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Structural Analysis of the Bacterial HPr Kinase/Phosphorylase V267F Mutant Gives Insights into the Allosteric Regulation Mechanism of This Bifunctional Enzyme*

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The HPr kinase/phosphorylase (HPrK/P) is a bifunctional enzyme that controls the phosphorylation state of the phospho-carrier protein HPr, which regulates the utilization of carbon sources in Gram-positive bacteria. It uses ATP or pyrophosphate for the phosphorylation of serine 46 of HPr and inorganic phosphate for the dephosphorylation of Ser(P)-46-HPr via a phosphorolysis reaction. HPrK/P is a hexameric protein kinase of a new type with a catalytic core belonging to the family of nucleotide-binding protein with Walker A motif. It exhibits no structural similarity to eukaryotic protein kinases. So far, HPrK/P structures have shown the enzyme in its phosphorylase conformation. They permitted a detailed characterization of the phosphorolysis mechanism. In the absence of a structure with bound nucleotide, we used the V267F mutant enzyme to assess the kinase conformation. Indeed, the V267F replacement was found to cause an almost entire loss of the phosphorylase activity of *Lactobacillus casei* HPrK/P. In contrast, the kinase activity remained conserved. To elucidate the structural alterations leading to this drastic change of activity, the x-ray structure of the catalytic domain of *L. casei* HPrK/P-V267F was determined at 2.6 Å resolution. A comparison with the structure of the wild type enzyme showed that the mutation induces conformation changes compatible with the switch from phosphorylase to kinase function. Together with nucleotide binding fluorescence measurements, these results allowed us to decipher the cooperative behavior of the protein and to gain new insights into the allosteric regulation mechanism of HPrK/P.

In Gram-positive bacteria, HPr kinase/phosphorylase (HPrK/P)⁶ is the central regulator of carbon catabolite repression, the paradigm of signal transduction (1). In response to certain metabolites that change their concentration in the presence of rapidly metabolizable carbon sources, HPrK/P either phosphorylates or dephosphorylates Ser-46 of the histidine-containing protein HPr. In Gram-positive bacteria, Ser(P)-46-HPr functions as co-repressor for carbon catabolite repression by interacting with the transcriptional regulator CcpA (catabolite control protein A) that regulates the expression of many genes (2, 3) (for a review, see Ref. 4). In the Gram-negative *Neisseria meningitidis* devoid of CcpA-mediated carbon catabolite repression, Ser(P)-46-HPr is involved in the cell adhesion process (5), suggesting that HPrK/P might constitute a potential target for new antimicrobial agents in pathogenic bacteria. Finally, the formation of Ser(P)-46-HPr inhibits PrfA, a transcription activator of several virulence genes in *Listeria monocytogenes* (6).

HPrK/P was first identified as a serine protein kinase, and this activity is enhanced in the presence of glycolytic intermediates such as fructose-1, 6-bisphosphate (FBP) and inhibited in the presence of inorganic phosphate (P_i) (7). Dephosphorylation of Ser(P)-46-HPr by HPrK/P was assumed to be hydrolytic. However, P_i was also reported to stimulate the HPrK/P-catalyzed dephosphorylation of Ser(P)-46-HPr, which was surprising as P_i was expected to be the product of the phosphatase reaction. In fact, Ser(P)-46-HPr dephosphorylation turned out to follow an unusual mechanism. P_i functions as substrate and carries out a nucleophilic attack on the phosphorous atom of Ser(P)-46-HPr, thus leading to the formation of pyrophosphate (PP_i) and HPr. Ser(P)-46-HPr dephosphorylation is therefore a phosphorolysis and not a hydrolysis reaction (8). Actually, it is the reversal of the PP_i-dependent HPr phosphorylation. *In vitro*, the HPrK/P kinase activity accepts both ATP and PP_i as phosphate donor. However, only the ATP-dependent kinase activity is stimulated by FBP.

The crystal structures of HPrK/P of *L. casei* (9), *Staphylococcus xylosum* (10), and *Mycoplasma pneumoniae* (11) showed similar tertiary and quaternary structures (12). HPrK/P is a homohexamer with subunits of ~300 residues folding in two

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The atomic coordinates and structure factors (code 2QMh) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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⁶ The abbreviations used are: HPrK/P, HPr kinase/phosphorylase; FBP, fructose-1, 6-bisphosphate; PP_i, pyrophosphate; P-loop, phosphate binding loop; FRET, fluorescence resonance energy transfer; r.m.s.d, root mean square deviation; MantADP, 2'-/3'-O-(N'-methylanthraniloyl) ADP.

distinct structural domains. The C-terminal domain carries both kinase and phosphorylase activities, whereas the N-terminal domain has no defined function yet. The C-terminal domain contains a Walker A motif forming the phosphate binding loop (P-loop) of the nucleotide-binding site. The HPrK/P fold is therefore different from that of eukaryotic protein kinases but resembles small molecule kinases from the P-loop-containing protein family (13). Thus, HPrK/P represents the first member of a novel family of protein kinases (14).

The structure of the complex between the *L. casei* HPrK/P C-terminal domain (Δ HPrK/P) and its protein substrate Ser(P)-46-HPr (15) allowed us to describe the catalytic mechanism of the phosphorylase reaction. The structure of the complex between *L. casei* Δ HPrK/P and the protein substrate of the ATP-dependent kinase activity, unphosphorylated HPr, was similar and has been obtained in the absence of a nucleotide (15). These two complexes adopt the phosphorylase conformation and prevent a modeling of ATP in the conserved nucleotide-binding site. They resemble the uncomplexed HPrK/P hexamer in which a conserved region called the central loop (residues 265–275) of a subunit clashes with the base moiety of ATP modeled into the P-loop of an adjacent subunit (9).

Kinetic measurements of ATP-dependent HPr phosphorylation provided sigmoid curves and therefore suggested that ATP binds to *Bacillus subtilis* HPrK/P with strong positive cooperativity (16). This result was confirmed by fluorescence studies using the unique tryptophan of *B. subtilis* HPrK/P in either intrinsic fluorescence measurements or fluorescence resonance energy transfer (FRET) experiments with the fluorescent nucleotide analogue MantADP (2'-/3'-O-(N'-methylanthraniloyl) ADP (16). Quenching of the tryptophan fluorescence by iodide further suggested that HPrK/P exists in two conformations. ATP binding, but not P_i binding, modifies the fluorescence properties of the unique tryptophan of the enzyme, indicating that the conformational change is induced by the binding of the base and sugar moieties of the nucleotide outside of the P-loop (17).

Positive cooperativity was also reported for FBP (16). The binding site of this effector is still unknown, but mutant studies (17, 18) suggest that it might bind in the capping motif (residues 197–222) corresponding to the signature sequence of HPrK/P (9). Addition of FBP does not increase the affinity of the enzyme for ATP and rather stimulates the kinase activity at low ATP concentrations (16). FBP binding is supposed to stabilize the kinase conformation (17) that still needs to be determined. Despite numerous co-crystallization or soaking attempts using ATP analogues, we were unable to obtain HPrK/P in complex with a nucleotide.

Random mutagenesis experiments permitted isolation of *L. casei* HPrK alleles encoding mutant HPrK/Ps exhibiting low phosphorylase but normal kinase activity (19). The mutations exclusively affected the P-loop and the central loop. The lowest phosphorylase activity was observed for the V267F mutation in the central loop, which led to a similar effect when introduced into *B. subtilis* HPrK/P (19). Here, we describe the crystal structure of the V267F mutant of *L. casei* Δ HPrK/P at 2.6 Å resolution. The conformation changes caused by the V267F replacement allowed the modeling of ATP into the active site.

Furthermore, fluorescence experiments aimed at studying the ATP binding mode of the mutant protein allowed us to gain insights into the allosteric behavior of the enzyme.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—A *L. casei* *hprK* allele encoding amino acid residues 128–319 (20) and carrying the V267F mutation (19) was cloned into the pQE30 plasmid as described for the corresponding wild type gene (9). The resulting protein (Δ HPrK/P-V267F) carries an N-terminal histidine tag. The plasmid was used to transform *Escherichia coli* M15, and transformants were grown in 2TY medium at 37 °C to an A_{600} of 0.8. The synthesis of Δ HPrK/P-V267F was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside for 3 h. Cells were harvested by centrifugation for 10 min at 6000 \times g and resuspended in lysis buffer (25 mM Na/Hepes, pH 7.5, 100 mM NaCl). After sonication and centrifugation for 30 min at 25,000 \times g, the clarified lysate was applied to a Fast-Flow nickel-nitrilotriacetic acid-agarose column (Qiagen). Δ HPrK/P-V267F was eluted with 25 mM Na/Hepes, pH 7.5, 150 mM NaCl, 300 mM imidazole, applied onto a Superdex 200 HL 16/60 gel filtration column (GE Healthcare), and eluted with 25 mM Na/Hepes buffer, pH 7.5, containing 100 mM NaCl.

The R245A *hprK* allele was obtained by PCR by using appropriate mutagenic oligonucleotides, and the presence of the mutation was confirmed by DNA sequencing. The R245A *hprK* allele was cloned into pQE30 by using the same enzymes as for the wild type and the V267F *hprK* alleles. The His-tagged protein Δ HPrK/P-R245A was synthesized in *E. coli* NM522 and purified by following the procedure described above.

Crystallization, Data Collection, and Structure Determination—Crystals were grown in hanging drops containing 150 μ M Δ HPrK/P-V267F, 10% (v/v) 2-methyl-2,4-pentanediol (MPD), 0.1 M sodium acetate, and 0.05 M sodium citrate, pH 5.4, over pits containing 20% (v/v) MPD, 0.2 M sodium acetate, 0.1 M sodium citrate, pH 5.4. Cryoprotection was achieved by soaking the crystals in a mother solution containing 25% (v/v) MPD prior to flash freezing in liquid nitrogen. Diffraction data were collected at 100 K to 2.6 Å resolution on an ADSC Quantum Q4R CCD detector from a single crystal on beamline ID14-H1 at the European Synchrotron Radiation Facility (Grenoble, France). Data processing was performed using MOSFLM (21) and the CCP4 suite (22). The structure was solved by molecular replacement using the program Phaser (23) and a trimer of *L. casei* wild type Δ HPrK/P (PDB code 1KKM) (15) lacking the C-terminal helix (residues 293–310) and the so-called flexible loop (residues 236–258) (9) as starting model. Four trimers forming two hexameric biological units compose the asymmetric unit. Model refinement was performed using REFMAC (24), and the electron density maps were evaluated using COOT (25). Overall statistics are given in Table 1. Figs. 1, 2, and 4 were produced using PyMol.

Fluorescence Measurements—All experiments were performed at 25 °C using a Cary Eclipse spectrofluorometer (Varian) with quartz cuvettes QS 180 (Hellma). Fluorescence measurements were carried out after appropriate dilution of Δ HPrK/P and equilibration for 10 min in 800 μ l of a buffer containing 25 mM Na/Hepes, pH 8.2, 100 mM NaCl. Increasing

Structural Analysis of Δ HPrK/P-V267F Mutant

TABLE 1
Crystallographic data

Data collection	
Space group	P1
Unit cell parameters (Å)	a = 69.1, b = 106.4, c = 106.5 $\alpha = 119.5^\circ, \beta = 90^\circ, \gamma = 89.9^\circ$
Resolution range (Å)	30–2.6 (2.74–2.6)
Unique reflections	80370 (10588)
Completeness (%)	99.3 (97.3)
I/σ	13 (1.9)
R_{sym} (%) ^a	13.9 (42)
Refinement	
R (%) ^b	21.5
R_{free} (%) ^c	29.8
R.m.s.d. of bonds (Å)	0.017
R.m.s.d. of angles (°)	1.9
Mean B factor (Å ²)	56.2

^a $R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of a reflection, and $\langle I \rangle$ is the average intensity of that reflection.

^b $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$.

^c 5% of the data were set aside for free R -factor calculation. The values for the last resolution shell are indicated in brackets.

concentrations of ADP, MantADP, or FBP were added such that the total volume of the sample was not significantly increased. Binding was monitored by the variation of protein intrinsic fluorescence, MantADP extrinsic fluorescence, or FRET. In the nucleotide binding experiments, equimolar nucleotide and magnesium concentrations were used. All spectra were corrected for buffer and/or protein fluorescence.

The intrinsic fluorescence resulting from the single tryptophan residue (Trp-237) of a 1- μ M *L. casei* Δ HPrK/P sample was measured at $\lambda_{\text{exc}} = 295$ nm and $\lambda_{\text{em}} = 350$ nm at 600 V with a 5 nm band pass. The inner filter effect due to nonspecific quenching of tryptophan fluorescence at high ligand concentrations was determined in control experiments with *N*-acetyltryptophanamide (NATA) (Sigma) and subtracted.

MantADP extrinsic fluorescence measurements were performed in the presence of 30 μ M Δ HPrK/P at $\lambda_{\text{exc}} = 350$ nm and $\lambda_{\text{em}} = 440$ nm at 600 V with a 2.5 nm band pass. FRET measurements between Trp-237 and MantADP-Mg were performed with 15 μ M Δ HPrK/P at $\lambda_{\text{exc}} = 295$ nm and $\lambda_{\text{em}} = 440$ nm at 600 V with a 5 nm band pass. For both FRET and MantADP experiments, the fluorescence of equivalent amounts of free MantADP in the absence of Δ HPrK/P was recorded under the same conditions, and the linear regression coefficient of the corresponding curve was used for correction of the binding data (27).

Dissociation constants were calculated using in-house software. After background correction, the concentration dependence of fluorescence intensity was fitted either to a sigmoidal (F_{sig}) (28) or a hyperbolic (F_{hyp}) (29) function following the equations $F_{\text{sig}} = F_{\text{max}} * [S]^n / (K_D^n + [S]^n)$ or $F_{\text{hyp}} = 0.5 * \gamma * (n[E] + [S] + K_D - \sqrt{((n[E] + [S] + K_D)^2 - 4 * n * [E] * [S])})$, where F is the relative intensity, $[E]$ is the concentration of Δ HPrK/P, $[S]$ is the concentration of nucleotide, n is the number of binding sites/molecule of protein, γ is the relative quantum yield coefficient, and K_D is the equilibrium dissociation constant. $[E]$ and $[S]$ were kept constant during the fitting procedure.

Mutant R245A Activity Measurements—Polyacrylamide gel electrophoresis under non-denaturing conditions was used to follow *B. subtilis* HPr phosphorylation and Ser(P)-46-HPr dephosphorylation, because under these conditions the two HPr forms migrate to different positions (30). 67 μ g/ml HPr or

Ser(P)-46-HPr were mixed with 6.7 or 83 μ g/ml HPrK/P in a solution containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and either 5 mM ATP or PPi for the phosphorylation or 5 mM P_i for the dephosphorylation experiments. The assay mixture was incubated for 25 min at 37 °C, and Δ HPrK/P was inactivated by keeping the solution for 5 min at 70 °C prior to separation on a 12.5% non-denaturing polyacrylamide gel.

RESULTS

Structure of *L. casei* Δ HPrK/P-V267F and Comparison with the Wild Type Enzyme—As for the wild type enzyme, Δ HPrK/P-V267F forms a hexamer composed of two trimers (9). The two hexamers present in the unit cell are equivalent with an r.m.s.d. of 0.5 Å for 980 residues excluding the region 236–250 and the C-terminal helix. The fold of the core of the twelve subunits is globally conserved with an r.m.s.d. of 0.49–0.9 Å over 140 C α atoms. Two subunits per hexamer are fully defined in the electron density map, except for a few residues at the N- and C-terminal extremities of the polypeptide chain. In the other subunits, an additional region is missing in the flexible loop. Due to the absence of bound phosphate, the electron density corresponding to the P-loop is poorly defined (11). The C-terminal helix that has already been reported to move upon HPr binding and crystal packing constraints (12) adopts various positions within a range of 15 Å.

The V267F mutation has little effect on the subunit fold. When compared with the wild type enzyme, the average r.m.s.d. value for 140 C α atoms is 1.2 Å. In contrast, it imposes an important conformation change on the quaternary structure leading to a compact hexamer with a global r.m.s.d. of 2.7 Å for 980 C α positions (excluding the region 236–250 and the C-terminal helix). Each trimer rotates along the 3-fold axis by $\sim 7^\circ$ toward the other trimer, thereby increasing the dimer interface by ~ 400 Å²/monomer (Fig. 1A). The trimer interface is unchanged, covering 2000 Å²/subunit. However, the central loops of the trimer undergo an important rearrangement (Fig. 1B).

Loss of Phosphorylase Activity—Superimposition of two subunits of *L. casei* Δ HPrK/P in complex with Ser(P)-46-HPr (PDB code 1KKM) (15) and two equivalently arranged subunits from the V267F mutant HPrK/P shows that the conformation change induced by the mutation leads to a 3 Å shift of Arg-245 away from the phosphoserine residue Ser(P)-46 of HPr (Fig. 2). The loss of the Arg-245-Ser(P)-46 interaction that was proposed to be important for phosphorylation (15) could be responsible for the almost entire loss of phosphorylase activity observed for HPrK/P-V267F (31).

To verify the importance of Arg-245, we purified the Δ HPrK/P-R245A mutant. Ser(P)-46-HPr dephosphorylation experiments followed by separation of the reaction products on non-denaturing polyacrylamide gels revealed that Δ HPrK/P-R245A had lost the phosphorylase function (Fig. 3A). In fact, no Ser(P)-46-HPr dephosphorylation could be detected in a pH range from 6.8 to 8.8 (data not shown). This result confirmed the catalytic role of Arg-245 in the phosphorylase reaction. In addition, HPr phosphorylation experiments showed that the R245A replacement causes a loss of PPi-dependent kinase activity (Fig. 3B) under conditions where the

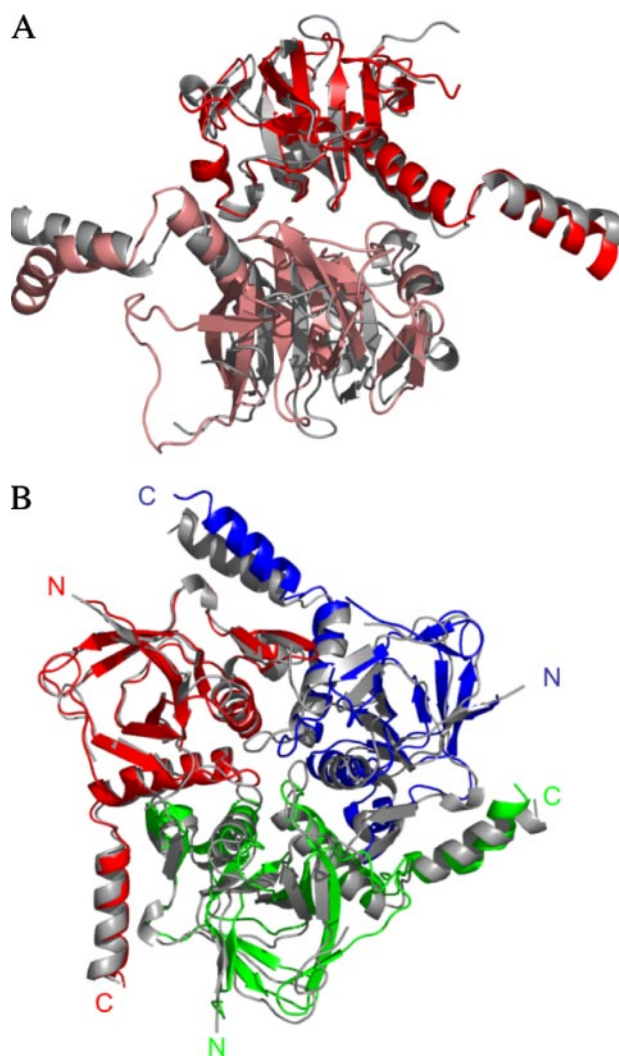


FIGURE 1. Quaternary structure changes induced by the V267F mutation. The red subunit of the V267F mutant protein (in color) has been superimposed on a subunit of the wild type *L. casei* Δ HPrK/P (PDB code 1JB1, in gray) (9). *A*, rearrangement of the dimerization interface. View along a 2-fold axis of the hexamer. The mutation induces a shift of the pink subunit toward the red subunit compared with the wild type dimer. *B*, rearrangement of the trimer. View along the 3-fold axis of the hexameric molecule. The major change concerns the central loops at the center of the molecule.

wild type enzyme completely phosphorylates HPr (data not shown). In contrast, the ATP-dependent kinase activity is not altered by the R245A mutation (Fig. 3*B*).

Nucleotide Binding Analysis—We superimposed each subunit of Δ HPrK/P-V267F with the nucleotide-binding domain of the ATP-bound PEP carboxykinase (PDB code 1AYL) (32), the closest structural homologue of HPrK/P (14). Contrary to the situation described for wild type *L. casei* Δ HPrK/P (9), the conformation changes observed with the Δ HPrK/P-V267F mutant allowed us to directly model bound ATP without any clashes between the base part of the nucleotide and the central loop of the enzyme (Fig. 4).

We used fluorescence measurements to test whether the V267F mutation would alter the affinity of the enzyme for the nucleotide. Using the unique tryptophan (Trp-237) situated at the N-terminal extremity of the flexible loop (residues 236–258), we carried out FRET experiments with the fluorescent

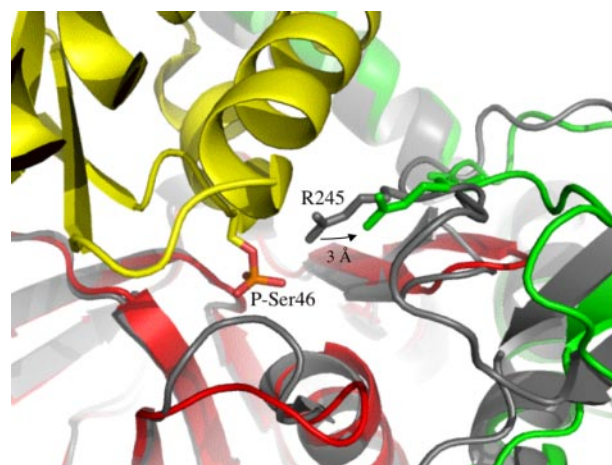


FIGURE 2. Mutation-induced shift of Δ HPrK/P-R245. The red and green subunits of the V267F mutant protein (Fig. 1*B*) are shown with the red subunit superimposed on an *L. casei* Δ HPrK/P subunit (in gray) in complex with Ser(P)-46-HPr (in yellow) (PDB code 1KKM) (15). Ser(P)-46 of HPr and residue Arg-245 of HPrK/P are highlighted in sticks and labeled.

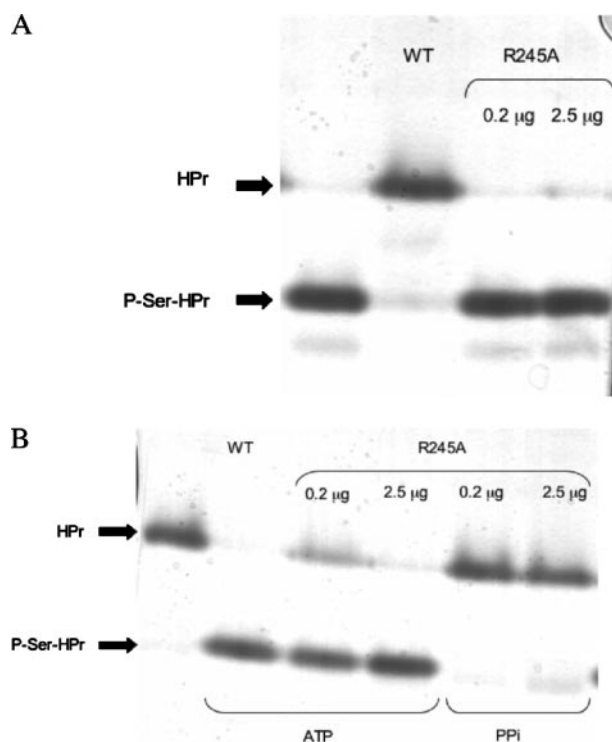


FIGURE 3. Activity assays of mutant Δ HPrK/P-R245A. *A*, phosphorylase assays. Lane *a*, Ser(P)-46-HPr standard; lane *b*, 2 μ g of Ser(P)-46-HPr incubated with 5 mM P_i and 0.2 μ g of wild type *L. casei* Δ HPrK/P; lanes *c* and *d*, 2 μ g of Ser(P)-46-HPr incubated with 5 mM P_i and 0.2 or 2.5 μ g of R245A mutant Δ HPrK/P, respectively. *B*, ATP- and PPI-dependent kinase assays. Lane *a*, HPr standard; lane *b*, 2 μ g of HPr incubated with 5 mM ATP and 0.2 μ g of wild type *L. casei* Δ HPrK/P; lanes *c* and *d*, 2 μ g of HPr incubated with 5 mM ATP and 0.2 or 2.5 μ g of R245A mutant Δ HPrK/P, respectively; lanes *e* and *f*, 2 μ g of HPr incubated with 5 mM PPI and 0.2 or 2.5 μ g of R245A mutant Δ HPrK/P, respectively.

analogue MantADP. Both *L. casei* wild type and V267F mutant Δ HPrK/P exhibit similar sigmoidal binding curves (Fig. 5*A*) with respective K_D values of 31 and 30 μ M, which is close to the dissociation constant of 17 μ M determined with the full-length *B. subtilis* enzyme (16). These results demonstrated that the truncated form of *L. casei* Δ HPrK/P exhibits a cooperative ATP binding behavior similar to that of the full-length *B. subtilis*

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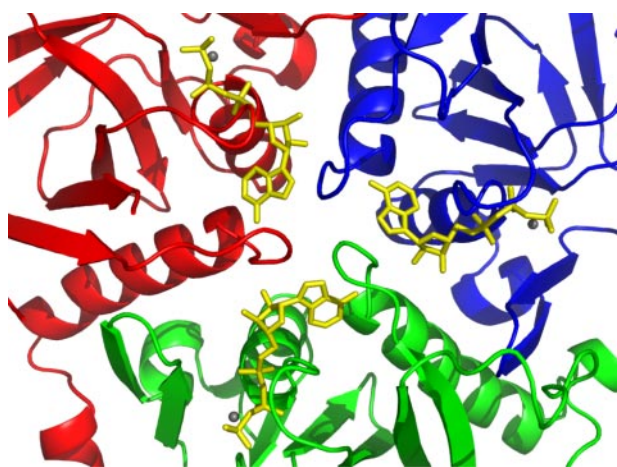


FIGURE 4. **Modeling of bound ATP.** The top trimer of Δ HPrK/P-V267F (Fig. 1B) is shown with bound ADP-Mg (in yellow sticks) obtained from a superimposition of each subunit with the liganded homologous *E. coli* phosphoenolpyruvate carboxykinase (PDB code 1AYL) (29).

enzyme. They further suggested that the conformation change induced by the V267F mutation does not modify the affinity of the enzyme for the nucleotide. We then investigated the exact significance of the sigmoidal response observed in the FRET experiments and in the phosphorylation assays (17).

Using intrinsic tryptophan fluorescence and ADP-Mg, we again obtained similar sigmoidal binding curves with both the wild type enzyme and the V267F mutant protein (data not shown). However, when extrinsic fluorescence of MantADP-Mg was used to follow nucleotide binding to *L. casei* Δ HPrK/P, the cooperative behavior was lost (Fig. 5B). This result clearly demonstrated that the sigmoidal character of the saturation curve reflected changes in the tryptophan environment. This experiment further revealed a 30-fold increased affinity of Δ HPrK/P-V267F for MantADP-Mg compared with the wild type enzyme, with a K_D value decreasing from 10 to 0.3 μ M.

The effect of FBP on the cooperative transition observed in the Trp-237/MantADP-Mg FRET experiments was also investigated. No effect was observed upon addition of 2 mM FBP with either *L. casei* wild type Δ HPrK/P or the V267F mutant protein (data not shown), suggesting that the conformation of the flexible loop is not affected by FBP binding.

DISCUSSION

So far, the wild type HPrK/Ps always adopted the phosphorylase conformation in the numerous known structures. This observation is explained by the absence of nucleotide in these structures. The structure of the *L. casei* Δ HPrK/P-V267F mutant protein presented here revealed that a global rearrangement of the Δ HPrK/P subunits accompanies the mutation, as expected from modeling of the V267F replacement. Indeed, the phenylalanine ring generated steric clashes when introduced in the wild type HPrK/P structure (data not shown). The quaternary structure rearrangement in the mutant enzyme allows the modeling of the base moiety of bound ATP, which was sterically hindered in the wild type protein. The mutant conformation is therefore compatible with the ATP-dependent activity of the enzyme.

Our fluorescence analysis demonstrated that this quaternary structure change induces an increased affinity of the protein for

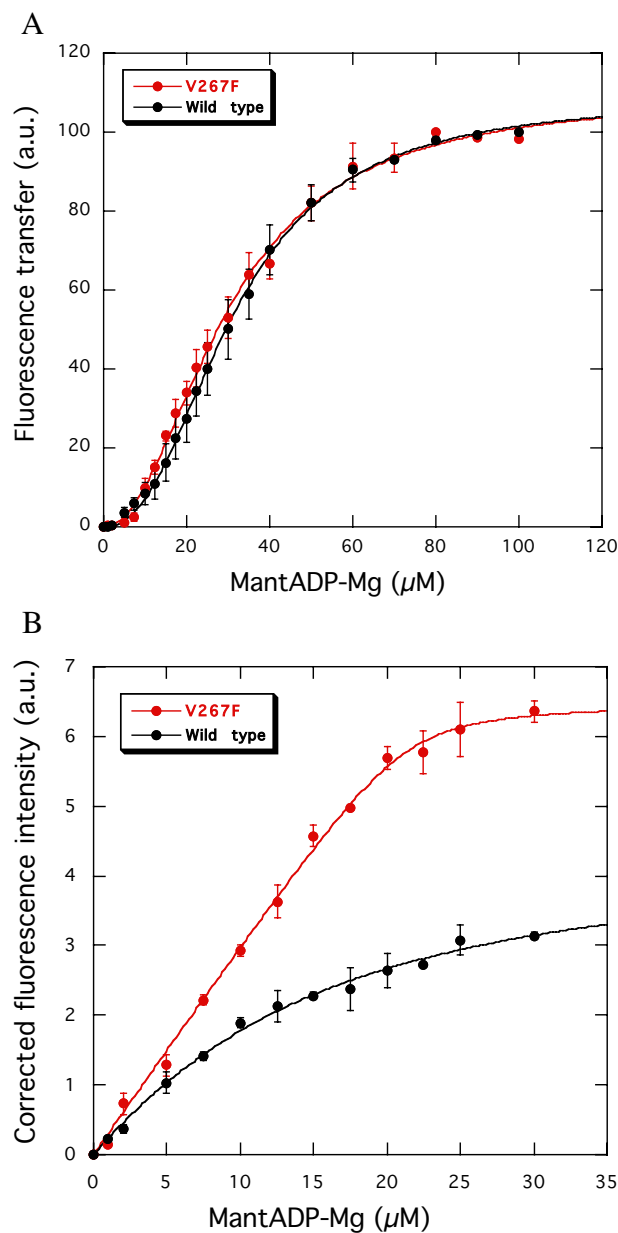


FIGURE 5. **Nucleotide binding fluorescence measurements.** A, fluorescence transfer from *L. casei* Δ HPrK/P W237 to MantADP plotted against the concentrations of MantADP-Mg. Data are presented as mean values \pm S.D. from five and three independent measurements for *L. casei* wild type (black markers) and V267F mutant Δ HPrK/P (pink markers), respectively. B, MantADP-Mg extrinsic fluorescence in the presence of 30 μ M *L. casei* Δ HPrK/P plotted against increasing concentrations of MantADP-Mg. Data are presented as mean values \pm S.D. from two independent measurements for *L. casei* wild type (black markers) and V267F mutant Δ HPrK/P (pink markers).

the nucleotide. On the other hand, the cooperative behavior of the protein is not affected by the mutation. The sigmoidal shape of the saturation curve therefore reflects a rearrangement of the Trp-237 environment induced upon nucleotide binding.

In agreement with the almost entire loss of phosphorylase activity (19), we thus propose that the quaternary structure of *L. casei* Δ HPrK/P-V267F corresponds to the kinase conformation. According to this hypothesis, ATP binding induces a rearrangement of the central loop similar to that observed in the mutant.

We also propose that ATP binding induces a rearrangement of the flexible loop carrying residue Arg-245 that we have shown to be directly involved in catalysis of phosphorolysis. This loop is disordered in the absence of a ligand but adopts a stable conformation in the presence of Ser(P)-46-HPr (15). The finding that Arg-245 is involved in the PPi-dependent phosphorylation mechanism suggests that this reaction requires the same HPrK/P conformation as the reverse reaction, *i.e.* the phosphorylase conformation. It also explains why Ser(P)-46-HPr cannot be dephosphorylated with ADP. The base moiety of ADP probably stabilizes the kinase conformation, preventing the Arg-245-phosphoserine interaction. The kinase conformation of the flexible loop, which might be stabilized by hydrophobic interactions between the base moiety of ATP and tryptophan Trp-237, seems to be implicated in catalysis of the ATP-dependent HPr phosphorylation rather than in nucleotide binding itself.

In contrast to most allosteric bifunctional proteins, such as aspartokinase I/homoserine dehydrogenase I (33), 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (34), or ATP sulfurylase/APS kinase (35) that possess two distinct active sites, both activities of HPrK/P are catalyzed by two conformations of the same active site. The two conformations of HPrK/P are probably in an ATP-regulated equilibrium where the absence of ATP favors the phosphorylase conformation and its presence the kinase conformation. This concept therefore corresponds to the equilibrium shift activation described for example for *E. coli* CheY (36). Applying this model to HPrK/P implies that the shift toward the kinase conformation is induced by the rearrangement of the central loops upon binding of the base moiety of ATP. The central loops are so close to each other that the rearrangement propagates from one subunit to the other in a global quaternary structure change where all HPrK/P subunits must exist in the same conformation. This corresponds to the symmetry model (or MWC model) of allostery (37). On the other hand, the rearrangement of the flexible loop may occur in a sequential way depending on the binding of each molecule of ATP, following the induced fit model of allostery where ligand binding and conformation switching are concomitant (38). This concerted collective motion associated to sequential local structural changes is certainly a more coherent mechanistic picture of the transition process, reconciling the induced fit and the population shift models for protein allostery by emphasizing the kinetic and thermodynamic aspects of allosteric transition (26).

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Structural Analysis of the Bacterial HPr Kinase/Phosphorylase V267F Mutant Gives Insights into the Allosteric Regulation Mechanism of This Bifunctional Enzyme

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