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Dual phosphorylation of *Mycoplasma pneumoniae* HPr by Enzyme I and HPr kinase suggests an extended phosphoryl group susceptibility of HPr

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Abstract

In Gram-positive bacteria, the HPr protein of the phosphoenolpyruvate:sugar phosphotransferase system can be phosphorylated at two distinct sites, His-15 and Ser-46. While the former phosphorylation is implicated in phosphoryl transfer to the incoming sugars, the latter serves regulatory purposes. In *Bacillus subtilis*, the two phosphorylation events are mutually exclusive. In contrast, doubly phosphorylated HPr is present in cell extracts of *Mycoplasma pneumoniae*. In this work, we studied the ability of the two single phosphorylated HPr species to accept a second phosphoryl group. Indeed, both Enzyme I and the HPr kinase/phosphorylase from *M. pneumoniae* are able to use phosphorylated HPr as a substrate. The formation of doubly phosphorylated HPr is substantially slower as compared to the phosphorylation of free HPr. However, the rate of formation of doubly phosphorylated HPr is sufficient to account for the amount of HPr(His~P)(Ser-P) detected in *M. pneumoniae* cells.

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Keywords: Phosphotransferase system; Carbon catabolite repression; Mollicutes

1. Introduction

In many bacteria, the carbon supply of the cell is monitored by the phosphotransferase system (PTS) and reflected by different phosphorylation statuses of individual PTS proteins. *Bacillus subtilis* and other firmicutes use HPr as an indicator of nutrient supply. In these bacteria HPr can be phosphorylated on two sites: His-15 is part of the PTS phosphorylation chain whereas Ser-46 serves as a regulatory phosphorylation site. His-15 is the target of Enzyme I of the PTS. Ser-46 is phosphorylated by the

HPr kinase/phosphorylase (HPrK/P) at the expense of ATP. HPr(His15~P) serves as phosphate donor for the sugar-specific enzymes II and can phosphorylate enzymes such as glycerol kinase and transcription regulators to stimulate their activity. HPr(Ser-P), in contrast, does not participate in sugar transport but acts as a cofactor for the transcriptional regulator CcpA that mediates carbon catabolite repression in the firmicutes [1,2].

In *B. subtilis*, HPr phosphorylation has been studied during growth with or without glucose. In the absence of glucose, HPr is phosphorylated on His-15 by Enzyme I whereas phosphorylation of Ser-46 is predominant in the presence of glucose. While non-phosphorylated HPr was detected under both conditions, only marginal

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amounts of doubly phosphorylated HPr were present upon growth in glucose [3]. This pattern of phosphorylation results from the control of HPrK/P activity in *B. subtilis*: If the intracellular concentrations of ATP and fructose-1,6-bisphosphate are high, the enzyme is active as a kinase, whereas phosphorylase activity prevails at low ATP and high phosphate concentrations [4]. In contrast to the observations with *B. subtilis*, substantial amounts of doubly phosphorylated HPr were found in rapidly growing cells of Streptococci [5]. This is astonishing, since the *Streptococcus salivarius* HPrK/P is controlled by ATP, fructose-1,6-bisphosphate and inorganic phosphate similar to the enzyme of *B. subtilis* [6].

Biochemical analyses with proteins from *B. subtilis* revealed that HPr(His~P) is a poor substrate for HPrK/P. Similarly, HPr phosphorylation at Ser-46 inhibits Enzyme I-dependent phosphorylation about 5000-fold [7,8]. In agreement with the in vivo results and in contrast to those obtained with proteins from *B. subtilis*, HPr(Ser-P) from *S. thermophilus* efficiently accepts a phosphate from Enzyme I in vitro [9].

We are interested in the control of carbon metabolism in the mollicute *Mycoplasma pneumoniae*. Based on in vivo phosphorylation patterns and the ability to use carbohydrates, the general components of the PTS and the permeases for glucose and fructose are functionally expressed whereas mannitol cannot be utilized [10]. The HPrK/P of *M. pneumoniae* differs in its activity from all other enzymes of this family studied so far in its extremely high affinity for ATP. This results in kinase activity even at very low ATP concentrations in the absence of any other effector [11,12]. In spite of these apparent differences in enzyme regulation, the known crystal structures of the HPrK/Ps including that of *M. pneumoniae* are all very similar to each other [13,14]. In vivo phosphorylation studies revealed that a significant portion of HPr (about 30%) was present in the doubly phosphorylated form [10]. This suggests that the HPrK/P of *M. pneumoniae* is not only peculiar in its regulation but also in its ability to phosphorylate HPr(His~P). In this work, we addressed the activities of the enzymes involved in HPr phosphorylation using phosphorylated HPr as a target. We demonstrate that unlike the enzymes from *B. subtilis* both Enzyme I and HPrK/P from *M. pneumoniae* are active on phosphorylated HPr.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli DH5 α , BL21(DE3)/pLysS [15] and M15 (Qiagen, Hilden, Germany) were used for over-

expression of recombinant proteins. The cells were grown in LB medium containing ampicillin (100 μ g ml⁻¹).

M. pneumoniae M129 in the 31st broth passage was used for preparation of cell extracts as a source of *M. pneumoniae* Enzyme I. Cells were grown at 37 °C in 150 cm² tissue culture flasks containing 100 ml of modified Hayflick medium which consists of 18.4 g PPLO broth (Difco), 29.8 g HEPES, 5 ml 0.5% phenol red, 35 ml 2 N NaOH and 10 g glucose per litre. Horse serum (Gibco) and penicillin were included to a final concentration of 20% and 1000 μ g/ml, respectively. Bacteria were cultivated for 96 h and cell extracts were prepared as described previously [10].

2.2. Protein purification

His₆-HPr (*M. pneumoniae*), His₆-Enzyme I (*B. subtilis*), and Strep-HPrK/P (*M. pneumoniae*) were overexpressed using the expression vectors pGP217 [11], pAG3 [16], and pGP611 [12], respectively. Expression was induced by the addition of IPTG (final concentration 1 mM) to exponentially growing cultures (OD₆₀₀ of 0.8). Cells were disrupted using a french press. After lysis the crude extracts were centrifuged at 10,000g for 30 min. For purification of His-tagged proteins the resulting supernatants were passed over a Ni²⁺NTA superflow column (5 ml bed volume, Qiagen) followed by elution with an imidazole gradient (from 0 to 500 mM imidazole in a buffer containing 10 mM Tris/HCl pH 7.5, 600 mM NaCl, 10 mM β -mercaptoethanol). For HPrK/P carrying a N-terminal Strep-tag, the crude extract was passed over a Streptactin column (IBA, Göttingen, Germany). The recombinant protein was eluted with desthiobiotin (Sigma, final concentration 2.5 mM). For the recombinant HPr protein the overproduced protein was purified from the pellet fraction of the lysate by urea extraction and renatured as described previously [11].

After elution the fractions were tested for the desired protein using 12.5% SDS-PAGE. The relevant fractions were combined and dialysed overnight. Protein concentration was determined using the Bio-rad dye-binding assay where Bovine serum albumin served as the standard.

2.3. Preparation of serine phosphorylated HPr

HPr (20 μ M) was phosphorylated at Ser-46 by *M. pneumoniae* HPrK/P (500 nM) and ATP (100 μ M) in a total reaction volume of 5 ml. The reaction was carried out at 37 °C for 1 h in 25 mM Tris-HCl 10 mM MgCl₂ 1 mM DTT and stopped using a heat step for 10 min at 95 °C which simultaneously leads to the denaturation of HPrK/P but does not denature the heat-stable HPr. Denaturated HPrK/P was sedimented by centrifugation

(10,000g, 10 min, 4 °C) and HPr(Ser-P) was enriched approximately 5-fold by passing the resulting supernatant through a Vivaspın 15 concentrator (Vivascience, Hannover, Germany). The elimination of HPrK/P and the phosphorylation status of HPr at Ser-46 were checked using denaturing SDS-PAGE and 10% native polyacrylamide gels [17], respectively.

2.4. Preparation of histidine phosphorylated HPr

HPr (20 µM) was phosphorylated at His-15 using *B. subtilis* Enzyme I (50 nM) and PEP (500 nM) as the phosphate donor in a total reaction volume of 4 ml. The phosphorylation reaction took place during an 1 h incubation step at 37 °C in a buffer containing 50 mM Tris-HCl, 10 mM MgCl₂ and 1 mM DTT. Subsequently, the reaction mixture was subjected to a buffer exchange procedure (i) to reduce the concentration of PEP and (ii) to concentrate the obtained HPr(His~P). For this purpose the reaction mixture was given on a Vivaspın 15 concentrator and centrifuged at 3000g at 4 °C until the original volume was reduced to 0.5 ml. The obtained solution was diluted 5-fold and concentrated to a volume of 0.5 ml again. All in all this step was repeated three times. The phosphorylation status of HPr was checked on a 10% native polyacrylamide gel.

2.5. Phosphorylation of HPr and HPr(Ser-P) on His-15

HPr or HPr(Ser-P) (each 20 µM) were used as the phosphoacceptor in a reaction requiring PEP (50 µM) and 5 µg of *M. pneumoniae* cell extracts as a source of mycoplasmal Enzyme I in a total volume of 20 µl. The phosphorylation reaction was allowed to proceed for a defined period of time at 37 °C and stopped immediately by the addition of 2 µl 0.5 M EDTA pH 8.0. The reaction mixture was separated on a 10% native polyacrylamide gel. Gels were stained with Coomassie stain and the resulting bands were quantified using the TotalLab™ v2003.03 software (Nonlinear Dynamics Ltd.).

2.6. Serine phosphorylation of HPr and HPr(His~P)

In a reverse experiment HPr and HPr(His~P) were the phosphoacceptors for HPrK/P dependent phosphorylation on Ser-46. To achieve serine phosphorylation of unphosphorylated or histidine phosphorylated HPr, HPr or HPr(His~P) (each 20 µM) were incubated in the presence of HPrK/P (400 nM) and ATP (100 µM) for a defined period at 37 °C. The reaction was stopped by adding 2 µl of 0.5 M EDTA pH 8.0. The reaction mixture was separated on 10% native gels and the proteins were visualized by Coomassie staining. Quantification was done as described above.

3. Results

3.1. Phosphorylation of HPr(Ser-P) by Enzyme I

In contrast to the situation observed in *B. subtilis*, large amounts of doubly phosphorylated HPr were detected in *M. pneumoniae* cells grown in the presence of glycerol. Therefore, *M. pneumoniae* Enzyme I may differ from that of *B. subtilis* in its ability to use HPr(Ser-P) as a target for phosphorylation. To test this hypothesis, we prepared HPr(Ser-P) and performed in vitro phosphorylation assays with cell extracts from *M. pneumoniae* as a source of Enzyme I. In a previous study, it was demonstrated that *M. pneumoniae* cells synthesize Enzyme I during growth in the presence of glucose (the relevant condition for this work) [10]. As a control, phosphorylation assays were performed with non-phosphorylated HPr. As shown in Fig. 1(a), HPr was completely phosphorylated after 20 min incubation in the presence of PEP and the cell extract. This phosphorylation was heat-labile and was not observed in the absence of PEP. These observations provide evidence that the phosphorylation occurred at His-15. Moreover, phosphorylation of HPr by Enzyme I seems to be very efficient since complete phosphorylation was detected after 2 min. As observed with non-phosphorylated HPr, HPr(Ser-P) was also used as a target of Enzyme I, since a heat-labile and PEP-dependent phosphorylation was detected (Fig. 1(b)). However, phosphorylation of HPr(Ser-P) by Enzyme I was significantly slower than that of non-phosphorylated HPr. After 20 min, only 40% were present as doubly phosphorylated HPr. The densitometric evaluation of the phosphorylation assays revealed that phosphorylation of HPr(Ser-P) by Enzyme I is about 25-fold slower than that of non-phosphorylated HPr (Fig. 1(c)). Thus, prior phosphorylation of *M. pneumoniae* HPr by HPrK/P inhibits Enzyme I-dependent phosphorylation. However, this inhibition is much weaker than that described for *B. subtilis*.

3.2. Phosphorylation of HPr(His~P) by HPrK/P

Doubly phosphorylated HPr may be formed by the phosphorylation of HPr(Ser-P) by Enzyme I (see above), but also by using HPr(His~P) as a substrate for HPrK/P. To test this hypothesis, we prepared HPr(His~P) and used it for in vitro phosphorylation assays with purified *M. pneumoniae* HPrK/P. Again, non-phosphorylated HPr served as a control. As shown in Fig. 2(a), HPr was readily phosphorylated. This phosphorylation was heat-stable as shown previously [11,12]. With HPr(His~P) as the substrate, the formation of doubly phosphorylated HPr was observed (Fig. 2(b)). As can be seen in Fig. 2(b), HPr(His~P) seems to be somewhat unstable. The preparation of HPr(His~P) gave rise to non-phosphorylated HPr, and after

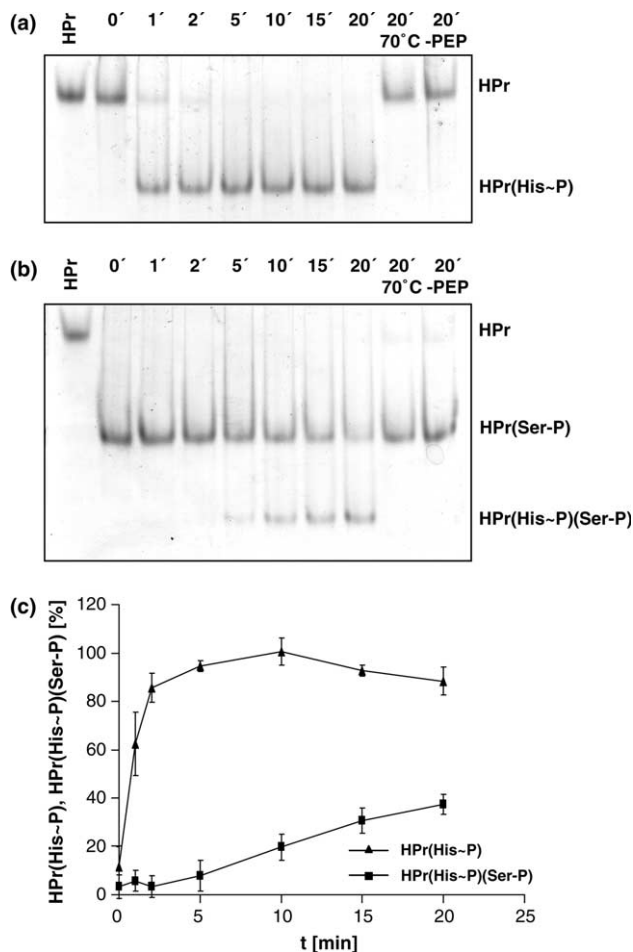


Fig. 1. Kinetics of HPr phosphorylation at His-15. (a) HPr phosphorylation at His-15 as a function of time using PEP and *M. pneumoniae* cell extracts as source of Enzyme I. The phosphorylation reactions were carried out as described in Section 2 for 0 to 20 min and stopped immediately by the addition of EDTA as a chelating agent. Reaction mixtures without added PEP and such ones that had been incubated for additional 10 min at 70 °C to hydrolyze the heat-labile HPr(His~P) served as negative controls. (b) Phosphorylation of HPr(Ser-P) at His-15 as a function of time as in (a). (c) The amounts of differently phosphorylated forms of HPr of both experiments were quantified by densitometry and displayed as ratio of total HPr plotted against time. Vertical bars indicate the standard deviation of three independent experiments.

the formation of doubly phosphorylated HPr, HPr(Ser-P) was formed. As judged from the amount of the different forms of HPr, the pool of HPr(Ser-P) was fed by the phosphorylation of free HPr and the decomposition of the doubly phosphorylated form. The quantitative evaluation of this experiment revealed that non-phosphorylated HPr was completely phosphorylated by HPrK/P after 5 min. With HPr(His~P) as the substrate, only about 20% of the protein were doubly phosphorylated after 20 min (Fig. 2(c)). The densitometric analysis indicated that the formation of doubly phosphorylated HPr with HPr(His~P) as the substrate is about 20-fold less efficient than the phosphorylation of non-phosphory-

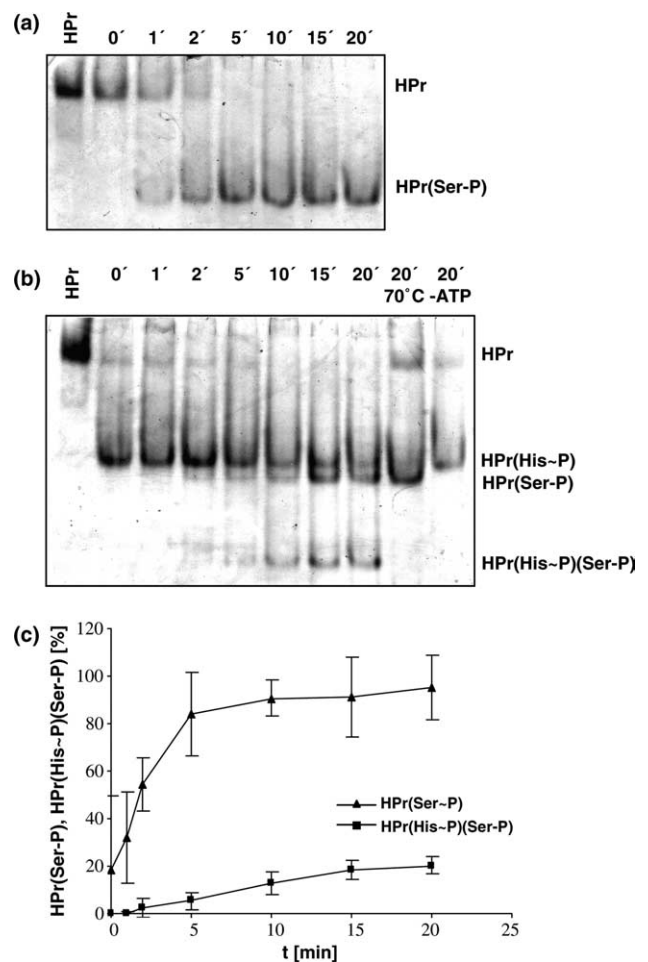


Fig. 2. Kinetics of HPr phosphorylation at Ser-46. (a) HPr phosphorylation at Ser-46 as a function of time. The phosphorylation reactions were carried out for 0–20 min and stopped by adding EDTA. (b) Phosphorylation of HPr(His~P) at Ser-46 as a function of time as in (a). Reaction mixtures where ATP had been omitted and parallel aliquots that had been incubated for additional 10 min at 70 °C to remove the histidine phosphoamidate served as negative controls. Note that HPr(His~P) and HPr(Ser-P) migrate to different positions in the gel suggesting that the two single phosphorylated forms of HPr have different conformations. (c) The amounts of differently phosphorylated forms of HPr of both experiments were quantified by densitometry and displayed as ratio of total HPr plotted against time. Vertical bars indicate the standard deviation of three independent experiments. Note that the serine residue phosphorylated by HPrK/P (Ser-46) is actually at position 47 in *M. pneumoniae* HPr.

lated HPr by HPrK/P. As seen with Enzyme I, HPrK/P from *M. pneumoniae* is much less inhibited by prior phosphorylation of HPr than the *B. subtilis* HPrK/P.

4. Discussion

Among the HPrK/P enzymes studied to detail, the *M. pneumoniae* protein is the only one from an organism that is highly adapted to nutrient-rich human tissues. In contrast to the other enzymes of the family, the

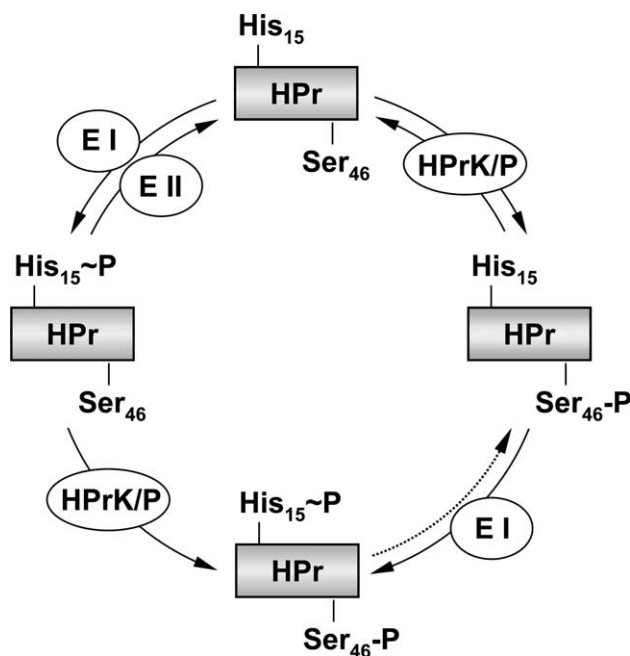


Fig. 3. HPr phosphorylation cycle in *M. pneumoniae*. HPrK/P phosphorylates HPr at Ser-46 and also catalyzes the dephosphorylation of HPr(Ser-P). Enzyme I (E I) mediates the formation of the phosphoamidate at His-15, whereas the dephosphorylation of HPr(His~P) occurs during phosphotransfer of the orthophosphate to sugar specific Enzymes II (E II). HPr(Ser-P) serves as substrate in a PEP-requiring reaction to form doubly phosphorylated HPr(His~P) (Ser-P). In a reverse manner HPr(His~P) can be phosphorylated at the serine residue by HPrK/P. The dotted arrow indicates the spontaneous dephosphorylation of doubly phosphorylated HPr at the histidine residue.

M. pneumoniae HPrK/P has several peculiarities: (i) It has a very high affinity for ATP allowing kinase activity even in the absence of glucose in the medium whereas kinase activity in *B. subtilis* and in Streptococci was only detected in glucose-grown cells [3–5,12]. (ii) The *M. pneumoniae* HPrK/P is unique in its glycerol requirement for in vivo activity suggesting a novel mechanism of control in addition to the residual regulation by glycolytic intermediates [10,11]. Finally, *M. pneumoniae* shares the high degree of double phosphorylation of HPr with the streptococci whereas the two phosphorylation events are essentially mutually exclusive in *B. subtilis* [5,7–9]. The complete phosphorylation/dephosphorylation cycle of *M. pneumoniae* HPr is depicted in Fig. 3.

Recently, the first HPrK/P from a phylogenetically distinct bacterium, the spirochaete *Treponema denticola*, was biochemically characterized. As observed for the *M. pneumoniae* HPrK/P, the enzyme from this organism has a high affinity for ATP [18]. Interestingly, *T. denticola* is also highly adapted to human tissues. It was proposed that the HPrK/P proteins from *M. pneumoniae* and *T. denticola* have the kinase activity as their default state as an adaptation to nutrient-rich environments [11,18].

The differences in the ability to form doubly phosphorylated HPr might originate from different properties of the phosphorylating enzymes, Enzyme I and HPrK/P, or from differences in the HPr structure that make the phosphorylation state of the second site irrelevant. We propose that the latter might be crucial for the acceptance or not of the second phosphorylation: First, both Enzyme I and HPrK/P of *B. subtilis* are unable to act upon phosphorylated HPr whereas the same set of two enzymes from *S. mutans*, *S. thermophilus* [5,9] and *M. pneumoniae* (this work) was active on phosphorylated HPr. Thus, subtle changes in the structure of HPr might affect the interaction between HPr and the phosphorylating enzymes to allow or prevent phosphorylation of a substrate molecule that had already been phosphorylated by the other enzyme. The second indication for our hypothesis is derived from the known structures of the complexes of HPr with Enzyme I or HPrK/P. Indeed, the helix capped by His-15 of *B. subtilis* HPr is in direct contact with HPrK/P [14]. On the other hand, the determination of the structure of the complex between the N-terminal domain of Enzyme I and HPr from *E. coli* revealed that Ser-46 directly interacts with Enzyme I [19]. For HPr from *Enterococcus faecalis*, loss of hydrophobic interaction with Enzyme I was described as the major structural effect of Ser-46 phosphorylation [20]. It will be interesting to determine the structure of *M. pneumoniae* HPr. A comparison with the known HPr structures is expected to reveal the distinct properties that determine whether the formation of doubly phosphorylated HPr is possible or not.

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