm) recorded in the absence and presence of iodide, respectively, and corrected for the fluorescence of the buffer alone containing the same concentration of iodide. The data are plotted using a Stern-Volmer representation (*A*) or a modified Stern-Volmer representation according to Lehrer (36) (*B*).

HprK oligomer (by sequential interaction (37)) or whether this reflected an equilibrium between two oligomer conformations of the enzyme (by concerted symmetry (38)) was not known. To decide between the two alternatives, the accessibility of tryptophan residues was assessed by using either acrylamide or iodide. As shown in Fig. 4*A*, addition of increasing amounts of iodide, a compound known to quench only surface accessible tryptophan residues (39), to native HprK caused a progressive quenching of intrinsic fluorescence. However, a downward curvature was observed in the Stern-Volmer plot for iodide concentrations above 80 mM. This curvature indicates that the population of tryptophan residues is heterogenous. This was confirmed by the modified Stern-Volmer plot (Fig. 4*B*), which allowed estimation of the fraction of tryptophan residues accessible to iodide, f_a, found to be about 0.5. The iodide effect was not caused by any denaturation of HprK, since the enzyme preincubation with 0.2 M potassium iodide for 1 h prior to measuring its activity by diluting it 1000-fold in an HPr kinase assay mixture did not alter HprK activity (data not shown here). Preincubation of HprK with 20 mM FBP tended to flatten the curve of the Stern-Volmer plot which was reflected by an increase in the fraction of tryptophan residues accessible to iodide (f_a = 0.61). In a control experiment, denatured HprK, obtained by incubation with 6 M guanidine hydrochloride for 16 h, was also found to give a linear Stern-Volmer plot, but under these conditions all the tryptophan residues became accessible to iodide (f_a of about 1). The same experiment was conducted by using acrylamide, a nonpolar quencher which have facilitated access to some of the buried tryptophans (39). In this case, however, the presence of FBP did not significantly change the fraction of tryptophan residues accessible to acrylamide (f_a of about 0.7), although it slightly increased the collisional quenching constant (K_Q = 6.63 M^{-1} as compared with 4.99 M^{-1}, data not shown). The f_a values, significantly lower than 1, indicate an inherent heterogeneity within the population of HprK oligomers. A f_a value of 1 would be expected if HprK followed a sequential model of cooperativity, since, in that case, all the monomers should be equivalent (40). Conversely, these results are consistent with a concerted-symmetry model for the HprK allosteric transition, where the popu-

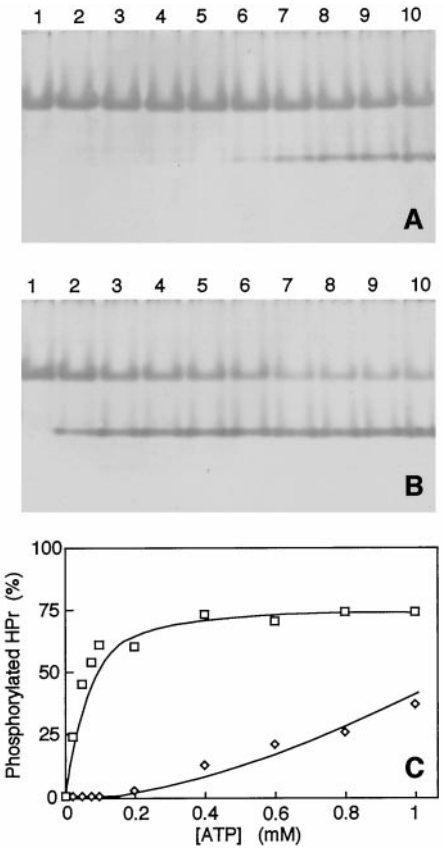

FIG. 5. **Effect of FBP on HPr phosphorylation by HprK.** 20 μl of a phosphorylation mixture contained 30 μM HPr, 350 nM HprK, 50 mM Tris/HCl, pH 8, 10 mM $MgCl_2$, and the following concentrations of ATP: 0 mM (*lane 1*), 0.025 mM (*lane 2*), 0.05 mM (*lane 3*), 0.075 mM (*lane 4*), 0.1 mM (*lane 5*), 0.2 mM (*lane 6*), 0.4 mM (*lane 7*), 0.6 mM (*lane 8*), 0.8 mM (*lane 9*), and 1 mM (*lane 10*). Phosphorylation activity was determined by electrophoresis on nondenaturing gels. *A*, incubation was carried out at 37 °C for 10 min in the absence of FBP. *B*, incubation was carried out at 37 °C for 5 min in the presence of 5 mM FBP. *C*, the gel was scanned as described in Fig. 2 and the percentage of phosphorylation is reported for each ATP concentration: in the absence of FBP (◇) or in the presence of 5 mM FBP (□).

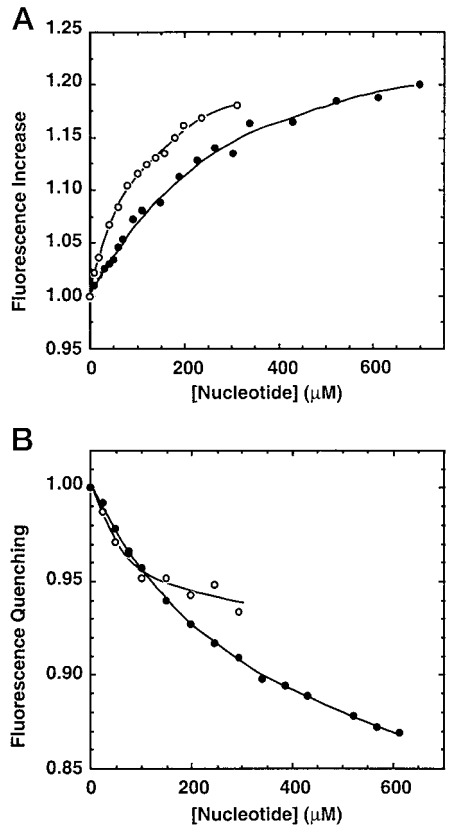

FIG. 6. **Effect of nucleotides on the HprK fluorescence.** Increasing concentrations of either ATP (●) or GTP (○) were added to a 2-ml mixture containing either 1 μM HprK (*A*) or 1 μM HprK preincubated for 10 min with 20 mM FBP (*B*), and the fluorescence intensity was recorded after each addition, as described under "Experimental Procedures." The ratio of the fluorescence intensity measured in the absence and presence of nucleotides was plotted against the nucleotide concentration.

lation of oligomers pre-exists in two different conformations (38).

FBP Binding Efficiently Stimulates Phosphorylation of HPr at Low ATP Concentrations without Apparently Changing the Affinity of HprK for ATP—In order to study in more detail the role of FBP in HPr phosphorylation by HprK, increasing concentrations of ATP were used in the presence or absence of 5 mM FBP. The kinase activity was determined by loading the assay mixtures onto nondenaturing polyacrylamide gels which permit separation of phosphorylated from nonphosphorylated HPr (30). The results clearly indicated that HPr phosphorylation was stimulated by FBP (Fig. 5, *A* and *B*). Nevertheless, the effect of FBP was more obvious at ATP concentrations lower than 0.4 mM (Fig. *5C*). For ATP concentrations between 0.075 and 0.2 mM, HPr phosphorylation was barely detectable in the absence of FBP whereas a strong proportion of HPr, around 60%, was phosphorylated in the presence of FBP (Fig. 5, *A* and *B*, *lanes 4–6*).

To further assess the role of FBP in the HprK phosphorylation activity, it was tested whether FBP was able to alter the affinity of nucleotide binding to HprK. In the absence of FBP, ATP binding to HprK produced an increase in HprK fluorescence intensity (Fig. 6*A*). Curve fitting of the results allowed estimation of the binding parameters for ATP binding, K_D = 270 ± 21 μM and F_{max} = 1.28 ± 0.008. Likewise, binding of GTP, which was previously shown to be an alternative phosphate donor for HPr phosphorylation by HprK (6), produced a similar increase in the kinase fluorescence intensity (F_{max} = 1.25 ± 0.008), with a somewhat higher affinity (K_D = 116 ± 11 μM). Conversely, when FBP was bound to the HprK, which already led to an increase of the fluorescence intensity by about 40% (*cf.* Fig. 3), the addition of either ATP or GTP produced a significant quenching of the fluorescence intensity (21 ± 0.5 and 8 ± 0.9%, respectively). Nevertheless, the binding affinity for both nucleotides was only slightly affected by the presence of FBP with a K_D of 363 ± 21 μM for ATP or 88 ± 29 μM for GTP. Therefore, the FBP enhancement of HPr phosphorylation at low ATP concentrations did not appear to be related to any increase in nucleotide affinity. However, as the fluorescence experiments had to be conducted in the absence of magnesium (see above), a pronounced effect of FBP on nucleotide binding in the presence of magnesium might have been overlooked.

HprK Exhibits a Positive Cooperativity for Nucleotide Binding—The results presented in Fig. 5*C*, obtained in the absence of FBP, suggested that ATP binding to HprK followed a positive cooperativity mechanism and this was further investigated. Fig. 7 shows no HprK-mediated HPr phosphorylation at low ATP concentrations. However, when the ATP concentration was raised above 0.1 mM, a progressive increase in activity was observed until a maximal level of phosphorylation was attained at about 5 mM ATP. A sigmoidal curve was readily seen when only low ATP concentrations were analyzed (*cf. inset,* Fig. 7*B*)

Positive cooperativity for nucleotide binding was further detected when carrying out fluorescence studies in the presence of

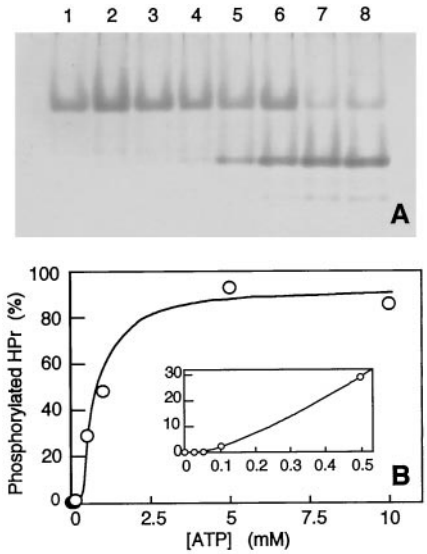

FIG. 7. **Phosphorylation of HPr in the presence of different amounts of ATP.** *A*, 20 μl of a phosphorylation mixture, which was incubated for 10 min at 37 °C, contained 30 μM HPr, 2.8 μM purified HprK, 50 mM Tris/HCl, pH 8, 10 mM $MgCl_2$ and the following concentrations of ATP, 0 mM (*lane 1*), 0.025 mM (*lane 2*), 0.05 mM (*lane 3*), 0.1 mM (*lane 4*), 0.5 mM (*lane 5*), 1 mM (*lane 6*), 5 mM (*lane 7*), 10 mM (*lane 8*). The phosphorylation reaction was stopped by adding 100 mM EDTA to the assay mixtures before loading them onto a nondenaturing 12.5% polyacrylamide gel gel. *B*, the gel was scanned as described in the legend of Fig. 2 and the percentage of phosphorylation is reported for each ATP concentration. *Inset* is a blow-up of the plot obtained at low ATP concentrations.

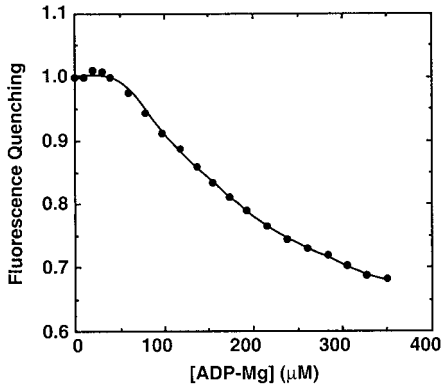

FIG. 8. **Effect of ADP-Mg on the HprK fluorescence.** Increasing concentrations of ADP-Mg (●) were added to a 2-ml mixture containing 1 μM HprK in 25 mM Hepes/KOH, pH 8.2, and the fluorescence intensity was recorded after each addition as described under "Experimental Procedures." The ratio of the fluorescence intensity measured in the absence and presence of nucleotides was plotted against the ADP-Mg concentration.

magnesium. To avoid the precipitation of HprK induced by the presence of magnesium alone, equimolar amounts of magnesium and ADP were used. Under these conditions no precipitation of HprK occurred. The results of such an experiment are reported in Fig. 8. No effect on the fluorescence emission spectrum was seen until the concentration of both magnesium and ADP reached about 50 μM. At higher concentrations, a quenching of tryptophan fluorescence was observed. Binding of fluorescent nucleotide analogues, *i.e.* Mant derivatives, was also found to follow a positive cooperativity mechanism, as can be seen from the experiment with MantADP shown in Fig. 9. Addition of increasing amounts of MantADP together with equimolar amounts of magnesium produced a drastic quenching of the fluorescence emission spectrum of HprK tryptophan residues (Fig. 9*A*, *left side*). Simultaneously, a new peak of fluorescence progressively developed, centered at approximately 430 nm, which is related to fluorescence resonance energy transfer between the tryptophan residues and the Mant group. The plot of fluorescence quenching as a function of MantADP-Mg concentration clearly shows that the nucleotide analogue binding followed a biphasic process, reflecting a positive cooperativity mechanism (Fig. 9*B*). Accordingly, the fluorescence resonance energy transfer plotted *versus* the concentration of MantADP-Mg also exhibited a biphasic dependence confirming a mechanism of positive cooperativity for MantADP binding. Curve fitting of the results obtained from either the quenching (Fig. 9*A*) or the energy transfer (Fig. 9*B*) experiments allowed estimation of the following binding parameters for the MantADP-Mg: $n_H = 2.06 \pm 0.17$ and apparent K_D = 21.87 μM, or $n_H = 2.26 \pm 0.13$ and apparent $K_D = 16.55$ μM, respectively. That the Mant analogue was actually able to bind to the nucleotide-binding site of HPrK was demonstrated by the ability of MantATP to efficiently replace ATP, although to a lower extent, during a HPr phosphorylation assay (not shown). When the MantGDP replaced MantADP, a similar positive cooperativity of binding was also obtained. Likewise, MantGTP proved to be an efficient phosphate donor for HPr phosphorylation by HprK confirming that MantGTP and -GDP do bind to the nucleotide-binding site of HprK (data not shown).

DISCUSSION

This paper describes the first thorough characterization of the enzymatic properties of the HprK from *B. subtilis*, a member of a recently identified new class of bacterial protein kinases unrelated to the eukaryotic protein kinase family (5, 6). The combination of biochemical and biophysical approaches used in this study allowed us to shed some light on the mechanistic properties of this enzyme. The new information reported in this paper includes (i) the oligomeric nature of *B. subtilis* HprK, which probably forms an octamer; (ii) the permanent asymmetry among the population of HprK oligomers; (iii) the pronounced positive cooperativity exhibited by HprK for the binding of either nucleotides or its allosteric activator, FBP, conceivably a consequence of the heterogeneity of HprK oligomers; (iv) the role of FBP at low ATP concentrations; (v) the properties of the unique tryptophan, Trp-235, present in the *B. subtilis* enzyme sequence and ideally located to sense the conformational changes induced by the binding of different effectors.

Homo-oligomeric Structure of HprK—The high oligomerization state (with an average molecular mass of 274 kDa) suggests that the native form of *B. subtilis* HprK is an octamer. An even higher oligomerization state has previously been reported for HprK from *Streptococcus salivarius*, compatible with a decamer structure (7). In that study, however, the molecular weight was only estimated from size exclusion chromatography and this result needs to be confirmed using a method independent of the shape of the molecule, such as equilibrium-sedimentation centrifugation. More surprising is the case of HprK isolated from *Enterococcus faecalis*, for which a dimeric structure has been determined by using gel filtration chromatography (8). Whether this apparent lower supramolecular structure may be related to an altered binding behavior toward effectors such as FBP (see here after) awaits to be tested.

Heterogeneity of the Enzyme Oligomers and Positive Cooperativity—The presence of a single tryptophan residue in the enzyme monomer allowed detection that the HprK oligomers exist in two different conformations. Addition of quenching agents such as iodide or acrylamide revealed a heterogeneity among the tryptophan residues. This heterogeneity could well explain the properties of positive cooperativity exhibited by the enzyme for the binding of either nucleotides (or analogues) or

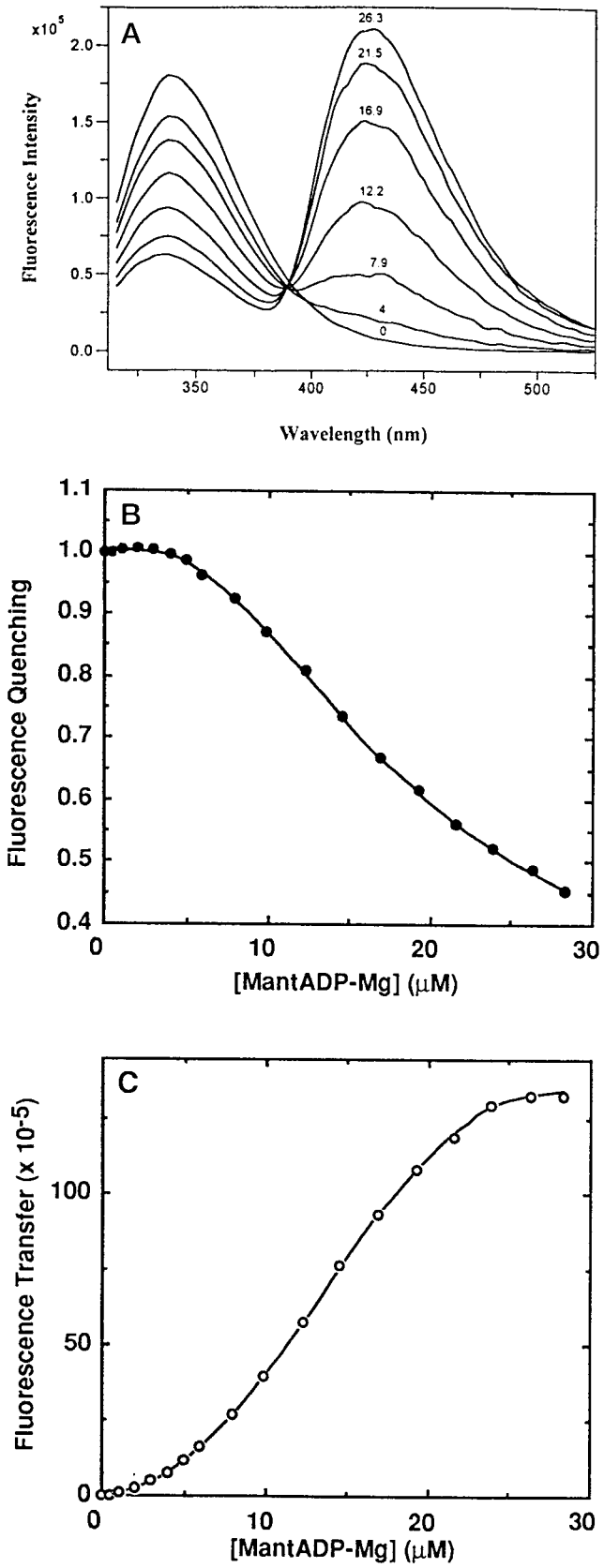

FIG. 9. **Effect of MantADP-Mg on HprK fluorescence.** Increasing concentrations of MantADP-Mg were added to a 2-ml mixture containing 1 μM HprK in 25 mM Hepes/KOH, pH 8.2, and the fluorescence intensity was recorded after each addition as described under "Experimental Procedures." *A*, the emission spectrum of HprK fluorescence was recorded at different micromolar concentrations of MantADP-Mg as indicated in the figure. *B*, the fluorescence quenching obtained from the integrated peak of fluorescence (between 310 and 380 nm), in the presence as compared with the absence of MantADP-Mg, was plotted against the concentration of MantADP-Mg, after correction for the inner filter effect of MantADP-Mg measured on *N*-acetyltryptophanamide. *C*, the fluorescence resonance energy transfer, taken as the increase in fluorescence between 400 and 530 nm, was plotted against the concentration of MantADP-Mg.

FBP. Additionally, this suggests that the mechanism of positive cooperativity exhibited by HprK falls into the category of concerted allosteric transition as first proposed by Monod *et al.* (38) and later demonstrated for aspartate transcarbamoylase (41). Two previous reports have dealt with the kinetic properties of HprK isolated from either *Streptococcus pyogenes* or *S. salivarius*, but in both studies it was concluded that the enzyme binds ATP following Michaelis-Menten (hyperbolic) kinetics (4, 7). This apparent discrepancy might, however, be explained by the different experimental conditions used in each report. In the first study, 4 mM FBP was added to the assay mixture used for HPr phosphorylation (4) which very likely precluded the detection of any positive cooperativity (see Fig. 5, in the presence of 5 mM FBP). Although FBP was omitted from the assay medium, in the second study, the concentration of ATP varied between approximately 0.6 and 5 mM (7) which might have hampered the finding of a lag at lower ATP concentrations. Accordingly, the K_m values reported for ATP were quite different for the two enzymes: 66 μM for *S. pyogenes* and 1 mM for *S. salivarius*.

FBP Is an Allosteric Activator of B. subtilis HprK at Low ATP Concentrations—The role of FBP as an activator of HPr phosphorylation has been previously determined by using either crude extracts or partially purified HprKs from many species including *B. subtilis* (10), *S. pyogenes* (42), *Streptococcus mutans* (43), *S. salivarius* (44), *E. faecalis* (3), and *Listeria monocytogenes* (45, 46). However, contradictory results have been reported with purified HprK. For instance, the phosphorylation activity of the *B. subtilis* recombinant enzyme has been shown to be strongly stimulated by FBP (5, 6), whereas that obtained from either *E. faecalis* (8) or *S. salivarius* (7) was not. A tentative explanation for the lack of FBP activation observed in the two latter studies is that the concentration of HprK, ATP, or both was too high thus preventing the detection of FBP stimulation. Indeed, the results of Fig. 5 show that the FBP effect is mostly observed at low ATP concentrations but also when a low concentration of HprK is used. It is also possible that the allosteric activation caused by FBP occurs only with certain members of the HprK family. It will be interesting to investigate whether the difference in the quaternary structure of some HprKs, a dimer in *E. faecalis* as opposed to an octamer in *B. subtilis*, might be related to different regulation mechanisms, *i.e.* activation of HprK by FBP for the *B. subtilis* but not for the *E. faecalis* enzyme.

The results reported here indicate that FBP is an allosteric activator and that its binding to *B. subtilis* HprK obeys a positive cooperative mechanism. This mechanism allows a rapid adaptation of metabolic enzymes to subtle variations of their intracellular effector concentrations. FBP is one of the first glycolytic intermediates and its concentration is 14-fold higher when *B. subtilis* is grown in the presence of glucose as compared with cells grown in the presence of malate (47). Consequently, this is a well suited activator to switch on the activity of the HprK, which then triggers the whole mechanism of carbon catabolite repression. It is noteworthy that in the microbiological world, FBP is often used as an allosteric effector of metabolic enzymes involved in sugar utilization (48), such as pyruvate kinase from yeast (49) or *Lactococcus lactis* (3, 50), *B. stearothermophilus* lactate dehydrogenase (51), or *Escherichia coli* glycerol kinase (52). FBP acts as an allosteric acti-

vator for pyruvate kinase and lactate dehydrogenase, which are both involved in glucose utilization. By contrast, the activity of glycerol kinase, which is repressed by the presence of glucose, is allosterically inhibited by high concentrations of FBP.

The molecular mechanism allowing the stimulation by FBP of *B. subtilis* HprK activity is presently unknown. In the absence of magnesium, FBP does not significantly alter the affinity for nucleotide binding, but we cannot rule out an effect on nucleotide affinity in the presence of magnesium. Alternatively, FBP can increase either the binding of the substrate, HPr, or the velocity of the phosphoryl-transfer step.

Strategic Location of Trp-235 in the HprK Structure—The single tryptophan residue present in the sequence of *B. subtilis* HprK appears to be ideally located in the enzyme to allow monitoring of the conformational changes associated with the binding of FBP, nucleotides, or nucleotide analogues. Comparison with other members of the HprK family revealed that a tryptophan residue is sometimes found at the same position such as in *T. pallidum* or *E. faecalis*, but it can be replaced by a phenylalanine, a leucine, or even an alanine residue in other species (5, 6). Nevertheless, the tryptophan residue in *B. subtilis* HprK must be located in close proximity to the nucleotide-binding site due to the quenching of fluorescence observed when either ATP or GTP were added to HprK in the presence of FBP, or when ADP-Mg was added to HprK in the absence of FBP. A drastic quenching effect was observed with MantADP-Mg (up to about 87%) accompanied by a high fluorescence resonance energy transfer leading to the appearance of a new emission peak and suggesting that the Mant moiety is also rather close to Trp-235.

Acknowledgments—We are grateful to J. Janin, in whose laboratory part of this work was carried out, for continuous encouragement and critical reading of the manuscript. We thank G. Batelier for the ultracentrifugation experiments as well as J. Philo and W. Stafford for making the computer programs available on the RASMB server. We are thankful to A. Bosch for help with iconography and S. Grael for excellent technical assistance.

REFERENCES

1. Kundig, W., Ghosh, S., and Roseman, S. (1964) *Proc. Natl. Acad. Sci. U. S. A.* **52,** 1067–1074
2. Gassner, M., Stehlik, D., Schrecker, O., Hengstenberg, W., Maurer, W., and Rüterjans, H. (1977.) *Eur. J. Biochem.* **75,** 287–296
3. Deutscher, J., and Engelmann, R. (1984) *FEMS Microbiol. Lett.* **23,** 157–162
4. Reizer, J., Novotny, M. J., Hengstenberg, W., and Saier, M. H., Jr. (1984) *J. Bacteriol.* **160,** 333–340
5. Galinier, A., Kravanja, M., Engelmann, R., Hengstenberg, W., Kilhoffer, M. C., Deutscher, J., and Haiech, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1823–1828
6. Reizer, J., Hoischen, C., Titgemeyer, F., Rivolta, C., Rabus, R., Stülke, J., Karamata, D., Saier, M. H., Jr., and Hillen, W. (1998) *Mol. Microbiol.* **27,** 1157–1169
7. Brochu, D., and Vadeboncoeur, C. (1999) *J. Bacteriol.* **181,** 709–717
8. Kravanja, M., Engelmann, R., Dossonnet, V., Blüggel, M., Meyer, H. E., Frank, R., Galinier, A., Deutscher, J., Schnell, N., and Hengstenberg, W. (1999) *Mol. Microbiol.* **31,** 59–66
9. Saier, M. J. (1996) *FEMS Microbiol. Lett.* **138,** 97–103
10. Galinier, A., Haiech, J., Kilhoffer, M. C., Jaquinod, M., Stülke, J., Deutscher, J., and Martin-Verstraete, I. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8439–8444
11. Galinier, A., Deutscher, J., and Martin-Verstraete, I. (1999) *J. Mol. Biol.* **286,** 307–314
12. Deutscher, J., Küster, E., Bergstedt, U., Charrier, V., and Hillen, W. (1995) *Mol. Microbiol.* **15,** 1049–1053
13. Jones, B. E., Dossonnet, V., Küster, E., Hillen, W., Deutscher, J., and Klevit, R. E. (1997) *J. Biol. Chem.* **272,** 26530–26535
14. Fujita, Y., Miwa, Y., Galinier, A., and Deutscher, J. (1995) *Mol. Microbiol.* **17,** 953–960
15. Gösseringer, R., Küster, E., Galinier, A., Deutscher, J., and Hillen, W. (1997) *J. Mol. Biol.* **266,** 665–676
16. Martin-Verstraete, I., Deutscher, J., and Galinier, A. (1999) *J. Bacteriol.* **181,** 2966–2969
17. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
18. Hunter, T. (1995) *Cell* **80,** 225–236
19. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* **1,** 945–951
20. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *Trends Biochem. Sci.* **15,** 430–434
21. Eccleston, J. F., Moore, K. J. M., Brownbridge, G. G., Webb, M. R., and Lowe, P. N. (1991) *Biochem. Soc. Trans.* **19,** 432–436
22. Jault, J.-M., Divita, G., Allison, W. S., and Di Pietro, A. (1993) *J. Biol. Chem.* **268,** 20762–20767
23. Kanazawa, T., Suzuki, H., Daiho, T., and Yamasaki, K. (1995) *Biosci. Rep.* **15,** 317–326
24. Bauer, C. B., Kuhlman, P. A., Bagshaw, C. R., and Rayment, I. (1997) *J. Mol. Biol.* **274,** 394–407
25. Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J.-M., Barron, D., and Di Pietro, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9831–9836
26. Hiratsuka, T. (1983) *Biochim. Biophys. Acta* **742,** 496–508
27. Woodward, S. K., Eccleston, J. F., and Geeves, M. A. (1991) *Biochemistry* **30,** 422–430
28. Stafford, W. F. (1992) *Anal. Biochem.* **203,** 295–301
29. Philo, J. S. (1994) in *Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems* (Schuster, T. M., and Laue, T. M., eds) pp. 156–170, Birkhausen, Boston
30. Deutscher, J., Kessler, U., and Hengstenberg, W. (1985) *J. Bacteriol.* **163,** 1203–1209
31. Divita, G., Goody, R. S., Gautheron, D. C., and Di Pietro, A. (1993) *J. Biol. Chem.* **268,** 13178–13186
32. Divita, G., Di Pietro, A., Deleage, G., Roux, B., and Gautheron, D. C. (1991) *Biochemistry* **30,** 3256–3262
33. Calhoun, D. B., Vanderkooi, J. M., and Englander, S. W. (1983) *Biochemistry* **22,** 1533–1539
34. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry* **15,** 672–680
35. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem.* **114,** 199–227
36. Lehrer, S. S. (1971) *Biochemistry* **10,** 3254–3263
37. Koshland, D. E. J., Nemethy, G., and Filmer, D. (1966) *Biochemistry* **5,** 365–385
38. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12,** 88–118
39. Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, 3rd Ed., Plenum Press, New York
40. Ricard, J., and Cornish-Bowden, A. (1987) *Eur. J. Biochem.* **166,** 255–272
41. Kantrowitz, E. R., and Lipscomb, W. N. (1990) *Trends Biochem. Sci.* **15,** 53–59
42. Deutscher, J., and Saier, M. H., Jr. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6790–6794
43. Mimura, C. S., Poy, F., and Jacobson, G. R. (1987) *J. Cell. Biochem.* **33,** 161–171
44. Thevenot, T., Brochu, D., Vadeboncoeur, C., and Hamilton, I. R. (1995) *J. Bacteriol.* **177,** 2751–2759
45. Mitchell, W. J., Reizer, J., Herring, C., Hoischen, C., and Saier, M. H. J. (1993) *J. Bacteriol.* **175,** 2758–2761
46. Christensen, D. P., Benson, A. K., and Hutkins, R. W. (1999) *Appl. Environ. Microbiol.* **65,** 2112–2115
47. Fujita, Y., and Freese, E. (1979) *J. Biol. Chem.* **254,** 5340–5349
48. Entian, K. D., and Barnett, J. A. (1992) *Trends Biochem. Sci.* **17,** 506–510
49. Murcott, T. H., Gutfreund, H., and Muirhead, H. (1992) *EMBO J.* **11,** 3811–3814
50. Mason, P. W., Carbone, D. P., Cushman, R. A., and Waggoner, A. S. (1981) *J. Biol. Chem.* **256,** 1861–1866
51. Cameron, A. D., Roper, D. I., Moreton, K. M., Muirhead, H., Holbrook, J. J., and Wigley, D. B. (1994) *J. Mol. Biol.* **238,** 615–625
52. Ormo, M., Bystrom, C. E., and Remington, S. J. (1998) *Biochemistry* **37,** 16565–16572