Properties and Regulation of the Bifunctional Enzyme HPr Kinase/Phosphatase in Bacillus subtilis*

Received for publication, September 4, 2002
Published, JBC Papers in Press, October 30, 2002, DOI 10.1074/jbc.M209052200

Helena Ramström‡§, Sarah Sanglier¶, Emmanuelle Leize-Wagner‡, Claude Philippe‡, Alain Van Dorselaer‡, and Jacques Haiech‡**

From the ¶Laboratoire de Spectrométrie de Masse Bio-Organique, UMR CNRS 7509, ECPM, Université Louis Pasteur de Strasbourg, 25 rue Becquerel, F-67087 Strasbourg, France.

The bifunctional allosteric enzyme HPr kinase/phosphatase (HPrK/P) from Bacillus subtilis is a key enzyme in the main mechanism of carbon catabolite repression/activation (i.e., a means for the bacteria to adapt rapidly to environmental changes in carbon sources). In this regulation system, the enzyme can phosphorylate and dephosphorylate two proteins, HPr/HPr(Ser(P)) and Crh/Crh(Ser(P)), sensing the metabolic state of the cell. To acquire further insight into the properties of HPrK/P, electrospray ionization mass spectrometry, dynamic light scattering, and BIACORE were used to determine the oligomeric state of the protein under native conditions, revealing that the enzyme exists as a hexamer at pH 6.8 and as a monomer and dimer at pH 9.5.

Using an in vitro radioactive assay, the influence of divalent cations, pH, temperature, and different glycolytic intermediates on the activity as well as kinetic parameters were investigated. The presence of divalent cations was found to be essential for both opposing activities of the enzyme. Furthermore, pH values equal to the internal pH of vegetative cells seem to favor the kinase activity, whereas lower pH values increase the phosphatase activity. Among the glycolytic intermediates evaluated, fructose 1,6-diphosphate and fructose 2,6-diphosphate were found to be allosteric activators in the kinase assay, whereas high concentrations inhibited the phosphatase activity, except for fructose 1,6-diphosphate in the case of HPr(Ser(P)).

Phosphatase activity was induced by inorganic phosphate as well as acetyl phosphate and glyceraldehyde 3-phosphate. Kinetic parameters indicate a preference for binding of HPr compared with Crh to the enzyme and supported a strong positive cooperativity. This work suggests that the oligomeric state of the enzyme is influenced by several effectors and is correlated to the kinase or phosphatase activity. The phosphatase activity is mainly supported by the hexameric form.

Protein kinases and phosphatases play a decisive role in many biological processes by phosphorylating and dephosphorylating target proteins. For humans, it was estimated in the mid-1990s that the genome may contain as many as 2000 protein kinases and 1000 protein phosphatases (1). Initial analysis, after decoding of the DNA that constitutes the human genome, indicates a somewhat lower number of predicted protein kinases and protein phosphatases (2, 3). Since protein kinases are not only involved in normal cell growth but also in malignant transformations, these enzymes have been in focus during recent years as new drug targets (4–6). Protein kinases and phosphatases also play a fundamental role in modulating signals in cellular processes in prokaryotic cells. In the Bacillus subtilis (B. subtilis strain 168) genome sequencing project (7) 4106 protein genes were identified encoding 52 known and 46 putative kinases and 26 known and 10 putative phosphatases (on the World Wide Web, see genolist.pasteur.fr/SubtiList/ genome.cgi).

The bifunctional HPr kinase/phosphatase (HPrK/P) in the low guanine and cytosine (low GC) Gram-positive bacteria, B. subtilis, is involved in the main regulatory mechanism for carbon catabolite repression/activation (CCR/CCA) (9–12). The enzyme possesses kinase activity in the presence of ATP and a favorable carbon source, such as glucose, which generates high concentrations of glycolytic intermediates (e.g., fructose 1,6-diphosphate (FBP)), and can phosphorylate two protein substrates (i.e., HPr (histidine-containing protein) and Crh (catabolite repression HPr)) on Ser-46 (10, 11, 13, 14). The presence of two substrates for HPrK/P seems to be unique among low GC Gram-positive bacteria, since Crh has only been detected, thus far, in species of Bacillus (15). High concentrations of inorganic phosphate and low concentrations of ATP, reflecting the intracellular state of cells at starvation (16), have been shown to trigger the phosphatase activity of the enzyme HPrK/P of B. subtilis and, thus, dephosphorylation of HPr(Ser(P)) and Crh(Ser(P)) (9). Furthermore, it has been demonstrated that HPrK/P is an allosteric homo-oligomeric enzyme yielding sigmoidal velocity curves and is strongly regulated by allosteric effector molecules such as FBP (17). In addition to being involved in the CCR/CCA regulatory mechanism, HPr is also a part of the bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS) and becomes phosphorylated on His-15 by enzyme I (EII) (18). The phosphoryl group can then be transferred to the sugar-specific enzymes II, which phosphorylate incoming PTS sugars (18). The other protein, Crh, which was discovered within the B. subtilis genome sequencing pro-

* 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.

§ Recipient of a grant from the Swedish Academy of Pharmaceutical Sciences.

¶ Supported by CNRS and Lilly.

‡ To whom correspondence should be addressed. Tel.: 33-3-90-24-42-70; Fax: 33-3-90-24-43-12; E-mail: haiech@pharma.u-strasbg.fr.
plasmid pREP4 (Qiagen, Courtabœuf, France) was transformed with ican Instrument Co.) at 500 p.s.i. Whole cells and cell debris were then ruptured by two passages through the French press (Amer-prise mixture was homogenized using Ultra-TURRAX for 2 minutes at high speed. An amount of deoxyribonuclease I type I (Sigma) was added, and the cell pellet was stored at −80 °C for at least 1 h before being resuspended in buffer solution. The cultures were transformed to 1 liter of medium containing the desired antibiotic drugs and were grown until the optical density at 600 nm was 0.6. Expression of HPr(His)_6, Crh(His)_6, and HPrK/P(Trx-His6-S-tag) was confirmed with SDS-PAGE separations using PhastGel (Amersham Biosciences) for HPr, and 50 μg/ml ampicillin and 170 μg/ml chloramphenicol for Crh. The following morning, the cultures were transformed to 1 liter of medium containing the desired antibiotic drugs and were grown until A600 was 0.6. Expression of the genes was induced by adding isopropyl-1-thio-β-D-galactopyranoside (Sigma) to a final concentration of 1 mM. Further incubation was performed at 37 °C for 2 h. Cells were harvested by centrifugation at 5000 rpm for 20 min at 4 °C. The supernatant was discarded, and the cell pellet was stored at −80 °C for at least 1 h before being resuspended in 5–10 volumes of 50 mM Tris buffer (pH 8), containing 0.3 M NaCl. A small amount of deoxyribonuclease I type I (Sigma) was added, and the cell mixture was homogenized using UltraTURRAX for 2–3 min. The cells were then ruptured by two passages through the French press (American Instrument Co.) at 500 p.s.i. Whole cells and cell debris were removed by centrifugation at 30,000 rpm for 20 min at 4 °C, and the resultant supernatant was then mixed during 30 min with Ni²⁺-NTA resin (Qiagen, Courtabœuf, France) (1 ml of resin/g of cells) pre-equilibrated with 10 column volumes of 100 mM Tris, pH 7.4. For HPrK/P, this was performed at 4 °C as well as all subsequent purification steps, whereas for HPr and Crh purification was performed at room temperature. The Ni²⁺-NTA resin was then transferred to a column, and the resin was washed with 50 ml Tris, pH 7.4, containing 50 mM NaSO₄ and 15% glycerol. The proteins were eluted with imidazole in 50 ml Tris containing 1 M KCl, pH 8.0, and devices with a molecular mass cut-off of 3000 Da were used for HPr(His)₆ and Crh(His)₆. The procedure for desalting was conducted following the protocol provided by the company and contained six dilution/concentration steps performed at 4 °C for 60 min. Finally, the concentration of the proteins was determined spectrophotometrically using the Bio-Rad protein assay kit (Bio-Rad). The samples were stored at −20 °C until ESI-MS measurements were performed.

**Instrumentation**—Mass spectrometry experiments were conducted using a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF II; Micromass, Altrincham, UK) equipped with a Z-Spray ESI source. All spectra were recorded in the positive ion mode. **ESI-MS Analysis under Denaturing Conditions**—Purity and homogeneity of HPr(His)_6, Crh(His)_6, and HPrK/P(Trx-His6-S-tag) were verified by mass analysis under denaturing conditions with the proteins diluted to 10 μM in a 1:1 water/acetonitrile (v/v) mixture acidified with 1% formic acid. Under these conditions, noncovalent interactions between subunits are disrupted, thus allowing for the accurate measurement of the molecular weight of the constituent enzyme subunits with a precision of better than 0.01%. Mass spectra were recorded in the positive ion mode in the mass range of 500–4000 m/z (mass to charge) after calibration of the instrument with horse heart myoglobin diluted to 2 μM in a 1:1 water/acetonitrile (v/v) mixture acidified with 1% formic acid.

**ESI-MS Analysis under Native Conditions**—Under native conditions, the mass measurements of HPr(His)_6, Crh(His)_6, and HPrK/P(Trx-His6-S-tag) were performed using 10 mM ammonium acetate buffer (pH 6.8). Samples were diluted to ~20 μM and continuously infused into the ESI ion source at a flow rate of 5 μl/min. To study the influence of pH variations on the stability of the oligomeric state of the proteins, the sequences of the proteins are as follows: HPr(His)_6, MGSHHSHHSHHSHGSAQKTPFENTPASSCHARPATLVQVTASKYDADVNLEYN GKTIVNLKSIIMVSGLIGAEGTATISSASSGADENDA LnETKSSERLGE; Crh(His)_6, MVQKVKVEVL KTGLQARPA LFVQ-EANRFRT SDVFLEKDGK VKNASIMGL MSLAVSTGTE VTLIAQ GEDEAEKLADY QVEVLQHIIIIIIHH, and HPrK/P(Trx-His6-S-tag), MSKHIIHTLD DSFDSTDLVKA DGAIVDFWDA EWCPCPKMIA FLDLIDAE QGKTTKLVDVK IODNQPSET ALEVVRKQHRI DAVDIDLF YHEDQDTLYNGA PELIEHELH RIGLGVINVTF LFAGAVGNSV R KTVIMNE LWEQQGQYDQ LGLEETMK TIDEITKVL TVPRGRRNLAV IEVAAANNFR LKRMGLNAE QFTNKLADVI EDREEE. The concentration of HPrK/P(Trx-His6-S-tag) was determined spectrophotometrically using the Bio-Rad protein assay (Bio-Rad) with Bio-Rad protein assay standard lyophilized bovine plasma γ-globulin (Bio-Rad) as a standard, and the concentrations of Crh and HPr were determined by UV spectrophotometry using the extinction coefficient for one and two tyrosine residues, respectively (1500 and 2900 × 1 cm⁻¹). Protein solutions were stored at −20 °C.

**Electrospray Ionization Mass Spectrometry (ESI-MS) Measurements**

Further Treatment of the Proteins—For ESI-MS analysis of HPrK/P(Trx-His6-S-tag), further purification was performed using a 1-ml HiTrap Q Sepharose high performance column (Amersham Biosciences). The HiTrap Q column was equilibrated with 5 column volumes of 25 mM Tris buffer (pH 8) followed by 5 column volumes of 25 mM Tris containing 1 mM KCl (pH 8) and finally 10 column volumes of 25 mM Tris buffer (pH 8). The protein solution was applied to the column, and fractions were eluted with 25 mM Tris buffer (pH 8), containing increasing concentrations of KCl (100, 300, 600, and 1000 mM). The purity of the fractions, after desalting was performed using an Ultrafree centrifugal filter unit with a molecular mass cut-off of 10,000 Da (Millipore), was confirmed with SDS-PAGE separation using PhastGel (Amersham Biosciences) and Coomassie staining. The pure fractions were then desalted through a PD-10 column (Amersham Biosciences) as described above.

Prior to mass spectrometry analysis, an additional desalting procedure was performed with Centricron (Millipore Corp.) using 10 mM ammonium acetate (pH 6.8) as reconstitution solution. Ammonium acetate was used, since this buffer preserves the native structure of proteins and is compatible with the ESI-MS analysis. Concentrators with a molecular mass cut-off of 10,000 Da were used for HPr/K/P(Trx-His6-S-tag), and devices with a molecular mass cut-off of 3000 Da were used for HPr(His)₆ and Crh(His)₆. The procedure for desalting was conducted following the protocol provided by the company and contained six dilution/concentration steps performed at 4 °C for 60 min. Finally, the concentration of the proteins was determined spectrophotometrically using the Bio-Rad protein assay kit. The samples were stored at −20 °C until ESI-MS measurements were performed.

Analysis of HPr and Crh purification was performed at room temperature.
enzyme, increasing amounts of NH3 were added, up to pH 9.5, to the 10 mM ammonium acetate buffer. HPr/K/P(Trx-His6-S-tag) was then diluted to 20 μM with the pH-adjusted buffer. All of the measurements were performed at an accelerating voltage equal to 200 V and with the pressure in the interface region of the mass spectrometer equal to 6.5 millibars. Mass data were acquired in the positive ion mode in the 2500–12,000 m/z mass range. Clusters of CsI (separate injections of 1 mg/ml CsI in 50% aqueous isopropanol alcohol) were used for the calibration of an extended mass range in the high m/z region. The molecular weight of the noncovalent complex of the enzyme was calculated as the mean of five peak maximum values ± S.D.

**Dynamic Light Scattering Measurements**

Dynamic light scattering data were obtained with the DynaPro-801 instrument (Protein Solutions Inc.) using a 30-milliwatt, 833-nm wavelength argon laser at 20 °C and equipped with a solid-state avalanche photodiode. During the illumination, the photons scattered by proteins were collected at 90 °C on a 10-s acquisition time and were filtered with the analysis software. Dynamics. Intensity fluctuations of the scattered light resulting from Brownian motion of particles were analyzed with an autocorrelator to fit an exponential decay function and then measuring a translational diffusion coefficient D. For polydisperse particles, the autocorrelation function was fit as the sum of contributions from the various size particles using the regularization analysis algorithm. D is converted to a hydrodynamic radius Rz through the Stokes-Einstein equation (Rz = kT/6πνD, where D represents the solution viscosity, k is the Boltzmann’s constant, and T is the temperature), and then to a molecular weight for a spherical particle. Apparent molecular weights were deduced from histograms of distribution of percentage mass versus Rz. HPr/K/P(Trx-His6-S-tag) was diluted in a 10 mM ammonium acetate buffer, similar to that used in mass spectrometry analysis under native conditions. All solutions were filtered with 0.22-μm Millipore filters prior to dilution of the protein. Sample preparations were achieved either by diluting the protein in the pre-equilibrated buffer or by adding increasing amounts of NH3 to a 10 μM HPr/K/P(Trx-His6-S-tag) solution in ammonium acetate until pH ranged from 6.8 up to 9.5. An aliquot was removed at the desired pH and kept at room temperature for at least 2 h before dynamic light scattering measurement.

**BIACore Surface Plasmon Resonance Analysis**

HPr/K/P(Trx-His6-S-tag) oligomerization was examined on a BIACore instrument (BIACore J; Amersham Biosciences). The principle of this technology relies on a surface plasmon resonance phenomenon that transforms the specific incident angle of the light reflected from a metal surface. The instrument (BIAcore J; Amersham Biosciences) used for the calibration of an extended mass range in the high m/z region. The molecular weight of the noncovalent complex of the enzyme was calculated as the mean of five peak maximum values ± S.D.

**Properties and Regulation of HPrK/P in B. subtilis**

The desired pH and kept at room temperature for at least 2 h before acetate until pH ranged from 6.8 up to 9.5. An aliquot was removed at the desired pH and kept at room temperature for at least 2 h before dynamic light scattering measurement.

**Different divalent cations for HPrK**

Different divalent cations, Mg2+, Mn2+, Co2+, Ca2+, and Cu2+ as chloride salts, were tested at a final concentration of 5 mM. To exclude possible influence from the buffer, 50 mM HEPES (pH 8) was used, since this buffer is known to form only weak complexes with Mg2+, Mn2+, Ca2+, and Cu2+ (33, 34).

**Effects of pH**

The activity of the enzyme was determined at 11 different pH values, ranging from 5.0 to 8.7, for the different substrates, Crh(Ser(P)), Crh(F1,6P) (Amer), and Crh(F2,6P), 7.0 for Crh(Ser(P)) and Crh(F1,6P) (Amer) and Crh(F2,6P). The buffer Tris (pH 7.4), containing, in general, 50 mM Tris buffer (pH 8), 5 mM MgCl2, 0.1% bovine serum albumin, 10 mM Tris buffer (pH 8), 7 mM MgCl2, 2 mM ATP, 3.3–5 Bq (leading to 200–300 cpm of [γ-32P]ATP/pmol of ATP) (Amersham Biosciences), 2 mM FBP, 0.1% bovine serum albumin, 10 μM HPr/Crh, and, to initiate the reaction, 100 mM HPr/K/P(Trx-His6-S-tag) in a final volume of 20 μl. The reaction was incubated at 37 °C for 10 min, and the phosphorylation reaction was then terminated by spotting samples onto 1 × 1-cm P81 phosphocellulose paper (Whatman, Maidstone, UK) and dropped immediately into a beaker containing 75 mM H3PO4. The total volume of phosphoric acid solution used was ~10 ml for each paper. Unreacted ATP was removed by washing three times with 75 mM H3PO4, for 15 min each, and once with ethanol, just covering the papers, for 5 min. The papers were dried and transferred to scintillation vials containing 6 ml of scintillation solution for water samples, Rotiszint ecopuro (Carl Roth, Karlsruhe, Germany), and the radioactivity was determined in a scintillation counter, LKB 1211 Rackbeta (PerkinElmer Life Sciences). Typically, each condition was tested in triplicate.

For the phosphatase assay, HPr(His)/Crh(His)6 was first phosphorylated in a kinase assay. The final volume was 20 μl and contained 50 mM Tris buffer (pH 8), 7 mM MgCl2, 2 mM ATP, 3.3–5 Bq (leading to 200–300 cpm of [γ-32P]ATP/pmol of ATP) (Amersham Biosciences), 5 mM FBP, 0.1% bovine serum albumin, 200 μM HPr/Crh, and, to initiate the reaction, 800 nM HPr/K/P(Trx-His6-S-tag). After incubation at 37 °C for 2 h, HPr(Ser(P)/Crh(Ser(P)) was purified on Ni2+-NTA resin using a column procedure. The resin was pre-equilibrated with 100 mM Tris buffer (pH 7.4) and then combined with the assay mixture for at least 30 min at room temperature. To remove ATP and FBP, the resin was washed extensively with 50 mM Tris buffer (pH 7.4) containing 50 mM Na2SO4 and 15% glycerol until no radioactivity was detected in the eluent. The enzyme and the phosphorylated protein were eluted with 50 mM Tris buffer (pH 7.4) containing 50 mM Na2SO4, 15% glycerol, and 300 mM imidazole. Desalting was performed using a PD-10 column (Amersham Biosciences). The column was pre-equilibrated with 25 ml of 10 mM Tris buffer (pH 8). After adding the sample (not more than 2.5 ml) to the column, the proteins were eluted with 3.5 ml of 10 mM Tris buffer (pH 8). Fractions containing radioactivity were collected and pooled. In most of the cases, remaining enzyme from the kinase reaction was removed by S-protein-agarose (Novagen, Madison, WI). After equilibration with 20 mM Tris buffer (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100 (Boehringer), 100 mM Tris buffer (pH 8), the S-protein-agarose was gently shaken with the protein solution for 30 min at room temperature. The phosphorylated protein was eluted with 20 mM Tris buffer (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100 as confirmed by radioactivity in the eluent. Desalting was then performed as described above. An Ultrafree centrifugal filter unit (molecular mass cut-off of 5000 Da) was used to concentrate the protein solution (4°C) according to the operating procedure provided by the company. The amount of phosphorylated protein, pmol/μL, was determined on the basis of cpm/pmol ATP. Amounts of HPr(ser(P))/Crh(Ser(P)) up to 10 μM were then included in a phosphatase assay containing, in general, 50 mM Tris buffer (pH 8), 5 mM MgCl2, 0.1% bovine serum albumin, 1 mM inorganic phosphate (K2HPO4 and KH2PO4, pH 5.0), and, to initiate the reaction, 100 nM HPr/K(P(Trx-His6-S-tag)). The reaction mixture was incubated at 37 °C for 2 h. Termination of the reaction and the following steps were the same for the phosphatase assay. Typically, each condition was tested in triplicate.

**Chemicals**

Pyruvic acid, n-fructose 2,6-diphosphate (F2,6BP), n-fructose 1,6-diphosphate (FBP), acetyl coenzyme A, β-nicotinamide adenine dinucleotide (β-NAD−), β-nicotinamide adenine dinucleotide reduced form (β-NADH), n-glucose 6-phosphate, n-glyceraldehyde 3-phosphate, CuCl2·6H2O, CuCl2·2H2O, MnCl2·4H2O, and acetyl phosphate were purchased from Sigma. MgCl2·6H2O and CuCl2·2H2O were obtained from Merck.

**Radioactive Kinase and Phosphatase Assay**

The assay mixture for in vitro phosphorylation of HPr(His)/Crh(His)6 contained 50 mM Tris buffer (pH 8), 5 mM MgCl2, 0.5 mM ATP, 3.3–5 Bq (leading to 200–300 cpm of [γ-32P]ATP/pmol of ATP) (Amersham Biosciences), 2 mM FBP, 0.1% bovine serum albumin, 10 μM HPr/Crh, and, to initiate the reaction, 100 mM HPr/K(P(Trx-His6-S-tag)) in a final volume of 20 μl. The mixture was incubated at 37 °C for 10 min, and the phosphorylation reaction was then terminated by spotting samples onto 1 × 1-cm P81 phosphocellulose paper (Whatman, Maidstone, UK) and dropped immediately into a beaker containing 75 mM H3PO4. The total volume of phosphoric acid solution used was ~10 ml for each paper. Unreacted ATP was removed by washing three times with 75 mM H3PO4, for 15 min each, and once with ethanol, just covering the papers, for 5 min. The papers were dried and transferred to scintillation vials containing 6 ml of scintillation solution for water samples, RotiZint ecopuro (Carl Roth, Karlsruhe, Germany), and the radioactivity was determined in a scintillation counter, LKB 1211 Rackbeta (PerkinElmer Life Sciences). Typically, each condition was tested in triplicate.

For the phosphatase assay, 10 mM inorganic phosphate was used, and the reaction was allowed to proceed for 10 min before termination.
Effects of Different Glycolytic Intermediates—Glycolytic intermediates were tested at the following concentrations: 1 mM acetyl phosphate, 2 mM FBP, 1 mM F2,6BP, 0.5 mM d-glucose 6-phosphate, 1 mM dL-glyceraldehyde 3-phosphate, 1 mM NAD', 0.1 mM NADH, 0.5 mM acetyl coenzyme A, and 1 mM pyruvate. Up to 40 mM FBP and F2,6BP were tested in a phosphatase assay. Inorganic phosphate was omitted when evaluating the potential of acetyl phosphate and dL-glyceraldehyde 3-phosphate as possible candidates to induce phosphatase activity.

Kinetic Parameters for HPK/Crh and ATP—To determine the kinetic parameters for HPK/Crh, the concentration of the protein was varied from 0.1 to 200 μM while keeping the concentration of the second substrate, ATP, at saturation (2 mM) with 7 mM MgCl₂. When the kinetic parameters for ATP were determined, the concentration of HPK/Crh was 200 and 160 μM, respectively, and the concentration of ATP was varied between 1 μM and 1 mM. The concentration of MgCl₂ was kept with an excess of 5 mM over the total ATP concentration to have a constant proportion of ATP existing as MgATP²⁻ and a constant concentration of free Mg²⁺ (35). The initial velocity was determined after 10 min of incubation.

Estimation of Kinetic Parameters—The program GraphPad Prism was used to determine Kₘ(ₗ) (the half-saturation constant in the Hill equation), the Hill coefficient (h), and Vₘₗₗₗ using the following equation: Y = Vₘₗₗₗ[S] / (Kₘ(ₗ) + [S]ⁿ), where S is the substrate concentration.

RESULTS AND DISCUSSION

Characterization of the Oligomeric State of the Purified Recombinant Proteins

ESI mass spectra were first recorded under denaturing conditions of the different proteins, HPK(His)₆, Crh(His)₆, and HPK/P(Trx-His₆-S tag), to verify purity and homogeneity, and to determine the molecular weight of constitutive subunits. The molecular mass determined for HPK(His)₆ was 10,688.5 ± 0.2 Da, which is in agreement with the theoretical value of 10,687 Da calculated from the amino acid sequence (data not shown). A minor compound (~10%) with a mass of 10,768.6 ± 0.2 Da was also detected, corresponding most probably to the phosphorylated form of the protein (ΔM = 80 Da). For Crh(His)₆, the main species detected (~85%) had a mass of 10,392.5 ± 0.2 Da, which is in agreement with the theoretical value of 10,390 Da (data not shown). About 15% of the signals were converted to a molecular mass of 10,261.6 ± 0.1 Da, which is probably due to the loss of the N-terminal Met (ΔM = 131 Da) from Crh. The different constructions of HP and Crh with a His tag at the N terminus preceded by a Met, Arg, Gly, and Ser for HP and a His tag on the C terminus and a Met followed by Val on the N terminus for Crh could be a reason for the differences observed in the mass spectra. A possible explanation for the findings that a part of HP was phosphorylated, but not Crh, may be due to the fact that HP was phosphorylated at His-15 by the EI during the purification procedure while the plasmid was expressed in Escherichia coli. In Crh the His in position 15 is exchanged by a Gln, and no phosphorylation has been demonstrated in vitro by phosphoenolpyruvate and EI (14). ESI-MS analysis of HPK/P(Trx-His₆-S tag) under denaturing conditions revealed a highly homogeneous sample with a single species of 51,700.7 ± 1.0 Da, which is in agreement with the theoretical value, 51,699 Da, taking into account the loss of the N-terminal Met (data not shown).

In addition to the use of ESI-MS for the evaluation of the purity and homogeneity of the proteins, the technique has also become a useful method to study noncovalent complexes (for recent reviews, see Refs. 36–38). ESI-MS analysis under native conditions of the proteins revealed only monomers of HPK(His)₆ and Crh(His)₆ (10 mM ammonium acetate buffer (pH 6.8), data not shown). Using other biophysical techniques, circular dichroism and NMR, HP was reported to be monomeric, whereas Crh was detected both as monomer and dimer (39). Substantially different experimental conditions presumably explain the different results obtained by ESI-MS and NMR. ESI-MS analysis unambiguously revealed that HPK/P(Trx-His₆-S tag) from B. subtilis is a specific noncovalent hexamer at pH 6.8 with a measured molecular mass of 310,337 ± 22 Da (Fig. 1A). The molecular mass obtained under native conditions is 0.05% higher than the mass predicted from the denatured analysis of HPK/P(Trx-His₆-S tag). This mass difference is very low compared with other mass measurements analyzing noncovalent subassemblies (40–42) and is not significant given the uncertainty of the measurements in native conditions. However, this difference is presumably due to the inclusion of water molecules or small cations that are not present in the denatured monomer. Despite the discrepancy, there is no doubt that HPK/P(Trx-His₆-S tag) exists as a hexamer at pH 6.8. The ESI-MS analysis is not in agreement with earlier reported results performed by size exclusion chromatography and analytical ultracentrifugation, which suggest that the enzyme is an octamer or possibly a heptamer (17). However, a precise quantification and an accurate determination of the molecular weight of oligomeric proteins are not directly and precisely determined with these latter techniques. The results from ESI-MS measurements are more precise and consistent with the x-ray crystallography experiments of HPK/P from Lactobacillus casei showing that the catalytic domain of the enzyme is a hexamer in the crystal, suggesting the same state in solution (43). The x-ray structure was solved at pH 5.2 with the truncated HPK/P with an N-terminal fragment missing. Whereas the N-terminal fragment is poorly conserved in the enzyme from different bacteria, the remaining residues, including the putative ATP- and HPK-binding domains, are highly conserved (43). Recently, full-length HPK/P from Staphylococcus xylosus was crystallized (pH 7.6), also supporting a hexameric structure of the enzyme (44). Furthermore, full-length Mycoplasma pneumoniae HPK/P(His)₆ was also reported to be a hexamer as revealed by biophysical and crystallographic data (pH 7.5) (45).

Since the conditions for ESI mass measurements differed from the radioactive kinase assay (see below), due to different buffer solutions and an additional desalting procedure, the kinase activity of HPK/P was also tested under the same conditions as those used for mass spectrometry. Using 10 mM ammonium acetate (pH 6.8) as buffer, the native enzyme was found to be fully active.

Divalent Cations Are Necessary for Both the Kinase and Phosphatase Activity

In the absence of divalent cations, neither kinase nor phosphatase activity was detected. The ion required to produce maximal kinase activity, when HP was included in the assay, was Mg²⁺ (Fig. 2A), which is in agreement with previously published results for HPK/P and HP from B. subtilis (11). When Crh was used as a substrate, Mg²⁺ gave the same degree of phosphorylation as the divalent cation Mn²⁺. However, for HP, a somewhat lower activity was observed with Mn²⁺ compared with Mg²⁺. Some activity was also observed with Co²⁺, more for HP than when Crh was used as a substrate. For Ca²⁺ and Cu²⁺, no activity was observed for either HP or Crh under the conditions used. The effect of divalent cations on HP phosphorylation by HP kinase from other Gram-positive bacteria with low GC content has also been investigated. For HP kinase from Streptococcus salivarius, Mg²⁺ was the preferred cation, but activity was also observed in the presence of Mn²⁺ and Cu²⁺ (46). No effect was seen for Ca²⁺ and Cu²⁺ (46). HP kinase from Streptococcus pyogenes was maximally activated by Mg²⁺ and Mn²⁺, and no activity was observed for the cations Sn²⁺, Ni²⁺, and Cu²⁺ (47). For the different HP kinases, Mg²⁺ seems to be the preferred divalent cation.

For the phosphatase assay, activity was observed in the pres-
ence of Mg\(^{2+}\) and Mn\(^{2+}\) (Fig. 2B). However, in this case, the same activity was obtained with either Mg\(^{2+}\) or Mn\(^{2+}\) when including HPr(Ser(P)) as the substrate, but in the case of Crh(Ser(P)) a more pronounced effect was observed with Mg\(^{2+}\) compared with Mn\(^{2+}\) on dephosphorylation of the proteins. For Co\(^{2+}\), Ca\(^{2+}\), and Cu\(^{2+}\), no major effect on the phosphatase activity was detected.

The HPrK/P was completely dependent on divalent cations for kinase activity. In general, protein kinases require metal ions, preferentially Mg\(^{2+}\), for catalysis (48). The metal ion neutralizes charge and orients and polarizes the \(-\text{phosphoryl}\) group, which facilitates the phosphorylation reaction (48, 49). Most protein kinases seem to bind two metal ions that surround the triphosphate of ATP (48). Although divalent metal ions other than Mg\(^{2+}\) can be involved, Mg\(^{2+}\) is considered the physiological activator in, at least, eukaryotes, due to its high concentration in the cell (48). For protein kinases in general, maximal activity is usually also observed with Mg\(^{2+}\) except for most tyrosine kinases, which are maximally activated by Mn\(^{2+}\) (50). The phosphatase activity of HPrK/P also required the addition of metal cations for catalysis, which is a common contributing factor for catalysis among Ser(P)/Thr(P) protein phosphatases (49).

Is pH Involved in the Switch between the Kinase and Phosphatase Activity?

Effect of pH on the Oligomeric State of the Enzyme Probed by Mass Spectrometry—Under carefully controlled operating conditions (accelerating voltage set to 200 V and the pressure in the interface region was 6.5 millibars).

Fig. 1. ESI mass spectra of HPrK/P (20 \(\mu\)M) under native conditions at pH 6.8 (A) and pH 9.5 (B). At pH 6.8, the major signals observed in the mass spectrum are due to multiply charged ions of the hexamer. Minor signals reveal the coexistence of small amounts of monomer and dimer. Increasing the pH to 9.5 results in destabilization of the hexamer, and most of the enzyme was detected as a monomer. Traces of dimer and hexamer are also observed. The accelerating voltage was equal to 200 V, and the pressure in the interface region was 6.5 millibars.

Effect of pH on Kinase and Phosphatase Activity by HPrK/P of HPr and Crh—The effect of pH on substrate binding and catalysis was studied using a mixed citric acid/Tris buffer covering the pH range between 5 and 8.7 (Fig. 3). The kinase activity versus pH shows an optimal activity between pH 6.6 and 8.7 for the enzyme and HPr and a maximal activity at pH 8.7 for the enzyme and Crh. To obtain further information regarding enzyme characteristics and to assess the pH stability
of HPrK/P, the enzyme was incubated for 10 min at 37 °C at the indicated pH values in 50 mM citric acid/Tris. After incubation, the residual activity was measured at pH 8.0 for HPr and pH 9.0 for Crh (final concentration of 250 mM Tris) at 37 °C and after incubation for 10 min. A decline in activity was observed at pH below 5.8, but above this value the enzyme retained its activity (data not shown). The decrease in activity between 5.8 and 6.6 when using HPr as substrate and between 5.8 and 8.7 when using Crh as substrate is probably due to improper ionic forms of the active site and/or substrate (51). The decline in activity below pH 5.8 can in part be related to irreversible denaturation of the enzyme (51). Thus, the activity loss between 6.6 and 8.7 for the enzyme and Crh seems to be related more to an improper ionic form of the substrate and/or substrate (51). The decline in activity below pH 5.8 can in part be related to irreversible denaturation of the enzyme (51). Thus, the activity loss between 6.6 and 8.7 for the enzyme and Crh seems to be related more to an improper ionic form of the substrate than to the enzyme, since the enzyme plus Crh does not show this reduction in activity. Regarding the influence of pH on HPr kinase activity from other species, the pH optima for HPr kinase from *S. salivarius* and *Streptococcus mutans* Ingbritt were reported to be 7.5 and 7.0, respectively (46, 52). The pH optimum for the stability of the enzyme from *S. salivarius* after preincubation for 30, 60, and 90 min prior to measuring its activity was 8.0 (46).

The activity and stability of HPrK/P when acting as a phosphatase were also investigated. Whereas the activity, when investigated with HPr(Ser(P)) as substrate, was optimal at pH 6.2 and then decreased successively above and below this value, a different activity was observed when using Crh(Ser(P)) as substrate (Fig. 3). In the latter case, optimal activity was observed between pH 6.2 and 7.5. Stability experiments measuring the activity at pH 6.2 for HPr(Ser(P)) and pH 7.0 for Crh(Ser(P)) after preincubating the enzyme did not influence the activity (data not shown).

FIG. 2. The effect of different divalent cations on kinase activity (Graph A) and phosphatase activity (Graph B) of HPrK/P for HPr (black bars) and Crh (white bars). The relative activity of the phosphorylated proteins from the kinase assay (100%), before adding the phosphate, is illustrated by the gray bar in graph B. The concentration of the divalent cations was 5 mM. The data represent the mean ± S.E. (n = 3).

A pH-dependent switch between the kinase and the phosphatase activities of the bivalent enzyme was observed. The kinase activity was predominant at higher pH, and increasing phosphatase activity was recorded at lower pH. The reason for this switch may be connected to the starvation metabolism of the bacterium. In general, the internal pH of neutralophilic bacteria, including *B. subtilis*, is between 7.5 and 8.0 (53). It has also been demonstrated that *B. subtilis* possesses a rather high cytoplasmic buffering capacity (54). However, entering the sporulation phase in response to starvation for a variety of nutrients, the internal pH of spores from *B. subtilis* was found to be ~6 (55). Thus, the kinase activity is high at pH around 8 when growing in the presence of certain nutrients, whereas decreasing pH favors phosphatase activity when the bacterium is about to enter sporulation due to starvation before metabolic dormancy. Favoring the phosphatase activity leads to relief of CCR/CCA. For another bifunctional enzyme, the 6-phospho-
fructo-2-kinase/fructose-2,6-bisphosphatase from bovine liver, the pH optimum for the kinase activity was 8.5, whereas the phosphatase reaction was maximal at pH 6.5 (56). Thus, a similar pH dependence for the two divergent opposing activities was observed, as for HPrK/P.

The ESI-MS results and the activity data indicate that the state of oligomerization may be an important factor in the switch between the kinase and phosphatase activity. Thus, when the enzyme exists as a hexamer, the phosphatase activity is favored.

In order to analyze this pH switch, we followed the oligomerization state of the enzyme as a function of pH using mass spectrometry, dynamic light scattering, and BIACore techniques. The pH titration by mass spectrometry was done using the previously described conditions.

We used dynamic light scattering to evaluate the changes in the quaternary structure of the HPrK/P as it has been observed from mass spectrometry analysis upon changes of pH. Interestingly, the analysis of histograms of distribution in percentage of mass shows that the apparent molecular masses were about 2-fold higher at pH 6.8 than at pH 9.5, respectively (343 and 143 kDa (data not shown). These average molecular weights calculated for a spherical particle and obtained from eight independent measurements are fully consistent with the existence of the HPrK/P as a hexamer at pH 6.8, whereas at pH 9.5 it more likely forms a trimer, although we cannot exclude the possibility that a dimer would be also compatible with the molecular weight of the particles at pH 9.5 with regard to the shape of the oligomers. We noted the existence of a mixture of particles in size as suggested by the high polydispersity value (around 40%) and the poor fit to a monomodal distribution. We assume that this polydispersity reflected a dynamic equilibrium of the HprK/P between different oligomeric states and a trend to form aggregates as well, since it persisted despite attempts to eliminate high size particles or possible dust particles by centrifugation, filtering, and increasing the ionic strength of the protein samples. Therefore, the regularization in bimodal distribution of radii by percentage of mass as a measurement of lower bound particle size appears to be a suitable approach for surveying the pH effect on the structure of the multimeric HPrK/P. In summary, our results suggest that HprK/P undergoes a transition in its oligomeric state upon the increase of basicity from pH 6.8 to pH 9.5. The trimer (and/or dimer) appears to be a predominant form of the HprK/P at pH 9.5, and the hexamer appears to be the main form at pH 6.8. Fig. 4C shows the pH titration experiments performed by mass spectrometry or by dynamic light scattering. There is a good agreement between the results obtained by those two techniques and a good correlation with the pH titration of the phosphatase activity using HPr as a substrate (Fig. 3A).

We used the BIACore technology and NTA sensor chip to capture pH-dependent different quaternary structures of the HPrK/P. The experiments were designed to measure a different amount of protein depending on whether the nickel was bound to the hexameric, trimeric, dimeric, or monomeric form of the protein. Thus, we generated NTA chips with low density of nickel so that each nickel was bound to only one hexamer of HprK/P at saturation. Under these conditions, we observed that for the same amounts of HprK/P injected on an NTA chip (regenerated with the same density of nickel), the amount of protein bound was about 2 times less when it was loaded in Tris buffer, pH 9.5, than when it was at pH 6.8. Accordingly to the previous results from mass spectrometry and dynamic light scattering studies, this difference was consistent with the binding of hexamer to the NTA-Ni2+ chip when HprK/P is loaded at pH 6.8 and the binding of trimer when loaded at pH 9.5. Furthermore, the analysis of the sensorgrams obtained from the protein loaded at pH 6.8 showed that the level of resonance units decreased rapidly after it had reached a maximum during the phase of binding. This dissociation phase was initiated at the switch between the loading buffer and the running buffer. We explained this effect as a dissociation of the hexamer at the change of pH from 6.8 to 7.4 between both buffers. In order to investigate more thoroughly the kinetic of dissociation, we performed experiments using running buffers similar to the loading buffers, pH 6.8 and 9.5, following each injection of the protein. The analysis of the sensorgram (data not shown) showed that only the protein loaded at pH 6.8 was subject to a sharp decrease of resonance units following the switch to the running buffer, pH 9.5. The absence of such a fast dissociation with the protein loaded at pH 9.5 indicated that this effect was due to the dissociation of the bound oligomers itself rather than to its dissociation from the Ni2+-NTA chip. Both the stoichiometry of the fixation of the HprK/P on the NTA-Ni2+ chip and the kinetics of its dissociation provided further evidence of a transition in the protein oligomeric state dependent on the pH that is consistent with the formation of a hexamer at pH 6.8 and a trimer at pH 9.5.
Altogether, these results strongly suggest that the kinase-phosphatase switch is linked to the oligomeric state of the enzyme. Moreover, BIACORE technology may be used to follow the oligomeric states of the enzyme under different physiological conditions.

The Enzyme Activities for the Two Substrates, HPr and Crh, Are Differentially Affected by Temperature

The temperature effect on enzyme activity was carried out over a temperature range between 4 and 70 °C using 50 mM MOPS (pH 8.0), since this buffer is more stable with temperature (ΔpKₐ°C = −0.006) (33). Using an incubation time of 10 min, the optimum temperature for the kinase activity was recorded to be between 37 and 45 °C for HPr and 45 °C for Crh (Fig. 4). Furthermore, the enzyme activity with HPr and Crh, respectively, differs with a higher degree of phosphorylation for HPr at temperatures below 45 °C than for Crh. The reverse is observed for temperatures above 45 °C (i.e., a higher degree of phosphorylation for Crh than for HPr). In the phosphatase assay, the optimal temperature appears to be 50 °C. No major differences in the behavior of the activity between the two different substrates were observed in this case.

Species of Bacillus are the only Gram-positive bacteria with low GC content, which in addition to HPr, is provided with a second protein substrate, Crh (15). The kinase activity was more stimulated at temperatures below 45 °C using HPr as a substrate, whereas temperatures above 45 °C favored phosphorylation of Crh. The proposed background for B. subtilis when provided with two different protein substrates for HPrKP may deal with CCR/CCA in different ecological environments. In this context, an observation worth noticing is that the gene encoding Crh has been localized in a genome area including operons dealing with catabolic degradation of complex substrates found in roots (7).

The Metabolic State of the Cell, through Different Glycolytic Intermediates, Modulates the Kinase/Phosphatase Balance

Several glycolytic intermediates were tested for potential allosteric effects of HPK/P (Fig. 5). The concentrations were chosen according to representative values recorded for B. subtilis as well as other Gram-positive bacteria. Thus, four intermediates were evaluated at concentrations found in vivo in Streptococcus mutans, another Gram-positive bacterium with low GC content: 0.5 mM α-glucose-6-phosphate, 1 mM NAD⁺, 0.1 mM NADH, and 1 mM pyruvate (57). FBP was initially tested at 2 mM, a concentration found in B. subtilis when grown on d-glucose (58), and F2,6BP was also initially tested at a similar concentration, 1 mM. D-Glyceraldehyde 3-phosphate was included at a concentration of 1 mM, since intracellular concentrations of this intermediate were found to be as high as 0.6 mM in Streptococcus bovis (59). Acetyl coenzyme A and acetyl phosphate were tested at concentrations of 0.5 and 1 mM, respectively. Intracellular concentrations of these two metabolites have been reported to be ~0.2 mM in the Gram-positive bacteria Corynebacterium glutamicum (60).

Fig. 5. The effect of different glycolytic intermediates on kinase (Graph A) and phosphatase activity (Graphs B and C) for HPr (black bars) and Crh (white bars). The intermediates were tested at the following concentrations: 1 mM acetyl phosphate, 2 mM FBP, 20 and 40 mM FBP in a phosphatase assay, 1 mM F2,6BP, 20 and 40 mM F2,6BP in a phosphatase assay, 0.5 mM α-glucose 6-phosphate, 1 mM α-glyceraldehyde 3-phosphate, 1 mM NAD⁺, 0.1 mM NADH, 0.5 mM acetyl coenzyme A, and 1 mM pyruvate. The reference samples in Graph A refer to a kinase assay without any glycolytic intermediate for the two different protein substrates. The reference samples in Graph B refer to the kinase assay (in gray) before proceeding to the phosphatase assay, and the phosphatase assay without any glycolytic intermediate (hatched). The reference sample in Graph C refers to the degree of phosphorylation (in gray) before proceeding to the phosphatase assay. When evaluating the effect of Mg²⁺, no inorganic phosphate was included. In the cases when testing acetyl phosphate and glyceraldehyde 3-phosphate for triggering the phosphatase activity, inorganic phosphate was also omitted. The values in Graphs B and C were normalized to the assay without any intermediate but with the addition of inorganic phosphate using the program GraphPad Prism. The data represent the mean ± S.E. (n ~ 3).
Regarding the kinase activity, FBP and F2,6BP were found to stimulate the phosphorylation reaction (Fig. 5A). FBP stimulated HPr phosphorylation more than F2,6BP, whereas in the case of Crh both intermediates caused the same degree of phosphorylation. On the contrary, inorganic phosphate, acetyl phosphate, and dL-glyceraldehyde 3-phosphate inhibited the kinase activity of HPrK/P. The inhibitory effect of inorganic phosphate on kinase activity has previously been reported for HPr kinases (46, 47, 61–63). To further evaluate the effect of inorganic phosphate, 1 mM inorganic phosphate was included in a kinase assay with increasing concentrations of FBP/F2,6BP. Whereas 10 mM FBP partly restored the kinase activity for both proteins, HPr and Crh, 10 mM F2,6BP had only a small effect regarding the phosphorylation of Crh, and no effect of F2,6BP was observed for the phosphorylation of HPr (data not shown). Similar results with FBP and the capacity of re-storing the kinase activity in the presence of inorganic phosphate were reported for HPrK/P from Lactobacillus casei (63). As a similar experiment was performed to evaluate the possibility to restore the kinase activity in an assay using ATP with 0.1 mM inorganic phosphate in the absence of effector molecules. Increasing concentrations of ATP up to 3 mM only partly re-stored the kinase activity (data not shown).

For the phosphatase activity, F2,6BP was found to inhibit the dephosphorylation of both HPr(Ser(P)) and Crh(Ser(P)) as well as high concentrations of FBP in the case of Crh(Ser(P)) (Fig. 5b). FBP alone only inhibited dephosphorylation of Crh(Ser(P)), and 2 mM ATP alone did not affect the dephosphorylation activity of either of the proteins, whereas 20 mM FBP with the addition of 2 mM ATP inhibited dephosphorylation of both HPr(Ser(P)) and Crh(Ser(P)) (data not shown). Similar results have been reported previously for HPrK/P from B. subtilis (9).

Since acetyl phosphate and glyceraldehyde 3-phosphate inhibited the kinase activity, their role in the phosphatase assay were investigated (Fig. 5c). Both substances seem to have similar potential as a source of inorganic phosphate, thus shifting the enzyme activity to a phosphatase. As in the case with inorganic phosphate, Mg2+ is required for activity. However, phosphatase activity was also observed in the absence of inorganic phosphate, acetyl phosphate, or glyceraldehyde 3-phosphate but in the presence of Mg2+, although not to the same extent. A similar observation, that the enzyme did not require inorganic phosphate to exhibit phosphatase activity, has also been reported for HPrK/P from S. xylosus (64).

Of the evaluated glycolytic intermediates, FBP as well as inorganic phosphate are known as an allosteric activator and inhibitor, respectively, not only for the kinase activity of HPrK/P from B. subtilis (11) but also for the enzymes from, for example, L. casei (63), Lactobacillus brevis (62), S. mutans (61), and S. pyogenes (47). Furthermore, F2,6BP was also found to be an allosteric activator for the kinase activity. The opposing phosphatase activity was found to be regulated by inorganic phosphate, acetyl phosphate, or glyceraldehyde 3-phosphate. Inorganic phosphate is known as an inhibitor of the metabolic status of the cell (16). In addition to the role of acetyl phosphate in acetate metabolism and in the overflow mechanism of excess carbohydrates (29), acetyl phosphate has been identified as a regulator of two-component signal transduction systems such as ComA in B. subtilis (65). Acetyl phosphate has been postulated as a good sensor reflecting the intracellular metabolic state of the cell as an indicator of glucose availability, and the synthesis of acetyl phosphate was found to be necessary for glucose-starved cells of E. coli to survive glucose starvation (66). Thus, reflecting the intracellular metabolic status of the cell in response to changing concentrations of PTS sugars, acetyl phosphate could shift the kinase activity of HPrK/P to a phosphatase. Regulatory properties were also found for glycer-aldehyde 3-phosphate, which induced the phosphatase activity of the enzyme. Recently presented data suggest multiple levels of control of the gapA operon, including the gene encoding glyceraldehyde 3-phosphate dehydrogenase, gapA, involved in the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (67). This enzyme catalyzes an irreversible reaction, and enzymes catalyzing irreversible reactions in the glycolysis were found to be inducible by glucose. Furthermore, the gapA operon was found to be regulated by a repressor, CggR, and CcPa (67). In view of the complex regulation of the gapA operon to modulate the needs of glycolysis and the tricarboxylic acid cycle under different conditions, the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate is strongly regulated. However, the exact role of glyceraldehyde 3-phosphate in the CCR/CCA mechanism requires further evaluation.

Taking into account the proposed relationship between the oligomeric state of the enzyme and its activity, FBP and F2,6BP are suggested to promote the dissociation of the hexamer into dimer and monomer. The presence of inorganic phosphate, acetyl phosphate, and/or glyceraldehyde 3-phosphate, on the other hand, are suggested to induce the formation of the hexamer.

The Different Substrate and Effector Sites are Strongly Coupled

The kinetic parameters for the three substrates, HPr/Crh and ATP, were determined by varying one substrate of interest and keeping the second substrate at a saturating level. When estimating the kinetic parameters for HPr and Crh, the concentration of HPr/Crh was varied between 0.1 and 200 μM, whereas the concentration of ATP, in general, was kept at 2 mM. Two graphs (Fig. 6) illustrate some of the results, and all of the kinetic parameters are listed in Table I.

Results presented in Fig. 6 (Graph B) are nearly identical to results presented in Fig. 5 of Jault et al. (17). This comparison validates the radioactive assay used in this work, whereas in most of the previous work, the enzymatic activity was measured by a gel assay (17).

The enzyme exhibited a positive homotropic response with respect to substrate binding. For the two substrates, HPr and Crh, the affinity was about 5 times higher for HPr, indicating the preference of HPr as a substrate. The addition of the effector molecule, FBP, resulted in increased values for Vmax whereas no major effect on K0.5 was observed. F2,6BP, on the other hand, increased both Vmax and K0.5. Values. Both activators increased the turnover numbers as well as kcat/K0.5. The degree of cooperativity decreased somewhat or was not affected in the presence of the effectors. The inclusion of the negative effector, inorganic phosphate, lowered all of the kinetic parameters except the Hill coefficient for Crh.

Furthermore, the possibility of an interaction between the HPr/Crh and ATP sites was also investigated. The concentration of ATP was decreased for the examination of whether lowered concentrations of the phosphate donor would alter the observed kinetic behavior of HPr/Crh. When the concentration of ATP was decreased 4-fold, the Hill coefficient decreased in the case with HPr and increased in the case with Crh. Thus, the binding of ATP to the enzyme seems to induce and/or influence the positive cooperative binding of HPr/Crh to HPrK/P.

To estimate the kinetic parameters for ATP, the concentration of ATP was varied between 1 μM and 1 mM, and the concentration of HPr/Crh was kept at 200 and 169 μM, respectively. In the absence of effector molecules, the enzyme exhibited low affinity for ATP. The addition of FBP/F2,6BP lowered both the K0.5 and Vmax when using HPr as the second sub-
strate. However, the opposite effect, with increasing values for $V_{\text{max}}$, was observed when including Crh in the assay. Whereas the $k_{\text{cat}}$ values decreased in the case with HPr, an increase was observed for Crh. In both cases, however, the quotient of $k_{\text{cat}}/K_{\text{m}}$ increased. For the Hill coefficient, no obvious explanation for either the increase or decrease in cooperativity after the addition of FBP/F2,6BP can be offered. Experiments to determine the kinetic parameters at pH 7.4 for the different substrates were also performed without effector molecules (data not shown). The results indicate that the positive cooperativity was more pronounced at pH 8.0 than at pH 7.4. In addition, the values for the turnover number and $k_{\text{cat}}/K_{\text{m}}$ were markedly lowered, except for the turnover number for HPr, which remained the same.

Attempts to determine kinetic parameters in a phosphatase assay failed. Under the different concentrations of phosphorylated substrates that we were able to use due to our incapacity to purify high concentrations of phosphorylated substrate, we were not able to obtain a simple and complete saturation curve. Therefore, we were not able to obtain kinetic parameters using the Hill equation (see “Experimental Procedures”).

CONCLUSION

A model for the proposed regulation of HPrK/P with HPr as substrate is illustrated in Fig. 7. The switch between kinase and phosphatase activity is probably correlated to the oligomeric state of the enzyme, where the hexamer favors the phosphatase activity and the trimeric form of the enzyme favors the kinase activity. Fieulaine et al. (43) proposed a model where the shift from kinase to phosphatase activity may be due to a direct competition between ATP and inorganic phosphate. An observation supporting an additional mechanism is that phosphatase activity was also observed even in the absence of inorganic phosphate but in the presence of Mg$^{2+}$. The same phenomenon, no requirement for inorganic phosphate to exhibit phosphatase activity, was reported for HPrK/P from S. xylosus (64). However, a close relationship seems to exist between the kinase and phosphatase activity supported by point mutations of Asp-176 (68). In addition, several mutants of HPrK/P from B. subtilis and L. casei affect phosphatase activity, but with almost normal kinase activity, and no mutant specifically affecting kinase activity has been found (8, 69). Some of the mutations, related with reduced phosphatase activity, are located on the surface in a direct contact with another subunit, suggesting that the mutation may influence the oligomeric state of the enzyme (8, 69).

The internal pH of vegetative cells, ~8, will dislocate the balance to the kinase activity, whereas starvation conditions,

Table I

| HPr | Crh | MgCl$_2$ | ATP | FBP | F2,6BP | $P_i$ | $V_{\text{max}}$ ± S.E. | $K_{\text{m}}$ ± S.E. | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{\text{m}}$ × 10$^4$ |
|-----|-----|---------|-----|-----|--------|-----|----------------|----------------|------------|----------------|
| μM | μM | mM | mM | mM | mM | mM | pmol/min | μM | s$^{-1}$ | μM$^{-1}$ s$^{-1}$ |
| Varied  | 5 | 0.5 | 18 ± 2 | 1.4 ± 0.2 | 31 ± 7 | 0.3 | 1.1 |
| Varied  | 7 | 2 | 33 ± 1 | 2.3 ± 0.2 | 34 ± 2 | 0.2 | 0.8 |
| Varied  | 7 | 2 | 14 ± 1 | 1.6 ± 0.2 | 25 ± 2 | 0.2 | 0.8 |
| Varied  | 7 | 2 | 100 ± 6 | 1.9 ± 0.3 | 42 ± 4 | 1.9 | 4.6 |
| Varied  | 7 | 2 | 160 ± 20 | 1.5 ± 0.2 | 100 ± 20 | 3.0 | 3.0 |
| Varied  | 7 | 2 | 2 | 140 ± 14 | 2.2$^*$ | 2900 ± 200 | 2.6 | 0.1 |
| 200 | 5 in excess | Varied  | 97 ± 2 | 3.1 ± 0.3 | 230 ± 7 | 1.8 | 0.8 |
| 200 | 5 in excess | Varied  | 29 ± 1 | 2.0 ± 0.1 | 580 ± 20 | 0.4 | 0.1 |
| Varied  | 5 | 0.5 | 47 ± 2 | 1.8 ± 0.1 | 63 ± 4 | 0.9 | 1.4 |
| Varied  | 7 | 2 | 120 ± 9 | 1.3 ± 0.1 | 140 ± 20 | 2.3 | 1.6 |
| Varied  | 7 | 2 | 0.5 | 52 ± 3 | 1.6 ± 0.2 | 63 ± 6 | 0.7 | 1.2 |
| Varied  | 7 | 2 | 200 ± 40 | 1.2 ± 0.2 | 140 ± 50 | 3.7 | 2.8 |
| Varied  | 7 | 2 | 2 | 400 ± 80 | 1.4 ± 0.1 | 330 ± 80 | 7.5 | 2.3 |
| 169 | 5 in excess | Varied  | 53 ± 3 | 2.3 ± 0.1 | 670 ± 40 | 0.8 | 0.1 |
| 169 | 5 in excess | Varied  | 100 ± 4 | 1.5 ± 0.1 | 200 ± 20 | 1.9 | 1.0 |
| 169 | 5 in excess | Varied  | 100 ± 1 | 3.2 ± 0.1 | 330 ± 3 | 1.5 | 0.4 |

$^*$ This parameter was held constant due to the low affinity of the phosphate donor to the enzyme and thus made the determination of kinetic parameters problematic.
Properties and Regulation of HPrK/P in B. subtilis

Fig. 7. A proposed model for the regulation of HPrK/P illustrated for the substrate HPr. The switch between kinase and phosphatase activity is probably correlated to the oligomeric state of the enzyme regulated by different glycolytic intermediates (e.g. FBP, acetyl phosphate and inorganic phosphate, as well as the pH reflecting the metabolic state of the cell). F2,6BP was further found to inhibit the dephosphorylation of HPr(Ser(P)) as well as Crh(Ser(P)), but regarding the enzyme, divalent cations were necessary for the reactions not only to neutralize the phosphate group of ATP or HPr(Ser(P))/Crh(Ser(P)) but possibly also through a specific site by modulating the conformational state of the enzyme. The enzyme, which shows a positive cooperativity for the binding of the two substrates, HPr and ATP, also proved to have a kinetic coupling between the two different binding sites.

In this study, proteins were expressed in E. coli and fused with different tags (His6 and Trx-His6-S) in order to facilitate their purification and to obtain a high yield of a homogenous population of proteins. The different tags were not removed, assuming that they were not going to interfere with the behavior of the enzyme. Comparison of some specific experiments from our laboratory and other groups using different constructions support this assumption (data not shown) (39). However, it can not be ruled out that the wild-type enzyme may behave in a somewhat different manner. Nonetheless, the proposed model is assumed to be also applicable for the wild-type enzyme.

In summary, the bifunctional HPrK/P from B. subtilis involved in the main mechanism of CCR/CCA is a strongly regulated allosteric enzyme. The differences in response obtained regarding phosphorylation and dephosphorylation of the proteins under different in vitro conditions (e.g. regulation by effector molecules and in cooperativity) could be a means by which bacteria “fine tune” the regulation of metabolic pathways. Thus, the enzyme HPrK/P is involved in a sophisticated regulation system, sensing environmental changes in the availability of carbon sources to adapt the uptake and use of nutrients in a hierarchical manner in at least two different ecological niches.

Acknowledgments—We thank Louis Cuccia for valuable linguistic advice and Mireille Gaillat for helpful scientific advice. We thank Virginie Lafont and Daniele Altschuh for technical support with the BIACORE system as well as Philippe Dumas and Bernard Lorber for support with the dynamic light-scattering apparatus.

REFERENCES

1. Hunter, T. (1995) Cell 80, 225–236
2. International Human Genome Sequence Consortium (2001) Nature 409, 860–921
3. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Wortman, J. R., et al. (2001) Science 291, 1304–1315
4. Noonberg, S. B., and Benz, C. C. (2000) Drugs 59, 753–776
5. Sedlacek, H. H. (2000) Drugs 59, 435–476
6. Traxler, P., Bold, G., Buchdunger, E., Caravatti, G., Fontet, P., Manley, P., O’Reilly, T., Wood, J., and Zimmermann, J. (2001) Mol. Rev. 21, 499–512
7. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bacino, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borris, R., Bouvier, L., Brans, A., Braun, M., Brignell, S. C., Brun, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capua, V., Carter, N. M., Choi, S. K., Codani, J. J., Conerton, I. F., Danchin, A. et al. (1997) Nature 390, 439–456
8. Hansson, K. G., Steinhauer, K., Reizer, J., Hillen, W., and Stulke, J. (2002) Microbiology 148, 1805–1811
9. Kravanja, M., Engelmann, R., Dosonnet, V., Bluegg, M., Meyer, H. E., Frank, R., Galinier, A., Deutscher, J., Schill, W., and Hengstenberg, W. (1999) Mol. Microbiol. 312, 59–66
10. 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
11. Reizer, J., Hoischen, C., Tsigemeyer, F., Rohrta, C., Rahnus, R., Stulke, J., Karamata, D., Saier, M. H., Jr., and Hillen, W. (1998) Mol. Microbiol. 27, 1157–1169
12. Turinsky, A. J., Grundy, F. J., Kim, J. H., Chambless, G. H., and Henkin, T. M. (1998) J. Bacteriol. 180, 5961–5967
13. Galinier, A., Deutscher, J., and Martin-Verastraete, I. (1999) J. Mol. Biol. 286, 307–314
14. Galinier, A., Haiech, J., Kilhoffer, M. C., Jaguinod, M., Stulke, J., Deutscher, J., and Martin-Verastraete, I. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 8439–8444
15. Darben, E., Galinier, A., Le Coq, D., and Deutscher, J. (2001) J. Mol. Microbiol. Biotechnol. 3, 439–444
16. Mason, P. W., Carbone, D. P., Cushman, R. A., and Waggner, A. S. (1981) J. Biol. Chem. 256, 1861–1866
17. Janss, J. M., Fierlaine, S., Nessler, S., Gonzalo, P., Di Pietro, A., Deutscher, J., and Galinier, A. (2000) J. Biol. Chem. 275, 1773–1780
18. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) FEMS Microbiol. Rev. 1157–1169
19. Jault, J. M., Fieulaine, S., Nessler, S., Gonzalo, P., Di Pietro, A., Deutscher, J., and Galinier, A. (1999) Mol. Microbiol. 312, 59–66
20. Grundy, F. J., Turinsky, A. J., and Henkin, T. M. (1994) Mol. Microbiol. 14, 275–287
21. Jacob, S., Allmansberger, R., Gartner, D., and Hillen, M. (1991) J. Mol. Microbiol. 1828–1823
22. Dahl, M. K., and Hillen, W. (1995) Mol. Microbiol. 17, 4527–4533
23. Miwa, Y., and Fujita, Y. (1999) Nucleic Acids Res. 28, 7049–7053
24. Fujita, Y., Miwa, Y., Galinier, A., and Deutscher, J. (1995) Mol. Microbiol. 17.
Properties and Regulation of the Bifunctional Enzyme HPr Kinase/Phosphatase in
*Bacillus subtilis*
Helena Ramström, Sarah Sanglier, Emmanuelle Leize-Wagner, Claude Philippe, Alain
Van Dorsselaer and Jacques Haiech

*J. Biol. Chem.* 2003, 278:1174-1185.
doi: 10.1074/jbc.M209052200 originally published online October 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209052200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 28 of which can be accessed free at
http://www.jbc.org/content/278/2/1174.full.html#ref-list-1