A novel mode of control of *Mycoplasma pneumoniae* HPr kinase/phosphatase activity reflects its parasitic lifestyle

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Among the few regulatory proteins encoded by *Mycoplasma pneumoniae* is HPr kinase/phosphatase (HPrK/P), the key regulator of carbon metabolism in low-GC Gram-positive bacteria. The corresponding gene, *hprK*, and the gene encoding the target protein HPr, *ptsH*, were overexpressed. *In vitro* analysis of the purified proteins confirmed ATP-dependent phosphorylation of HPr by HPrK/P. In contrast to HPrK/P of *Bacillus subtilis*, which is by default a phosphatase and needs high ATP concentrations for kinase activity, the *M. pneumoniae* enzyme exhibits kinase activity at very low ATP concentrations and depends on P_i for phosphatase activity. This inverted control of enzymic activity may result from the adaptation to very different ecological niches. While the standard activities of HPrK/P from *M. pneumoniae* and other Gram-positive bacteria differ, they are both modulated by the concentration of ATP, P_i and glycolytic intermediates. Site-directed mutagenesis of a potential ATP-binding site and of the HPrK/P signature sequence resulted in four different activity classes: (i) inactive proteins, (ii) enzymes with reduced kinase and phosphatase activities, (iii) enzymes that had lost phosphatase, but not kinase activity, and (iv) enzymes that exhibited increased phosphatase activity.

Keywords: phosphorylation, Walker A box, mutagenesis, catabolite repression

INTRODUCTION

Mycoplasmas are pathogens of mammals that cause respiratory and urogenital diseases. They belong to the low-GC Gram-positive bacteria, even though they lack a cell wall (Woese, 1987). Their parasitic lifestyle is reflected by the fact that they contain the smallest genomes of any self-replicating organisms known so far (Razin *et al.*, 1998). Over the past years, mycoplasmas have attracted considerable and growing interest owing to three considerations: (i) to understand the biology of an important pathogen (Razin *et al.*, 1998); (ii) to identify the minimum genetic equipment necessary for independent cellular life and to construct such minimal genomes (Hutchinson *et al.*, 1999); and (iii) to develop and refine methodology in the post-genomic era, such as proteomics and transcriptomics (Balasubramanian *et al.*, 2000; Wasinger *et al.*, 2000; Weiner *et al.*, 2000).

We are interested in the control of carbon catabolism in the human pathogen *Mycoplasma pneumoniae*. This bacterium contains a small genome of about 816 kb (Himmelreich *et al.*, 1996). The metabolic capacities of *M. pneumoniae* are rather limited. There are enzymes of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) required for the transport of glucose, fructose and mannitol and all the enzymes of the glycolytic pathway. Glycerol and glycerol 3-phosphate may be transported by the glycerol facilitator and an ABC-type transporter, respectively, and are further catabolized by glycolysis. In contrast, the enzyme complement for the pentose-phosphate pathway is incomplete. Since *M. pneumoniae* lacks enzymes for the tricarboxylic acid cycle, quinones and cytochromes, ATP generation is restricted to substrate-level phosphorylation (Razin *et al.*, 1998; Himmelreich *et al.*, 1996; Miles, 1992). In addition to carbohydrates, *M. pneumoniae* can probably catabolize arginine, yielding ammonia and ATP (Himmelreich *et al.*, 1996).

Knowledge of the regulation of carbon catabolism in *M. pneumoniae* and other mollicutes is very limited. The complete genome of *M. pneumoniae* encodes only a few

Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; HPrK/P, HPr kinase/phosphatase; FBP, fructose 1,6-bisphosphate.

regulatory proteins. Two-component systems or alternative sigma factors commonly found in other bacteria are absent (Himmelreich *et al.*, 1996; Himmelreich *et al.*, 1997). *M. pneumoniae* and *Mycoplasma genitalium* encode an HPr kinase/phosphatase (HPrK/P), the key regulatory enzyme of carbon catabolism in low-GC Gram-positive bacteria (Himmelreich *et al.*, 1996; Fraser *et al.*, 1995). Moreover, HPrK/P activity is also present in mollicutes such as *Mycoplasma capricolum* and *Acholeplasma laidlawii* (Hoischen *et al.*, 1993; Zhu *et al.*, 1997). In *Bacillus subtilis*, HPrK/P senses the metabolic state of the cell and reversibly phosphorylates HPr of the PTS and an HPr homologue, Crh, at seryl residues (Galinier *et al.*, 1997, 1998; Reizer *et al.*, 1998). HPr-Ser-P and Crh-Ser-P serve as cofactors for the transcriptional regulator, CcpA. HPr-Ser-P is a poor substrate for phosphorylation by Enzyme I of the PTS (Galinier *et al.*, 1997; Reizer *et al.*, 1998; Deutscher *et al.*, 1995). The regulatory consequences of HPr phosphorylation by HPrK/P have been reviewed in detail (Stülke & Hillen, 1999). The presence of an *hprK* gene in *M. pneumoniae* and other mollicutes suggests that carbon metabolism in these bacteria may be governed by this master regulator. The assumed involvement of HPrK/P in regulation of carbon and energy metabolism is reinforced by the accumulation of fructose 1,6-bisphosphate (FBP), a trigger of HPr kinase activity in *B. subtilis*, in glycolytically active cells of *Mycoplasma gallisepticum* (Reizer *et al.*, 1998; Egan *et al.*, 1986; Mason *et al.*, 1981).

We have studied the activity of HPrK/P isolated from *M. pneumoniae*. Our results demonstrate that the protein has both kinase and phosphatase activities. In contrast to HPrK/P of *B. subtilis* which is active as a phosphatase in the absence of any metabolic intermediates, the *M. pneumoniae* enzyme exhibits kinase activity. The activity of the protein *in vivo*, and thus the phosphorylation state of HPr, is adjusted by the ratio of ATP, FBP and P_i . Mutagenesis experiments indicate that a conserved Walker A box nucleotide-binding motif and the conserved HPr kinase signature sequence (Reizer *et al.*, 1998) are required for both activities. Several mutations in the nucleotide-binding motif of HPrK/P completely eliminate phosphatase activity without affecting the kinase activity.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used for cloning experiments and overexpression of recombinant proteins. The cells were grown in LB medium and transformants were selected on plates containing ampicillin (100 μ g ml⁻¹).

DNA manipulation and plasmid constructions. Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and DNA polymerase were used as recommended by the manufacturers. DNA fragments were purified from agarose gels using a Nucleotrap Gel Extraction kit (Macherey & Nagel). DNA sequences were determined using the dideoxy chain-termination method (Sambrook *et al.*, 1989).

To overexpress the wild-type HPrK/P protein as well as the HPr protein fused to a hexahistidine sequence at the N terminus, plasmids were constructed as follows. DNA fragments corresponding to the *hprK* and *ptsH* ORFs, respectively, were amplified by PCR using the cosmids G07 and D09 bearing the *M. pneumoniae hprK* and *ptsH* sequences (Himmelreich *et al.*, 1996). The primer pair used for amplification of the *hprK* gene was KS9 (5'-AAAGTCGACATGA AAAAGTTATTAGTCAAGGAG-3') and KS10 (5'-ATTAA GCTTGGTCTGCTACTAACACTAGGATTCAT CTTTT TTACG-3'). The primers used for amplification of the *ptsH* gene were KS 34 (5'-AAAGTCGACATGAAGAAGATTCAA GTAGTCGTTAAAGAC-3') and KS 35 (5'-AAAAAGCTTT TAAATAACTTGGTGTCTTTTCTAAAAGTGC-3'). The PCR products were digested with *SalI* and *HindIII*, and the resulting fragments were cloned into the expression vector pWH844 (Schirmer *et al.*, 1997) cut with the same enzymes. The resulting plasmids were pGP204 (for *hprK*) and pGP217 (for *ptsH*).

Site-directed mutagenesis of the *M. pneumoniae hprK* gene was performed by a two-step PCR approach as described previously (Hanson *et al.*, 2002). The mutant alleles were cloned into pWH844 as described for the wild-type.

Protein purification. *E. coli* DH5 α was used as host for the overexpression of recombinant proteins. Expression was induced by the addition of IPTG (final concentration 1 mM) to exponentially growing cultures (OD₆₀₀ 0.8). Cells were lysed using sonication (8 $\times$ 30 s, 4 °C, 50 W) or a French press cell (2000 p.s.i. = 13.8 MPa). After lysis the crude extracts were centrifuged at 15 000 g for 30 min. For purification of the HPrK/P proteins the resulting supernatants were passed over a Ni²⁺ HiTrap chelating column (Pharmacia) followed by elution with an imidazole gradient (in a buffer containing 10 mM Tris/HCl, pH 7.5, 200 mM NaCl).

For the recombinant HPr protein the overproduced protein was detected in the pellet fraction of the lysate. Therefore, after centrifugation as described above, the supernatant was discarded and the pellet was resuspended using 6 M urea. After an additional centrifugation of the resuspended pellet at 15 000 g for 30 min, the resulting supernatant containing the solubilized HPr protein was passed over the Ni²⁺ HiTrap chelating column (Pharmacia). The protein was renatured while attached to the column by using an extended wash-out, followed by elution via an imidazole gradient. Renaturation of the HPr was assayed by using it as a substrate for *in vitro* phosphorylation by Enzyme I and HPrK/P. Complete phosphorylation was taken as an indication that the renatured protein was present in a native form.

After elution the fractions were tested for the desired protein using 12.5% SDS-PAGE gels for HPrK/P and 10% Tris/Tricine gels (Schägger & von Jagow, 1987) for HPr. The relevant fractions were combined and dialysed overnight.

Purified proteins were concentrated using Microsep Microconcentrators with a molecular mass cut-off of 3 and 10 kDa for HPr and HPrK/P, respectively (Pall Filtron). Protein concentration was determined according to the method of Bradford (1976) using the Bio-Rad dye-binding assay where bovine serum albumin served as the standard.

(His₆)HPr and (His₆)HPrK/P of *B. subtilis* were purified as described previously (Hanson *et al.*, 2002).

Activity assays of HPrK/P. Activity assays were carried out with purified HPrK/P in assay buffer (10 mM MgCl₂, 25 mM Tris/HCl, pH 7.6, 1 mM dithiothreitol) using purified (His₆)HPr or (His₆)HPr-Ser-P. ATP, potassium phosphate and FBP were added as indicated. The assays were carried out at

37 °C for 15 min followed by thermal inactivation of the enzyme (4 min at 95 °C). The assay mixtures were analysed on 10 % native polyacrylamide gels as described previously (Hanson *et al.*, 2002). Proteins were visualized by Coomassie staining.

For the radioactive assay, HPrK/P activity was determined in a mixture containing 10 mM MgCl₂, 25 mM Tris/HCl, pH 7.6, 1 mM dithiothreitol, 0.1 mM [³²P]ATP (0.8 µCi nmol⁻¹), 20 mM FBP and purified (His₆)HPr from *M. pneumoniae* or HPr from *Bacillus megaterium* in a volume of 20 µl. Incubation was performed for 15 min at 37 °C. The reaction was stopped by adding 5 µl SDS 'quenching buffer' (Reizer *et al.*, 1998) followed by boiling for 3 min. Proteins were separated by SDS-PAGE on a 15 % gel. HPr-Ser-³²P was analysed by autoradiography using the TINA software of a Phosphorimager (Fujifilm BAS 1500).

(His₆)HPr-Ser-P of *M. pneumoniae* was prepared as follows. After incubation of purified (His₆)HPr with HPr kinase in assay buffer in the presence of 10 mM ATP and 20 mM FBP the enzyme was thermally inactivated. The reaction led to complete phosphorylation of (His₆)HPr which was confirmed on a 10 % native gel. To separate the phosphorylated protein from low-molecular-mass effector molecules used in the phosphorylation reaction, the assay mixture was passed over a HiTrap Desalting column (Sephadex G25, Pharmacia). (His₆)HPr-Ser-P of *B. subtilis* was prepared as described previously (Hanson *et al.*, 2002).

RESULTS

Purification of *M. pneumoniae* HPr and HPrK/P

The *M. pneumoniae* HPr (NCBI NP_109741) and HPrK/P (NCBI NP_109911) proteins were identified in the course of the sequencing of the *M. pneumoniae* genome (Himmelreich *et al.*, 1996). To assess the activities of HPr and HPrK/P from *M. pneumoniae* the corresponding genes were cloned into the expression vector pWH844, allowing the expression and purification of the proteins carrying an N-terminal hexahistidine sequence (see Methods). Cloning and expression of the *M. pneumoniae* proteins was facilitated by the fact that both the *ptsH* and *hprK* genes do not contain UGA triplets (which encode tryptophan in *M. pneumoniae*, but are used as stop codons in *E. coli*). The recombinant proteins were purified to apparent homogeneity by Ni-NTA affinity chromatography.

His₆-HPr was phosphorylated by Enzyme I and HPrK/P in a phosphoenolpyruvate or ATP-dependent manner, respectively (data not shown). His₆-HPr was also recognized by polyclonal antibodies raised against HPr from *B. megaterium* (data not shown). The purified His₆-HPrK/P *in vitro* phosphorylated HPr from *B. megaterium* and *M. pneumoniae*; however, the efficiency of phosphorylation was about 20-fold higher for the cognate HPr (data not shown).

Regulation of kinase activity of *M. pneumoniae* HPrK/P

The effect of increasing concentrations of ATP on kinase activity of *M. pneumoniae* HPrK/P was tested. All HPr in the reaction was phosphorylated at ATP con-

Fig. 1. Kinase activity of *M. pneumoniae* HPrK/P in the presence of various effectors. *M. pneumoniae* HPr (20 µM) was incubated with HPrK/P (500 nM) in assay buffer in a final volume of 20 µl. After 15 min incubation at 37 °C, the enzyme was inactivated. The proteins were analysed using 10 % native PAGE. (a) Effect of increasing ATP concentrations on HPr phosphorylation by HPrK/P. The control lane (CO) contains HPr(Ser-P). (b) Effect of increasing concentrations of FBP on kinase activity in the presence of 50 µM ATP and 5 mM P_i. The control lanes contain HPr and HPr(Ser-P).

centrations of 25 µM and above. An ATP concentration as low as 1 µM did yield kinase activity (note that ATP was the limiting component in this reaction) (Fig. 1a).

Kinase activity of some HPrK/P proteins, like that of *B. subtilis* and *Lactobacillus casei*, is stimulated by glycolytic intermediates such as FBP (Galinier *et al.*, 1998; Reizer *et al.*, 1998; Jault *et al.*, 2000; Dossonnet *et al.*, 2000). We analysed the response of the kinase activity of HPrK/P of *M. pneumoniae* to FBP in the presence of low ATP concentrations (1, 5 and 10 µM). Even at the lowest ATP concentration tested, no stimulatory effect of FBP was observed (data not shown).

Both activities of HPrK/P may be controlled by the concentration of P_i, as shown for the enzyme from *Enterococcus faecalis* (Kravanja *et al.*, 1999). The addition of 1 mM P_i to the *M. pneumoniae* kinase reaction in the presence of 50 µM ATP resulted in partial inhibition of kinase activity (data not shown). At 5 mM P_i, kinase activity was completely inhibited (Fig. 1b). We investigated whether FBP as a major glycolytic

Fig. 2. Phosphatase activity of *M. pneumoniae* HPrK/P in the presence of increasing concentrations of P_i . HPr(Ser-P) (20 μ M) was incubated with HPrK/P (500 nM) in assay buffer to which increasing concentrations of P_i were added as indicated. After 15 min of incubation at 37 °C, the enzyme was inactivated by heating at 95 °C for 4 min. The proteins were analysed using 10% native PAGE.

intermediate might counteract the inhibitory effect of P_i . Indeed, FBP prevented kinase inhibition by P_i (see Fig. 1b).

Regulation of phosphatase activity of *M. pneumoniae* HPrK/P

Phosphatase activity of His₆-HPrK/P from *M. pneumoniae* was analysed using His₆-HPr-Ser-P from *M. pneumoniae* as a substrate. Significant phosphatase activity was detected only if the P_i concentration in the reaction mixture exceeded 1 mM (Fig. 2). The observed

antagonistic effects between FBP and P_i regarding kinase activity of HPrK/P from other bacteria prompted us to investigate the influence of FBP on phosphatase activity as well. In the presence of 5 mM P_i , no effect of FBP on phosphatase activity was observed (data not shown). However, if ATP (50 μ M) was included in the assay mix, FBP shifted the activity towards the kinase reaction.

Comparison of activities of HPrK/P from *M. pneumoniae* and *B. subtilis*

The data presented above indicate that the HPrK/P of *M. pneumoniae* is active as a kinase at very low ATP concentration, irrespective of the presence or absence of FBP. In contrast, HPrK/P from *B. subtilis* was reported to exhibit kinase activity only at high ATP concentrations or at intermediate ATP concentrations in the presence of FBP (Jault *et al.*, 2000). Thus, the *M. pneumoniae* enzyme may have an inverted default activity. To test whether this difference is significant we assayed both enzymic activities under physiological conditions (5 mM P_i , 0.2 or 2 mM ATP, increasing amounts of FBP). The enzyme from *B. subtilis* has no kinase activity at 0.2 mM ATP and requires the presence of FBP (at least 10 mM, indicative of a high glycolytic activity) for kinase activity at 2 mM ATP. In contrast, the *M. pneumoniae* HPrK/P exhibits kinase activity at low concentration of ATP (Fig. 3). As expected in the presence of P_i (Jault *et al.*, 2000), *B. subtilis* HPrK/P exhibits phosphatase activity in the presence of both low and high ATP concentrations. Only in the presence of high FBP concentrations, was inhibition of phosphatase activity observed (Fig. 4a). Again, the enzyme from *M. pneumoniae* shows a different pattern of activity: FBP was a very potent inhibitor of phosphatase activity, even

Fig. 3. Kinase activity of HPrK/P in the presence of various effectors. The assays were performed as described in the legend to Fig. 1. (a) Kinase activity of HPrK/P from *B. subtilis* in the presence of increasing FBP concentrations. The enzyme was incubated with HPr from *B. subtilis*. The control lane (CO) shows *B. subtilis* HPr. Lanes: 2–6, reactions contained 0.2 mM ATP and 5 mM P_i ; 7–11, reactions contained 2 mM ATP and 5 mM P_i . (b) Kinase activity of HPrK/P from *M. pneumoniae* in the presence of increasing FBP concentrations. The enzyme was incubated with HPr from *M. pneumoniae*. The control lane (CO) contains HPr(Ser-P). Lanes: 1–5, reactions contained 0.2 mM ATP and 5 mM P_i ; 7–11, reactions contained 2 mM ATP and 5 mM P_i .

Fig. 4. Phosphatase activity of HPrK/P in the presence of various effectors. The assays were carried out under the same conditions as described in the legend to Fig. 1, except for the fact that the enzyme was incubated with HPr(Ser-P) instead of HPr. (a) Phosphatase activity of HPrK/P from *B. subtilis* in the presence of increasing FBP concentrations. The enzyme was incubated with HPr(Ser-P) from *B. subtilis*. Lanes: 1–5, reactions contained 0.2 mM ATP and 5 mM P_i ; 7–11, reactions contained 2 mM ATP and 5 mM P_i . The control lane (CO) shows HPr(Ser-P). (b) Phosphatase activity of HPrK/P from *M. pneumoniae* in the presence of increasing FBP concentrations. The enzyme was incubated with HPr(Ser-P) from *M. pneumoniae*. Lanes: 1–5, reactions contained 0.2 mM ATP and 5 mM P_i ; 6–10, reactions contained 2 mM ATP and 5 mM P_i . The control lane (CO) shows HPr(Ser-P).

1 mM FBP resulted in complete and partial inhibition of phosphatase activity in the presence of either 0.2 or 2 mM ATP (Fig. 4b).

Functional analysis of conserved regions of HPrK/P

HPrK/P of *M. pneumoniae* shares two conserved regions with other members of this class of bifunctional protein kinases/phosphatases: the Walker A box nucleotide-binding site (Walker *et al.*, 1982; Saraste *et al.*, 1990; residues G154–E162) and the HPrK/P specific signature sequence (Reizer *et al.*, 1998; residues E202–V211). We studied the functional importance of these regions by site-directed mutagenesis. Mutant alleles of *hprK* were constructed, and the resulting proteins were overexpressed and purified with an N-terminal hexahistidine sequence. The results of the activity assays are summarized in Fig. 5.

Two residues in the nucleotide-binding site were essential for both enzymic activities of HPrK/P. The corresponding mutations (G159A, K160A, K160R) completely prevented activity. Mutations of the two other universally conserved glycine residues in the Walker A box motif (G154A and G157A) resulted in a fourfold decrease of kinase activity in comparison to the wild-type protein under the conditions of assay. Moreover, kinase activity of these proteins was no longer regulated by P_i and FBP. Both mutations also severely affected phosphatase activity: whereas the G154A exchange

resulted in complete loss, the G157A mutant exhibited a very weak phosphatase activity. Mutations of S156, which is also strongly conserved in HPrK/P, had opposing effects. The S156A mutation did not affect kinase activity (Fig. 5), whereas the phosphatase activity was about fourfold greater than that of the wild-type enzyme. Interestingly, this mutant enzyme also showed an increased inhibition of kinase activity by P_i . By contrast, the S156T mutation led to a fourfold decrease of kinase and a complete loss of phosphatase activity. These findings suggest that the size of the amino acid at this position is important rather than the hydroxyl group. Based on studies with other ATP-binding proteins (Vertommen *et al.*, 1996), S161 may be involved in Mg^{2+} binding. A mutation of this residue in HPrK/P (S161A) resulted in a tenfold decrease of the kinase activity and abolished any phosphatase activity. Moreover, P_i very efficiently inhibited the residual kinase activity of this mutant enzyme. An S161T mutation had less pronounced effects: the kinase activity was only slightly reduced, but phosphatase activity was barely detectable. The regulation of this mutant by P_i and FBP was similar to that of the wild-type protein. Finally, an E162D mutant protein exhibited a constitutive kinase activity which was no longer inhibited by P_i . The E162D mutant protein did not show any phosphatase activity.

In addition to the three conserved glycine residues in the Walker A box motif, another universally conserved glycine was analysed by site-directed mutagenesis.

Fig. 5. Location of the mutations introduced in the *hprK* gene by site-directed mutagenesis. Alignment of the conserved regions of HPrK/P from *M. pneumoniae* (Himmelreich *et al.*, 1996), *M. genitalium* (Fraser *et al.*, 1995), *B. subtilis* (Galinier *et al.*, 1998; Reizer *et al.*, 1998), *E. faecalis* (Kravanja *et al.*, 1999) and *Streptococcus salivarius* (Brochu & Vadeboncoeur, 1999) containing the Walker A box nucleotide-binding motif and the HPrK/P signature sequence. Numbering corresponds to *M. pneumoniae* HPrK/P. Effects of the mutations as compared to the wild-type protein were grouped as follows: mutations which did not affect kinase activity, mutations which diminished kinase activity, mutations which led to complete loss of phosphatase activity, 'super phosphatases' which show even better phosphatase activity as compared to the wild-type, mutations which strongly reduced phosphatase activity and mutations which led to a complete loss of function.

This mutation, G140A, had no effect on kinase activity; however, the phosphatase activity was observed even at low concentrations of P_i (1 mM).

Two mutations of the signature sequence were introduced and their effects studied. An R204K mutation affected only phosphatase activity, which was strongly reduced as compared to the wild-type protein. The G207A mutation completely abolished all activities of HPrK/P.

DISCUSSION

The regulation of carbon catabolism has been the subject of intensive studies in different groups of bacteria, such as the enteric bacteria and the low-GC Gram-positive bacteria with their model organisms, *E. coli* and *B. subtilis*, respectively (Stülke & Hillen, 1999). These bacteria often have to cope with a scarcity of nutrients in their natural habitats. *M. pneumoniae*, in contrast, lives attached to mammalian cells and depends on the host for many nutrients. For these bacteria, nutrient limitation may thus be an exception rather than the rule. Yet, HPr and HPrK/P are among the few regulatory proteins encoded in the genome of *M. pneumoniae*, suggesting that they serve a role in the control of carbon metabolism.

HPrK/P from *B. subtilis* is active as a phosphatase in the absence of any metabolites if P_i is present. Kinase activity occurs only if the concentration of ATP is high (> 0.2 mM) and glycolytic intermediates such as FBP are

present (Jault *et al.*, 2000). Similarly, the enzymes from other low-GC Gram-positive bacteria are active as a kinase only at high ATP concentrations that indicate a good supply of nutrients (Kravanja *et al.*, 1999; Brochu & Vadeboncoeur, 1999; Dossonnet *et al.*, 2000; Huynh *et al.*, 2000). In contrast, *M. pneumoniae* HPrK/P exhibits an inverse mode of regulation: this enzyme needs P_i for phosphatase activity, whereas the kinase activity occurs at ATP concentrations as low as 1 μ M. Thus, this protein is by default a kinase rather than a phosphatase. *M. pneumoniae* HPrK/P phosphorylates HPr at Ser-46 as the standard reaction (see Figs 3 and 4). This may result in permanent carbon catabolite repression of genes required for the utilization of secondary carbon sources, and in controlled sugar uptake. This correlates well with the nutrient-rich environment of *M. pneumoniae*, in which there is no limitation of preferred sources of carbon and energy. On the other hand, the standard phosphatase activity of the *B. subtilis* HPrK/P results in absence of catabolite repression as a standard regulatory mechanism in poor environments.

In the course of this study it turned out that *M. pneumoniae* HPrK/P phosphorylated the cognate HPr much more efficiently than its counterpart from *B. megaterium*. This finding is in good agreement with the identification of specificity determinants required for the interaction between HPrK/P and HPr of *M. capricolum*. Residues 48, 49 and 51–53 of HPr are important for kinase-HPr recognition (Zhu *et al.*, 1998). While the latter three residues are shared by the HPr sequences of *M. pneumoniae* and *B. megaterium* (Himmelreich *et al.*,

1996; Wagner *et al.*, 2000), the former are not conserved and may be responsible for the weak phosphorylation of *B. megaterium* HPr by *M. pneumoniae* HPrK/P.

There are two strongly conserved sequence motifs in HPrK/P: the potential ATP-binding site and the signature sequence. A mutational analysis of these sequences revealed that both motifs contain residues that are indispensable for the enzymic function of HPrK/P. The recent elucidation of the three-dimensional structure of *L. casei* HPrK/P indicated that both motifs are located in close proximity (Fieulaine *et al.*, 2001). The GKSE cluster in the ATP-binding site is most important for both kinase and phosphatase activities. Even conservative substitutions (G159A, K160R) result in complete inactivation. Similarly, a replacement at the corresponding position in *B. subtilis* and *L. casei* HPrK/P (G158A and G160S, respectively) results in a strong decrease of kinase and abolition of phosphatase activity (Monedero *et al.*, 2001; Hanson *et al.*, 2002). The mutational separation of the enzymic activities is also observed with *M. pneumoniae* HPrK/P. The E162D substitution results in complete loss of phosphatase activity without affecting kinase activity. A corresponding mutation of *L. casei* HPrK/P may have similar consequences, since the mutant strain exhibits constitutive catabolite repression indicative of constitutive phosphorylation of HPr and loss of dephosphorylation (Monedero *et al.*, 2001). In HPrK/P of *L. casei*, amino acids of the KSE motif interact with P_i (Fieulaine *et al.*, 2001). Moreover, the phosphatase reaction was shown to be enzymically distinct from the kinase reaction for HPrK/P of *L. casei* (Monedero *et al.*, 2001). Thus, the specific effect of mutations in this region on phosphatase activity may result from loss of P_i binding. In contrast, the G140A and S156A mutant proteins exhibit normal kinase activity, but their phosphatase activity is strongly enhanced as compared to the wild-type protein. The fully conserved G207 residue of the signature sequence is essential for HPrK/P function in *M. pneumoniae*. The corresponding mutant of the *B. subtilis* enzyme exhibits very weak kinase and no phosphatase activity (Hanson *et al.*, 2002). Of the mutations studied so far, no substitutions that reduce kinase activity without affecting phosphatase activity have been identified, whereas mutations abolishing phosphatase activity but allowing kinase activity have been found in *M. pneumoniae*, *B. subtilis* and *L. casei* (Monedero *et al.*, 2001; Hanson *et al.*, 2002). Interestingly, a mutational analysis of another bifunctional protein kinase/phosphatase from *E. coli*, isocitrate dehydrogenase kinase/phosphatase (IDH K/P), yielded results similar to those reported here: mutations of the ATP-binding site inactivate both kinase and phosphatase activities (Stueland *et al.*, 1989). In addition, mutations selectively abolishing phosphatase activity were found (Ikeda *et al.*, 1992).

To the best of our knowledge, HPrK/P is the first bifunctional enzyme for which opposing basal activities have been found in orthologues from different organisms. It will be interesting to analyse the structural basis

of this phenomenon. The protein has been crystallized (Steinhauer *et al.*, 2002) and analysis of the structure is under way.

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