Mechanistic and physiological consequences of HPr(ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: studies with site-specific mutants of HPr

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Communicated by M. Hofnung

The bacterial phosphotransferase system (PTS) catalyzes the transport and phosphorylation of its sugar substrates. The protein-kinase-catalyzed phosphorylation of serine 46 in the phosphocarrier protein, HPr, inhibits PTS activity, but neither the mechanism of this inhibition nor its physiological significance is known. Site-specific HPr mutants were constructed in which serine 46 was replaced by alanine (S46A), threonine (S46T), tyrosine (S46Y) or aspartate (S46D). The purified S46D protein exhibited markedly lower V_{max} and higher K_m values than the wild-type, S46T or S46A protein for the phosphoryl transfer reactions involving HPr(His ~ P). Interactions of HPr with the enzymes catalyzing phosphoryl transfer to and from HPr regulated the kinase-catalyzed reaction. These results establish the inhibitory effect of a negative charge at position 46 on PTS-mediated phosphoryl transfer and suggest that HPr is phosphorylated on both histidyl and seryl residues by enzymes that recognize its tertiary rather than its primary structure. *In vivo* studies showed that a negative charge on residue 46 of HPr strongly inhibits PTS-mediated sugar uptake, but that competition of two PTS permeases for HPr(His ~ P) is quantitatively more important to the regulation of PTS function than serine 46 phosphorylation.

Key words: sugar transport/protein phosphorylation/phosphotransferase system/HPr(ser) kinase/site-specific mutants

Introduction

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) catalyzes the concomitant detection, uptake and phosphorylation of its sugar substrates (Saier, 1985; Reizer and Peterkofsky, 1987). Several phosphoryl transfer proteins which themselves are phosphorylated catalyze these processes as follows:

- (i) Phosphoenolpyruvate + Enzyme I → phospho-Enzyme I + pyruvate
- (ii) Phospho-Enzyme I + HPr → phospho-HPr + Enzyme I
- (iii) Phospho-HPr + Enzyme III → phospho-Enzyme III + HPr

(iv) Phospho-Enzyme III + Enzyme II → phospho-Enzyme II + Enzyme III

(v) Phospho-Enzyme II + sugar_{out} → sugar-phosphate_{in} + Enzyme II

Previous studies have shown that HPr of the PTS in Gram-positive bacteria can be phosphorylated on a regulatory seryl residue in addition to the catalytic histidyl residue (Deutscher and Saier, 1983; Reizer *et al.*, 1983, 1984a, 1988b; Mimura *et al.*, 1987; Reizer *et al.*, 1985, 1988a; for recent reviews see Cozzzone, 1988; Reizer and Peterkofsky, 1987; Deutscher and Saier, 1988; Reizer, 1989). This phosphorylation event results in inhibition of the Enzyme-I-catalyzed phosphorylation of histidyl residue 15 in HPr, and the reciprocal inhibitory effect of His15 phosphorylation on the kinase-catalyzed phosphorylation of seryl residue 46 has also been demonstrated (Deutscher *et al.*, 1984; Reizer *et al.*, 1984a). Both the HPr(Ser) kinase and the HPr(Ser-P) phosphatase which reverses the action of the kinase have been purified (Deutscher and Engelmann, 1984; Reizer *et al.*, 1984a, 1988b; Deutscher *et al.*, 1985), and *in vitro* studies have indicated that the presence of an Enzyme III of the PTS could partially overcome the inhibitory effect of serine 46 phosphorylation on the Enzyme-I-catalyzed phosphorylation of the active histidyl residue (His15) in HPr (Deutscher *et al.*, 1984). It has been suggested that this ATP-dependent, protein-kinase-catalyzed phosphorylation of seryl residue 46 is important to the regulation of PTS function *in vivo* (sugar uptake) (Deutscher *et al.*, 1984). To date no experiments have been conducted which serve to test this postulate.

In this communication, we report the construction of site-specific mutants of the *Bacillus subtilis* HPr in which the active histidyl residue (H15) and the regulatory seryl residue (S46) are replaced with other amino acids. The resultant mutant proteins have been purified to apparent homogeneity and used to characterize by kinetic means the functional interactions of other PTS proteins with HPr. In addition, plasmids bearing these mutant *ptsH* genes were used to transform a *ptsH*-negative mutant of *Staphylococcus aureus* to allow examination of the physiological consequences of serine-46 phosphorylation under *in vivo* conditions. The results showed that in *S. aureus*, under the conditions employed, HPr(ser) phosphorylation is not necessary for the exclusion of a PTS sugar by another PTS sugar, although serine phosphorylation, or merely the presence of a negative charge on residue 46, is sufficient to strongly inhibit PTS function. Further studies revealed that the ATP-dependent kinase is capable of phosphorylating a threonyl residue in HPr when this residue replaces serine at position 46. An inhibitory effect of Enzyme I and a stimulatory effect of the lactose-specific Enzyme III (III^{lac}) on the activity of the kinase was also demonstrated. These results serve to define the conditions influencing ATP-dependent HPr phosphorylation and to evaluate the physiological significance of this reaction with respect to PTS function in a relevant *in vivo* system. A preliminary account of this work was

presented at the FEMS Symposium on Group Translocation in Bacterial Membrane Transport at the Pasteur Institute, Paris, France, September 7–9, 1988 (Reizer, 1989).

Results

Confirmation of the nucleotide sequence of the *ptsH* gene in *B.subtilis*

Gonzy-Tréboul *et al.* (1989) have cloned and sequenced part of the *pts* operon in *B.subtilis*. In the present study, the *ptsH* gene was subcloned into M13mp8 and M13mp19 by inserting the 0.6Kb *Hind*III–*Eco*RI fragment from plasmid pTS21, obtained from Dr G.Gonzy-Tréboul (Gonzy-Tréboul and Steinmetz, 1987), and both strands of this fragment were sequenced as described in Materials and methods. The results were in full agreement with the sequence reported by Gonzy-Tréboul *et al.* (1989). Comparisons of the sequences of both Gram-positive and Gram-negative HPrs have been published (Reizer *et al.*, 1988a; Gonzy-Tréboul *et al.*, 1989; Reizer, 1989; Schnierow *et al.*, 1989).

PEP- and ATP-dependent phosphorylation of site-specific HPr mutant proteins

Several site-specific mutants of the *B.subtilis* HPr were generated by altering the *ptsH* gene in M13 as described in Materials and methods. The catalytic histidyl residue was replaced by alanine (H15A), and the regulatory seryl residue was replaced by alanine (S46A), threonine (S46T), tyrosine (S46Y) or aspartate (S46D). A double mutant lacking both the active histidyl residue and the regulatory seryl residue (H15A S46A) was also produced. An *Nde*I site was created at the initiation codon of the mutant and wild-type *ptsH* genes, and they were then cloned into the *Nde*I and *Sma*I sites of the pRE overexpression vector (P.Reddy, A.Peterkofsky and K.McKenney, in preparation). Employing this vector in *Escherichia coli* strain MZ1 (Zuber *et al.*, 1987), the wild-type HPr, as well as the mutant proteins S46A, S46D, S46T and H15A, were overproduced and purified using the procedure described in Materials and methods. From 4 l of late logarithmic cells, 100–200 mg of the nearly homogeneous (> 95% pure) HPrs were obtained.

The relative electrophoretic mobilities of the purified HPrs on native polyacrylamide gels are revealed in Figures 1 and 2. Lanes 1–5 in Figure 1 show the mobilities of these HPrs in the absence of phosphorylation. S46T, S46A and H15A migrated as did the wild-type protein (H15;S46), but S46D migrated substantially faster due to the increased negative charge of this protein (lane 2). Lanes 6–10 show the migratory behavior of these same proteins after exposure to phosphoenolpyruvate (PEP) and Enzyme I from *Streptococcus faecalis*. It can be seen that a major fraction of the wild-type protein as well as the S46T and S46A mutant proteins was phosphorylated and consequently exhibited increased mobility. Similar results were obtained for the S46Y protein (data not shown). By contrast, S46D (lane 7) was not appreciably phosphorylated, and as expected, H15A (lane 10) could not be phosphorylated at all. The inactivity of H15A in the Enzyme-I-catalyzed phosphorylation reaction confirms the suggestion of Kalbitzer *et al.* (1982) that Gram-positive HPrs are phosphorylated by PEP on histidyl residue 15.

Figure 2 shows the electrophoretic mobilities of these same proteins following exposure to [γ - 32 P]ATP and the HPr

Fig. 1. Electrophoretic mobilities of free and phosphorylated wild-type and mutant HPr proteins. **Lanes 1–5** show migration of proteins without phosphorylation; **lanes 6–10** show migration following exposure to PEP and Enzyme I. The HPr species depicted are indicated below the gel. Purification of the proteins, conditions of phosphorylation and conditions of electrophoresis on a native nondenaturing polyacrylamide gel were described in Materials and methods. Top, cathode; bottom, anode.

Fig. 2. Phosphorylation of the wild-type and mutant HPr species by ATP and the HPr kinase. **Left:** Coomassie-blue-stained native gel of the various HPrs; **right:** autoradiogram of the same gel. The experiment was conducted employing the conditions described in Materials and methods. The HPr species are indicated below the gel. A control without HPr is shown in **lane 1** (none).

kinase from *Lactobacillus brevis* (Romano *et al.*, 1987; Reizer *et al.*, 1988b). Both the wild-type and the H15A mutant proteins were readily phosphorylated in the kinase-catalyzed reaction. As revealed in the figure, a higher degree of phosphorylation was observed with the H15A mutant protein than the wild-type HPr. S46T was also phosphorylated by the ATP-dependent kinase, albeit at a slower rate. By contrast, neither S46A nor S46D was phosphorylated in this reaction. Similar results were obtained for the S46Y protein (data not shown). It should be noted that the ATP-dependent phosphorylation of S46T is not unique to the kinase isolated from *L.brevis* since it was also observed with partially purified kinase preparations isolated from *Streptococcus pyogenes*, *S.faecalis*, *S.aureus* and *B.subtilis* (Figure 3).

Fig. 3. Phosphorylation of wild-type HPr and S46T mutant HPr by various Gram-positive HPr kinases. The autoradiogram in lanes 1, 5, 9 and 13 shows the phosphorylated wild-type HPr. Lanes 4, 8, 12 and 16 show controls in which HPr was omitted. Other lanes contain the S46T mutant HPr. The bacterial sources of the partially purified kinases are indicated at the bottom of the autoradiogram. Experimental procedures were as described in Materials and methods.

Fig. 4. Phosphoamino acid analysis of the wild-type HPr (left) and the S46T mutant HPr (right) following phosphorylation of the proteins with [32 P]ATP and the *L.brevis* kinase. Acid hydrolysis and two-dimensional electrophoresis were conducted as described in Materials and methods. The solid circles in these autoradiograms show the positions of the origins while the dashed circles show the positions of standard phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) as detected with ninhydrin reagent.

The 32 P-phosphorylated wild-type (H15;S46) and S46T mutant HPrs were subjected to acid hydrolysis, and the phosphorylated amino acids were identified as outlined in Materials and methods. As shown in Figure 4, the wild-type protein was phosphorylated exclusively on serine whereas the S46T mutant was phosphorylated exclusively on threonine. These findings strongly suggest that the ATP-dependent HPr kinase can catalyze the phosphorylation of either a serine or a threonine residue at position 46 in this phosphocarrier protein.

Fermentation characteristics of a *S.aureus* *ptsH* mutant bearing the plasmid-encoded wild-type or mutant *ptsH* genes from *B.subtilis*

In order to determine the physiological function of HPr(Ser) phosphorylation in a relevant *in vivo* situation, an *S.aureus* *lacR ptsH* double mutant was transformed with the pCPP shuttle vector carrying either the wild-type HPr gene or one of the various mutant *ptsH* genes. The characteristics of all of these strains on a variety of sugar-fermentation plates are summarized in Table I. While the wild-type *S.aureus* strain fermented all of the sugars tested, the *ptsH* mutant fermented only the non PTS carbohydrates maltose and glycerol and the PTS sugar glucose. In contrast to *ptsH* mutants of enteric bacteria, fructose was not fermented, suggesting the absence of a functional FPr-like protein in *S.aureus* (Sutrina *et al.*, 1988). Introduction of the wild-type, plasmid-encoded *ptsH* gene from *B.subtilis* into this *S.aureus* *ptsH* mutant restored fermentation on all PTS sugars although lactose and sucrose

were fermented less efficiently than the wild-type parent. Substituting the wild-type *B.subtilis* *ptsH* gene with the H15A S46A double mutant did not promote sugar fermentation showing that the catalytic activity of HPr as a phosphocarrier protein, and not merely the presence of the protein in the cell, was responsible for sugar utilization. When the regulatory serine 46 was replaced by other neutral amino acids (alanine or threonine) the fermentation pattern was essentially the same as when the wild-type *B.subtilis* HPr was synthesized. By contrast, when serine 46 was replaced by the negatively charged aspartyl residue, a markedly reduced fermentation response on all PTS sugars was observed (Table I). Interestingly, a presumed non-PTS sugar, maltose (Button *et al.*, 1973), also showed reduced fermentation.

Fermentation characteristics of an *E.coli* *ptsH* mutant bearing the plasmid-encoded wild-type and mutant *ptsH* genes from *B.subtilis*

The same plasmids carrying the wild-type and mutant *ptsH* genes of *B.subtilis* were transferred to an *E.coli* *ptsH* microdeletion mutant, LBG1650 (Bolshakova *et al.*, 1979; Waygood *et al.*, 1987), and the fermentation responses on a variety of carbohydrates were recorded. Of these sugars, the *ptsH* mutant fermented only fructose efficiently, but lactose and melibiose were fermented weakly (Table II). Inclusion of the plasmid-encoded wild-type *ptsH* gene from *B.subtilis* fully or partially restored fermentation of all sugars (Table II) except glycerol (data not shown). Inclusion of the H15A single mutant (data not shown) or the H15A S46A double mutant did not restore fermentation, and, surprisingly, it depressed fermentation of lactose and melibiose. The S46A and S46T mutants exhibited fermentation responses comparable to those observed when the wild-type *B.subtilis* *ptsH* gene was present. In agreement with the results obtained with *S.aureus*, the S46D mutant showed diminished fermentation responses on all sugars except fructose (Table II). These results suggest that although the Gram-negative HPr cannot be phosphorylated on serine 46 by Gram-positive kinases (Reizer *et al.*, 1984a, 1988b), and although no *E.coli* HPr(Ser) kinase has yet been identified (Reizer *et al.*, 1984a), a PTS protein (probably Enzyme I) in *E.coli* is nevertheless responsive to the regulatory consequences of a negative charge at position 46.

Transport characteristics of *S.aureus* strains bearing the wild-type and mutant *ptsH* genes from *B.subtilis*

The uptake of four metabolizable sugars by the *ptsH* mutant of *S.aureus* bearing the wild-type or mutant forms of the *B.subtilis* *ptsH* gene is depicted in Figure 5. As can be seen, the plasmid-encoded wild-type HPr from *B.subtilis* as well as the S46A mutant promoted sugar uptake in the chromosomally impaired *ptsH* strain. In some cases, the S46A mutant actually appeared to function slightly better in uptake of the radioactive sugars than did the wild-type protein. The S46D mutant, however, showed markedly depressed rates of uptake for all four carbohydrates (between 15 and 40% of the rates observed with the wild-type *B.subtilis* HPr protein). As expected, the H15A mutant HPr did not promote sugar uptake. An early report suggested that maltose is a non-PTS carbohydrate in *S.aureus* (Button *et al.*, 1973), but the other sugars are clearly substrates of the PTS (Egan and Morse, 1965; Friedman and Hays, 1977).

Inhibition of thiomethyl β -galactoside (TMG) uptake by metabolizable sugars in *S.aureus* strains

Previous *in vitro* studies (Deutscher *et al.*, 1984) had indirectly implicated the ATP-dependent phosphorylation of HPr(Ser) in the exclusion of certain PTS sugar(s) by preferable carbon sources. We examined this possibility under *in vivo* conditions by measuring the degree of the inhibition of [14 C]TMG uptake via the lactose PTS by metabolizable sugars in three isogenic strains of *S.aureus*, all of which synthesized the lactose Enzyme II–III pair and the lactose catabolic enzymes constitutively, due to a mutation in the *lacR* gene which encodes the repressor of the *lac* operon (Breidt *et al.*, 1987; Oskouian and Stewart, 1987). The relevant genotypes of these three strains were (i) *lacR*, (ii) *lacR ptsH/H15;S46* and (iii) *lacR ptsH/S46A* (Table III). These strains were grown in media containing various carbon sources, and the uptake of [14 C]TMG was measured in the presence and absence of various sugars (Table III). TMG uptake in the *ptsH* mutant strain carrying either the wild-type (H15;S46) or the mutant (S46A) *B.subtilis ptsH* plasmid was inhibited to about the same extent by glucose, mannitol or fructose after growth in the presence of any one of these carbon sources. While inhibition by glucose was independent of growth conditions, inhibition by mannitol or fructose was strongest after growth in the presence of mannitol or fructose respectively. Results with a *lacR S.aureus* strain lacking the *B.subtilis* HPr but possessing the staphylococ-

cal phosphocarrier protein were comparable, although inhibition was generally weaker. Non-PTS sugars (maltose and glycerol) were not strongly inhibitory (data not shown, see footnote a of Table III). The results clearly indicate that competition for HPr(His ~ P) rather than ATP-dependent phosphorylation of HPr at serine 46 is responsible for exclusion of TMG by other PTS sugars.

In vivo phosphorylation of *B.subtilis* HPrs in an *S.aureus ptsH* mutant

In agreement with previously published results with *Streptococcus pyogenes* (Reizer *et al.*, 1983, 1984a), phosphorylation of the wild-type *B.subtilis* HPr (on serine) in *S.aureus* could be demonstrated *in vivo* upon addition to the growth medium of any one of a variety of carbon sources (data not shown). It is likely that the HPr(Ser) kinases from *S.pyogenes* and *S.aureus* exhibit similar regulatory properties and that the *S.aureus* kinase recognizes the *B.subtilis* HPr *in vivo* as suggested by previously published *in vitro* experiments (Reizer and Peterkofsky, 1987). In the absence of a carbon source, the *B.subtilis* HPr was not appreciably phosphorylated on serine. Inclusion in the growth medium of one of the various carbohydrates (glucose, fructose, maltose, glycerol or lactose) stimulated phosphorylation of HPr and other cellular proteins to differing extents. Lactose, maltose and glucose were most stimulatory in these uninduced cells while fructose and glycerol exerted smaller stimulatory

Table I. Fermentation of sugars by a *lacR* mutant of *S.aureus* (w.t.) or an isogenic *lacR ptsH* mutant (*ptsH*) of *S.aureus* carrying plasmids bearing the wild-type or mutant *ptsH* genes derived from *B.subtilis*^a

Sugar	w.t.	<i>ptsH</i> ^b	Fermentation response by strain <i>ptsH/H15;S46</i>	<i>ptsH/S46A</i> ^c	<i>ptsH/S46D</i>
Glucose	+	±	+	+	±
Fructose	+	—	+	+	±
Mannitol	+	—	+	+	±
Lactose	+	—	±	±	—
Sucrose	+	—	±	±	—
Maltose	+	±	+	+	±
Glycerol	+	±	+	+	+

^aFermentation responses were recorded on neutral red-dehydrocholate plates containing 2% sugar as described previously (Morse and Alire, 1958). The plasmids bore the wild-type *ptsH* gene of *B.subtilis* (H15;S46) or site-specific mutants of the same *ptsH* gene in which His15 was replaced by alanine (H15A) and/or Ser46 was replaced by alanine (S46A), threonine (S46T) or aspartate (S46D).

^bThe fermentation responses of this strain were the same as those of this strain bearing the plasmid carrying the H15A or H15A S46A mutant *ptsH* gene.

^cThe fermentation responses of the same *S.aureus ptsH* mutant bearing the S46T mutation were the same as those of this strain bearing the S46A mutation.

Table II. Fermentation of sugars by wild-type *E.coli* and a *ptsH* mutant lacking or containing the plasmids bearing the wild-type or mutant *ptsH* genes derived from *B.subtilis*^a

Sugar	w.t.	<i>ptsH</i>	<i>ptsH/H15;S46</i>	<i>ptsH/H15A S46A</i>	<i>ptsH/S46A</i> ^b	<i>ptsH/S46D</i>
Glucose	+	—	±	—	±	—
Fructose	+	+	+	+	+	+
Mannose	+	—	+	—	+	—
N-acetylglucosamine	+	—	±	—	±	—
Mannitol	+	—	±	—	±	—
Glucitol	+	—	±	—	±	—
Lactose	+	±	+	—	+	±
Maltose	+	—	+	—	±	—
Melibiose	+	±	±	—	±	±

^aFermentation responses were recorded on eosin–methylene blue (EMB) agar medium containing 1% sugar as described previously (Saier *et al.*, 1976). Plasmids were as indicated in the text.

^bThe fermentation responses of this strain were the same as those of this *ptsH* mutant bearing the S46T mutation.

effects (data not shown). The poor stimulatory effect of fructose presumably relates to the low basal activity of the fructose PTS in *S. aureus* (unpublished observations). Although the glycerol uptake system is constitutive in *S. aureus* (Saier and Simoni, 1976), glycerol may be a poor precursor for the synthesis of fructose-1,6-diphosphate, the principal allosteric activator of the ATP-dependent HPr kinase (Reizer *et al.*, 1984a). Control experiments showed that the S46A mutant protein was not phosphorylated *in vivo* under the same conditions. The results establish that the *B. subtilis* HPr is readily phosphorylated by the *S. aureus*

HPr(Ser) kinase in intact cells and validate the use of this heterologous system for *in vivo* studies of the physiological significance of HPr(Ser) phosphorylation.

Kinetic parameters reflecting interactions of wild-type and mutant HPrs with Enzymes I and III^{lac}

Figure 6 depicts results of *in vitro* PEP-dependent sugar phosphorylation assays in which an extract from a *ptsH* *S. aureus* mutant was supplemented with wild-type HPr or with the S46A, S46T or S46D mutant HPr. Similar activities were observed for the wild-type HPr and the S46A and S46T mutant proteins when TMG phosphorylation (lactose PTS; panel A) or methyl α -glucoside (α MG) phosphorylation (glucose PTS; panel B) was measured. In contrast, the S46D mutant HPr exhibited only 20% of the wild-type activity when TMG phosphorylation was measured (A) and < 1% of the wild-type activity when α MG phosphorylation was examined (B). Results similar to those reported for TMG were obtained using the semi-quantitative colorimetric assay with ONPG as the sugar substrate: comparable activities were observed for the wild-type, S46A and S46T HPrs whereas the activity of the S46D mutant was markedly lower (data not shown).

In a similar experiment with TMG as the sugar substrate, the activity of HPr(Ser-P) was compared with that of unphosphorylated HPr. This assay was performed using Tris-HCl in place of the standard phosphate buffer and a *ptsH* extract which had been dialyzed extensively against Tris-HCl to remove P_i derived from the bacteria. In contrast to results observed using purified PTS proteins (see below), no significant difference in activity between the phosphorylated and unphosphorylated HPrs was observed (data not shown). The results suggest that even in the absence of P_i , a protein-phosphate phosphatase present in the *ptsH* mutant extract hydrolyzed the HPr(Ser-P).

In order to determine the effects of serine phosphorylation and of amino acid substitutions on the kinetic parameters of the PTS-mediated phosphorylation reactions, the phosphorylation of [¹⁴C]TMG was studied as a function of HPr concentration with either Enzyme I or III^{lac} (both purified to near homogeneity) present in limiting amounts. Exper-

Fig. 5. Uptake of metabolizable sugars by an *S. aureus ptsH* mutant bearing plasmids that carry the wild-type or mutant *ptsH* genes of *B. subtilis*. The figures show the uptake of fructose (A); mannitol (B); glucose (C) and maltose (D). Plasmids carry the wild-type *ptsH* gene (dashed line; $\square$) or the mutant *ptsH* genes (solid lines) as follows: S46A ($\circ$); S46D ($\blacktriangle$); H15A ($\times$). The uptake experiments were conducted as described in Materials and methods.

Table III. Relative rates of [¹⁴C]TMG uptake in the presence of inhibitory sugars after growth in the presence of the indicated carbon source^a

Carbon source for growth	Residual [¹⁴ C]TMG uptake (%) ^b in the presence of								
	Glucose			Mannitol			Fructose		
	w.t. ^c	<i>ptsH</i> /H15;S46	<i>ptsH</i> /S46A	w.t.	<i>ptsH</i> /H15;S46	<i>ptsH</i> /S46A	w.t.	<i>ptsH</i> /H15;S46	<i>ptsH</i> /S46A
None	56	32	26	110	82	61	36	71	65
Glucose	40^d	22	26	108	97	106	96	75	94
Mannitol	52	27	27	40	33	31	82	76	74
Fructose	60	21	27	115	98	84	35	28	27
Maltose	34	31	23	85	76	77	90	83	77
Glycerol	48	15	35	109	76	92	96	67	96

^a*Staphylococcus aureus* strains were grown in LB broth containing the indicated carbon source at a concentration of 1%, harvested during exponential growth, washed, and resuspended in medium 63 containing 0.5% bactopectone and 0.1% yeast extract for uptake measurements at 37°C employing 2 mM [¹⁴C]TMG, 10 mM of the inhibitory sugar, and a cell density of ~ 2 mg dry wt/ml. Non-PTS sugars (maltose and glycerol) were only weakly inhibitory regardless of growth conditions (inhibition never in excess of 50%; data not shown).

^bData are expressed as percentages of initial rates relative to those obtained in the absence of sugar during the transport reaction. A [¹⁴C]TMG uptake rate of 100% (uptake in the absence of an inhibitory sugar) corresponds to a value of between 13 and 29 nmols/min/mg, dry wt for all three strains regardless of the carbon source present during growth.

^cAll strains carried the *lacR* mutation and consequently expressed the *lac* regulon constitutively.

^dNumbers in bold represent values obtained under conditions of maximal inhibition (see text).

imental conditions were as described in Materials and methods. The results of these experiments are depicted in Figure 7, and the kinetic parameters obtained from the plots are summarized in Table IV. Substitution of serine 46 with alanine (S46A) had little effect on the kinetic parameters obtained when either the Enzyme I–HPr interaction or the III^{lac} –HPr interaction was studied, and substitution of serine 46 with threonine exerted little effect on the latter

Fig. 6. Kinetic analyses of the overall PTS-catalyzed phosphorylation of methyl β -thiogalactoside (TMG; A) and methyl α -glucoside (α MG; B) as a function of HPr concentration. The data obtained with the wild-type HPr (H15;S46) are indicated by the dashed lines (●). Those obtained with the mutant proteins are indicated by the solid lines: S46A (○), S46T (□) and S46D (△). The experiment was conducted with a crude extract of an *S.aureus ptsH* mutant as described in Materials and methods. Each value represents an initial rate measurement obtained from the time course of phosphorylation.

Fig. 7. Kinetic analyses characterizing the phospho-Enzyme I–HPr interaction (A) and the phospho-HPr– III^{lac} interaction (B). The experiments were conducted with free, wild-type HPr (●; dashed lines), wild-type HPr(Ser-P) (×; dotted lines) and the mutant HPr proteins (solid lines) as follows: S46A (○), S46T (□) and S46D (△). Purified Enzymes I and III^{lac} as well as washed membranes as a source of Enzyme II^{lac} were used as described in Materials and methods. In (A) Enzyme I was rate limiting while III^{lac} and II^{lac} were present in excess; in (B) III^{lac} was rate limiting while Enzyme I and II^{lac} were present in excess.

reaction. Thus, the V_{max} values were the same within experimental error for the three substrate proteins while the K_m values differed only by a factor of two to three. Interestingly the S46A mutant protein exhibited higher apparent affinity than the wild-type counterpart for both PTS enzymes in agreement with the uptake result (Figure 5).

The substitution of serine 46 by aspartate (S46D) resulted in a dramatic change in the kinetic parameters when either the Enzyme-I-limiting or the III^{lac} -limiting reaction was studied. Thus, these reactions were characterized by a 4- to 6-fold decrease in the apparent affinities of the enzymes for HPr as well as a 2- to 10-fold decrease in the maximal velocities (Table IV). Phosphorylation of serine 46 in the wild-type HPr protein resulted in a similar but more dramatic reduction of activity which precluded accurate estimation of the kinetic parameters. It should be noted that the low activity of HPr(Ser-P) was not due to loss of activity during its isolation since dephosphorylation led to complete reactivation (data not shown). The results suggest that serine phosphorylation can control the overall PTS phosphorylation cascade by reducing the affinities of both Enzyme I and III^{lac} for HPr as well as the maximal velocities of these phosphoryl transfer reactions. The mere presence of a negative charge at position 46 can apparently effect this change. These results substantiate by an independent method the results shown in Figure 1 which led to the conclusion that S46D is a poor substrate of Enzyme I.

Effects of PTS proteins on the ATP-dependent phosphorylation of HPr

Figure 8 shows the effects of Enzymes I and III^{lac} on the phosphorylation of wild-type HPr (A) and the H15A mutant protein (B) by the HPr(Ser) kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Lane 1 (controls) in both A and B show the phosphorylation of the wild-type and mutant HPrs respectively, in the absence of PEP and Enzyme I. In lane 2, Enzyme I and PEP were added 15 min prior to the addition of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the kinase. It can be seen for both the wild-type HPr and the mutant protein which cannot be phosphorylated by Enzyme I, that the amounts of incorporation of ^{32}P into each of the HPrs were significantly diminished. As shown in lane 3, PEP alone had no significant affect. By contrast, Enzyme I alone inhibited phosphorylation by ATP both in the wild-type and the H15A mutant forms of HPr (lane 4). Interestingly, addition of III^{lac} appeared to have the opposite effect, stimulating the kinase-catalyzed phosphorylation of the two HPrs (lane 5 in Figure 8A; data not shown for the H15A protein). These results suggest (i) that the binding of Enzyme I or III^{lac} to HPr induces a conformational change

Table IV. Kinetic parameters characterizing the interactions between the various HPr proteins and both Enzyme I and III^{lac}

	Enzyme I–HPr interaction		III^{lac} –HPr interaction	
	K_m (μM)	V_{max} (relative values)	K_m (μM)	V_{max} (relative values)
H15;S46	4	1.0	2.0	1.0
S46A	1.4	1.0	1.0	1.0
S46T	— ^b	— ^b	1.0	2.0
S46D	16	0.1	11	0.5

^aThe kinetic constants were calculated from Lineweaver–Burk plots of the data shown in Figure 7 as described in Materials and methods.

^b—, not determined.

in HPr, which alters its interaction with the kinase; (ii) that inhibition by Enzyme I might be partially explained by competition for the kinase binding site on HPr; and (iii) that the kinase does not bind to the site on HPr which interacts with III^{lac} .

Discussion

In the present study we have isolated site-specific mutants of the *ptsH* gene of *B. subtilis* and used these mutants for both *in vitro* and *in vivo* studies aimed at defining the mechanism and physiological significance of seryl residue 46 phosphorylation in HPr by the ATP-dependent, fructose-1,6-diphosphate-activated HPr kinase of Gram-positive bacteria. Previous studies using the purified general energy-coupling proteins and sugar-specific Enzyme III of the PTS led to the suggestion that phosphorylation of seryl residue 46 resulted in strong inhibition of HPr phosphorylation on the active histidyl residue (residue 15) by Enzyme I (Deutscher *et al.*, 1984, 1985). However, the kinetic parameters affected by Ser46 phosphorylation *in vitro*, the influence of PTS protein binding to HPr, the mechanistic basis for the inhibition of Enzyme I function, and the physiological significance of this reaction *in vivo* were not defined. In the present study, we attempt to provide answers to each of these uncertainties. Our *in vitro* studies have shown that replacement of seryl residue 46 with alanine or threonine has no major effect on the kinetic parameters which characterize the interactions of HPr with either Enzyme I or III^{lac} . By contrast, replacement of seryl residue 46 with a negatively charged aspartyl residue resulted in strong inhibition of both the Enzyme-I- and III^{lac} -catalyzed phosphorylation reactions. Phosphorylation of seryl residue 46 by the kinase led to a similar but more dramatic effect. These observations lead to the conclusion that the presence of a negative charge on residue 46 rather than the addition of a bulky phosphoryl group is the cause of inhibition.

The detailed mechanism by which a negative charge at position 46 in HPr reduces both the affinities and maximal velocities of the Enzyme-I- and III^{lac} -catalyzed reactions is unknown. However, the three-dimensional structure of the *E. coli* HPr has been determined both by X-ray diffraction

(El-Kabbani *et al.*, 1987) and by two-dimensional NMR (Klevit and Waygood, 1986), and the three-dimensional structures of both the *B. subtilis* wild-type HPr and the S46D mutant are currently under study (R. Klevit and J. Reizer, unpublished results). These studies have shown that the Gram-positive HPr is structurally similar to that of Gram-negative bacteria. Further, the S46D mutant protein shows striking similarities to HPr(Ser-P). The structural consequences of the presence of a negative charge on residue 46 may well be revealed by these studies.

Only one other bacterial protein kinase is as well characterized at the present time as the HPr(Ser) kinase (Cozzzone, 1988; Deutscher and Saier, 1988). Isocitrate dehydrogenase kinase of *E. coli* catalyzes the phosphorylation of regulatory seryl residue 113 in the dehydrogenase (Borthwick *et al.*, 1984; Thorsness and Koshland, 1987). This reaction results in complete inhibition of the target dehydrogenase (LaPorte and Koshland, 1983; Nimmo, 1987). Studies from Koshland's laboratory showed that replacement of the regulatory seryl residue in isocitrate dehydrogenase with aspartate resulted in virtually complete inhibition of the enzyme (Thorsness and Koshland, 1987). Thus, isocitrate dehydrogenase of *E. coli* seems to be regulated by a charge-induced conformational change similar to that by which the interactions of HPr with other PTS proteins is regulated in Gram-positive bacteria.

The low specificity of eukaryotic protein kinases does not seem to be a characteristic of the Gram-positive bacterial HPr kinases. Thus, while the latter kinases can phosphorylate any one of the purified Gram-positive HPrs, they do not phosphorylate the conserved seryl residue 46 in the structurally similar *E. coli* HPr. Moreover, replacement of Ser46 in the *B. subtilis* protein with threonine substantially reduced the kinase activity. Recently, we have synthesized a 29-amino-acyl residue peptide, corresponding to the middle third of the *B. subtilis* HPr (residues 28–56), and showed that it was not phosphorylated by the HPr(Ser) kinase (S.L. Sutrina, T. Paulson, J. Reizer, J. Tomich and M.H. Saier, Jr, unpublished results). Evidently, the kinase recognizes the three-dimensional structure of HPr rather than a restricted amino acyl sequence within the protein. This conclusion is in agreement with the observation that His15 phosphorylation by PEP and Enzyme I strongly inhibits Ser46 phosphorylation by ATP and the kinase (Reizer *et al.*, 1984a). Since a large N-terminal peptide (residues 1–31) derived from the *B. subtilis* HPr was poorly phosphorylated by Enzyme I and PEP (S.L. Sutrina *et al.*, unpublished results), we must conclude that Enzyme I, like the kinase, recognizes the three-dimensional structure of HPr.

A surprising result of our *in vitro* studies was the observation that the presence of Enzyme I in the HPr(Ser) kinase assay inhibited the kinase while the presence of III^{lac} stimulated it. Our previous studies (Reizer *et al.*, 1984a), showing that Enzyme I and PEP strongly inhibited the protein-kinase-catalyzed phosphorylation of seryl residue 46, was interpreted solely in terms of an inhibitory effect by His15 phosphorylation. It now appears that there are two components of this inhibition, the first attributed merely to the binding of Enzyme I to HPr, the other due to HPr histidine phosphorylation and consequent depletion of the natural substrate of the kinase, i.e. free HPr. Since the presence of III^{lac} in the HPr(Ser) kinase assay was shown to stimulate the kinase, it must be concluded that the kinase and III^{lac}

Fig. 8. Effects of Enzyme I and III^{lac} on the ATP-dependent, kinase-catalyzed phosphorylation of the wild-type HPr (A) and the H15A mutant HPr (B). [^{32}P]ATP and the kinase were added 15 min after addition of Enzyme I (80 $\mu\text{g/ml}$), PEP (1 mM) and/or III^{lac} (120 $\mu\text{g/ml}$) as indicated below the autoradiogram. The experiment was conducted several times employing conditions described in Materials and methods to ensure reproducibility. In the experiment shown, a 15-min incubation at 37°C with ATP and the kinase was employed prior to electrophoretic separation on a native gel.

can simultaneously bind to the monomeric HPr protein. The inhibitory effect of Enzyme I could be due to either direct competition of the two enzymes for HPr, in which case overlapping binding sites are implied, or to the formation of an Enzyme I–HPr complex that induces a conformational change in HPr which inhibits the kinase. These two mechanisms are not mutually exclusive.

In order to ascertain the physiological significance of HPr(Ser) phosphorylation, a *lacR ptsH* mutant of *S.aureus* was transformed with plasmids carrying either the wild-type or one of the mutant *ptsH* genes of *B.subtilis*. Fermentation analyses of these strains (Table I) showed that with the exception of sucrose and lactose, the *B.subtilis* HPr functioned as effectively in the *S.aureus* PTS *in vivo* as did the *S.aureus* HPr. Moreover, all of the site-specific mutants, in which seryl residue 46 was replaced by an amino acid with a neutral side chain, gave fermentation responses comparable to those shown by wild-type *S.aureus*. In contrast, replacement of seryl residue 46 with a negatively charged aspartyl residue gave diminished fermentation of most carbohydrates tested including the non-PTS carbohydrate, maltose (Button *et al.*, 1973). Similar behavior was noted in a comparable *E.coli* genetic background (Table II) showing that Gram-negative Enzyme I can be regulated by the presence of a negative charge at position 46. The conclusions reached from these fermentation studies were substantiated by transport studies.

Two mechanisms have been proposed to explain the exclusion of one PTS sugar by another: (i) competition for HPr(His ~ P) and (ii) inhibition by HPr(Ser-P) (Saier, 1985; Reizer and Peterkofsky, 1987). Since mutagenesis of S46 in HPr to alanine does not diminish the exclusion response (Table III), we conclude that competition for HPr(His ~ P) is largely, if not solely, responsible for the inhibition of the uptake. The *in vitro* studies showed that seryl residue 46 phosphorylation strongly inhibited Enzyme I activity and overall PTS function, and the *in vivo* studies showed that our PTS sugar competition study was performed under conditions that resulted in enhancement of serine 46 phosphorylation. Consequently, we must conclude that a fraction of the cellular HPr was phosphorylated by the kinase which was insufficient to cause inhibition. This conclusion applies to wild-type *S.aureus* as well as the *ptsH* mutant strain harboring various *B.subtilis ptsH* genes (Table III).

During preparation of this paper, Hengstenberg and co-workers published a study describing the isolation and some properties of site-directed *B.subtilis* HPr mutants in which the protein was modified at the ATP-dependent phosphorylation site (Eisermann *et al.*, 1988). Four major findings and conclusions described by Eisermann *et al.* differ from those presented in this report.

- (i) We have successfully over-expressed the *ptsH* gene product following substitution of seryl residue 46 with aspartate in both *S.aureus* and *E.coli* showing that expression of this protein is not a lethal event. The cause for the failure of Eisermann *et al.* (1988) to express this mutant derivative of HPr is not known.
- (ii) Figures 2–4 in this report show that the ATP-dependent HPr kinases of *L.brevis*, *S.pyogenes*, *S.aureus*, *B.subtilis* and *S.faecalis* phosphorylate a threonyl residue in the S46T mutant. Because the rate of this reaction is low relative to that for the wild-type

protein, the failure of Hengstenberg and co-workers to detect this reaction may have been due to insufficient sensitivity of their assay.

- (iii) The kinetic analyses presented in Figures 6 and 7 and summarized in Table IV indicate that the S46A and S46T mutant HPrs exhibit phosphotransfer activities comparable to those of wild-type HPr. These results were substantiated by the uptake results reported in Figure 5 and Table III. Eisermann *et al.* (1988) using a semi-quantitative assay reported up to 80% reduction in the phosphoryl transfer activities of the same mutant HPrs relative to the wild-type protein. A detailed kinetic analysis was not reported, and no *in vivo* studies were conducted. We have no explanation for the discrepant results.
- (iv) Indirect evidence, i.e. growth yield of a *B.subtilis* mutant in which the chromosomally encoded HPr was replaced with HPr species that lacked a phosphorylatable amino acyl residue at position 46, i.e. S46A, led Eisermann *et al.* (1988) to conclude that accumulation of glucose and mannitol by this mutant strain occurs in an uncontrolled manner which differs in rate or extent from the modulated accumulation of these sugars by the wild-type bacillus. The data shown in Figure 5 of the present paper do not reveal significant differences in the rate of uptake of these carbohydrates in an *S.aureus* strain bearing the S46 mutant or the wild-type HPr. Nevertheless, the conditions and bacterial strains used in the two laboratories were different and may provide an explanation for the differing results.

As a result of these studies we propose that in *S.aureus*, and perhaps in other Gram-positive bacteria as well, the primary physiological function of HPr(Ser) phosphorylation is not regulation of carbohydrate influx via the PTS, but rather regulation of some other metabolic or transport process (Reizer and Peterkofsky, 1987; Reizer *et al.*, 1984b, 1988b; Saier, 1989). This suggestion is supported by the recent discovery of HPr and an HPr(Ser) kinase with properties similar to those found in *B.subtilis* and *S.aureus* in the heterofermentative lactobacilli which do not possess an intact PTS (Reizer, *et al.*, 1988b). We are currently engaged in studies to establish the physiologically relevant target(s) of HPr(Ser) phosphorylation in Gram-positive bacteria.

Materials and methods

Materials

Restriction endonucleases, T₄ DNA ligase, T₄ DNA polymerase, T₄ polynucleotide kinase and the large fragment of DNA polymerase I (Klenow), were obtained from Bethesda Research Laboratories, Gaithersburg, MD, New England Biolabs, Beverly, MA, or International Biotechnologies, Inc., New Haven, CT. The deoxyadenosine 5'-(α -³²S)thiotriphosphate (1335 Ci/mmol), [γ -³²P]ATP (4500 Ci/mmol) and ¹⁴C-radiolabeled sugars were purchased from the New England Nuclear Corp., Boston, MA, or the Amersham Corp., Arlington Heights, IL. Isopropyl- β -D-thiogalactopyranoside, 5-bromo-4-chloro-3-indolyl- β -D-galactoside and lysozyme were purchased from Boehringer Mannheim Biochemicals. Acrylamide, bisacrylamide and N,N,N',N'-tetramethylethylenediamine were purchased from Bio-Rad Laboratories. Agarose was purchased from Bethesda Research Laboratories. Deoxynucleotides and dideoxynucleotides were obtained from Pharmacia and P-L Biochemicals, Inc. Sephadex G-50 and DEAE–Sephacel were from Pharmacia, and cellulose-coated thin-layer (0.1 mm) chromatography plates were obtained from E. Merck, Darmstadt, FRG. All other chemicals and enzymes used were of the highest quality available commercially.

Bacterial strains and plasmids

Escherichia coli strains. LBG1650 (*ptsH5*) was obtained from Dr V.N. Gershanovitch (Bolshakova *et al.*, 1979; Rusina and Gershanovitch, 1983). C600 (an $r^{-}m^{+}$ lambda lysogen) and MZ1 (a lambda lysogen carrying C1875ts) (Zuber *et al.*, 1987) were obtained from Dr D. Court of the National Cancer Institute. Strain TG1 was provided by Dr T. Gibson of the Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK (Carter *et al.*, 1985).

Staphylococcus aureus strains. S797A (*lacR ptsH*) and KUS104 (*lacR*) have been described previously (Hengstenberg *et al.*, 1969; Oskouian and Stewart, 1987).

Plasmids. pTS21 (Gonzy-Tréboul and Steinmetz, 1987), carrying the *ptsH* structural gene, was kindly provided by Dr G. Gonzy-Tréboul of the Jacques Monod Institute, University of Paris, France. The shuttle vector pCPP (Band *et al.*, 1983) that contains the *spac-1* promoter (Yansura and Henner, 1984) and a multilinker cloning site 3' to the *lac* operator, was obtained from Dr P.S. Lovett, Department of Biological Sciences, University of Maryland. The pRE expression vector (P.Reddy, A. Peterkofsky and R. McKenney, in preparation) was used to overproduce the wild-type and mutated *ptsH* gene products.

Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides were synthesized from β -cyanoethyl phosphoramidite precursors on a Model 380B Applied Biosystems DNA synthesizer. The oligonucleotides were synthesized as trityl-off derivatives which were deblocked at 58°C for 16 h. The solutions were evaporated to 0.25 ml under a stream of nitrogen, and the oligonucleotides were then precipitated by the addition of cold ethanol. The precipitates were dried under vacuum and then dissolved in Tris (10 mM)–EDTA (0.1 mM) buffer (pH 7.0). The oligodeoxynucleotides used to change HPr at position 46 to (i) alanine, (ii) threonine, (iii) tyrosine or (iv) aspartate, were (i) 5' GTT AAC CTT AAA GCT ATT ATG GGT GTT 3', (ii) 3' GTT AAC CTT AAA ACT ATT ATG GGT GTT 3', (iii) 5' GTT AAC CTT AAA TAT ATT ATG GG 3' or (iv) 5' GTT AAC CTT AAA GAC ATT ATG GGT 3' respectively. The oligodeoxynucleotide used for modification of position 15 of HPr to alanine was 5' GAT TCT GGA ATC GCT CGT CCT GCG 3'. The oligodeoxynucleotide used to create an *NdeI* site at the initiation codon of the *ptsH* gene, was 5' TGA TAC ATA TGG CAC 3'.

DNA procedures

Escherichia coli strains C600 and TG1 carrying the pCPP or pRE plasmids were grown at 37°C in Luria–Bertani (LB) medium (Miller, 1972) containing chloramphenicol (25 μ g/ml) or ampicillin (50 μ g/ml) respectively. The lysozyme/heat method (Holmes, 1984) was used for miniprep preparation of plasmid DNA. Single-stranded M13mp8 and M13mp19 DNA were prepared from *E. coli* TG1 cultures infected with M13 phage as described by Sanger *et al.* (1980). Large-scale preparation of phage replicative form and plasmid DNAs was accomplished by the procedure of Birnboim (1983). Competent cells of *E. coli* strain TG1 were prepared by the CaCl_2 method (Messing, 1983). Competent cells of *E. coli* strains C600 and MZ1 were prepared as described by Hanahan (1983). Transformation of *S. aureus* strains was conducted as previously described (Oskouian and Stewart, 1987).

Site-directed mutagenesis was performed as described by Zoller and Smith, (1983). Briefly, the phosphorylated synthetic oligonucleotides were annealed with an M13-recombinant DNA template containing the *ptsH* gene of *B. subtilis*, and the second strand was synthesized in the presence of deoxyadenosine 5'- $(\alpha\text{-}^{35}\text{S})$ thiotriphosphate, dCTP, dGTP, dTTP, the large fragment of DNA polymerase I and T4 DNA ligase. Closed circular DNA was isolated by centrifugation on an alkaline sucrose gradient (5–20%) and was used to transform a competent TG1 strain of *E. coli*. Phage DNA containing the desired base change were characterized by dideoxynucleotide sequencing (Sanger *et al.*, 1977) after isolation of the single-stranded DNA template.

Protein purification

Cultures of *E. coli* MZ1 containing plasmid pRE with the wild-type or mutated *ptsH* gene fused to the lambda *cII* ATG were grown to the mid logarithmic phase at 30°C in LB medium containing ampicillin (25 μ g/ml), and expression of the *ptsH* gene products was induced by a 2 h incubation period at 42°C. Cells were harvested by centrifugation and washed twice with 20 mM Tris–HCl buffer (pH 7.2) containing 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (TTP buffer). They were then resuspended in a minimal volume of the same buffer and ruptured by two passages through an Aminco French pressure cell at 10 000 p.s.i. Whole cells and cell debris were removed by centrifugation for 15 min at 10 000 r.p.m. and the wild-type or mutated HPrs were purified by ion-exchange

chromatography on a DEAE–Sephacel column (20 ml bed volume) using a linear salt concentration gradient (0–0.4 M NaCl) in TTP buffer. Fractions that contained HPr were pooled, concentrated by ultrafiltration (Amicon Corp., YM-2) and purified further on a Sephadex G-50 column equilibrated with TTP buffer containing 0.1 M NaCl. Mutant complementation assays with the *S. aureus ptsH* mutant and the chromogenic substrate *o*-nitrophenyl β -D-galactoside (ONPG) (5 mM) was used to determine HPr activity during purification of this protein (Hengstenberg *et al.*, 1969). Purification of the H15A mutant HPr was monitored by SDS–PAGE. This two-step procedure yielded nearly homogeneous HPrs (>95% pure) as judged by isoelectric focusing on a PhastGel IEF 3–9 (Phastsystem, Pharmacia Inc., Piscataway, NJ) and by nondenaturing (Figure 1) as well as SDS–PAGE. Enzymes I, III^{lac} and the ATP-dependent kinase of HPr were purified from *S. faecalis*, *S. aureus* and *L. brevis* respectively, as detailed previously (Alpert *et al.*, 1985; Deutscher *et al.*, 1982; Reizer *et al.*, 1988b respectively).

HPr(Ser-P) was purified following phosphorylation of HPr in a standard reaction mixture (see below) that contained 1 mM unlabeled ATP, 4–6 mg of HPr and 50 μ l of the partially purified HPr(Ser) kinase from *L. brevis* in a final volume of 1 ml. HPr(Ser-P) was then separated from HPr by native polyacrylamide (13.5%) preparative gel electrophoresis on a 6.5 $\times$ 1 cm column (Bethesda Research Laboratories, Gaithersburg, MD) at 2 mA and 500 V. Elution was carried out with Tris (25 mM)–glycine (192 mM) buffer (pH 8.3) at a flow rate of 14 ml/h, and fractions (0.7 ml) containing HPr(Ser-P) were pooled and dialyzed against 20 mM Tris–hydrochloride buffer (pH 7.2). This procedure yielded electrophoretically pure HPr(Ser-P) as confirmed by analytical nondenaturing PAGE.

Protein phosphorylation assays

The standard assay mixture for *in vitro* phosphorylation of HPr contained 50 mM Tris–acetate buffer (pH 7.5), 1 mM dithiothreitol, 2 mM MgCl_2 , 5 mM fructose 1,6-diphosphate, 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (approximate sp. act. 500–2000 c.p.m./pmol), 5–10 μ g (12.5–25 μ M) of HPr and 2–3 μ g of the purified kinase preparation from *L. brevis* in a final volume of 40 μ l. The assay for HPr(His) phosphorylation was performed under similar conditions except that fructose 1,6-diphosphate was omitted, and PEP (5 mM) and Enzyme I (2 μ g) replaced ATP and the HPr(Ser) kinase. The reaction mixture was incubated at 37°C for 15–60 min, and proteins were then separated by SDS– or nondenaturing-polyacrylamide (15%) gel electrophoresis as described previously (Reizer *et al.*, 1983, 1984a). The gels were stained (for 30 min at 55°C) with Coomassie blue R in 40% methanol, 10% acetic acid and destained (for 2.5 h at 55°C) in the same solvent. Phosphorylated HPr species were identified by autoradiography or by their shift in electrophoretic mobility in nondenaturing stained gels (Reizer *et al.*, 1984a).

The *in vivo* phosphorylation of proteins in an *S. aureus ptsH* mutant bearing the wild-type form of the *B. subtilis* HPr carried on the pCPP shuttle vector was performed, and products were analyzed essentially as previously described (Reizer *et al.*, 1983, 1988b).

Transport assays

Cells of the *S. aureus ptsH* mutant bearing the wild-type or mutant forms of the *B. subtilis ptsH* gene carried on the shuttle vector pCPP were grown with shaking at 37°C in LB medium containing chloramphenicol (25 μ g/ml) and the indicated sugar (1%). Midlogarithmic cultures were harvested by centrifugation, washed twice with Medium 63 (Miller, 1972) and resuspended in the same medium to an absorbance of 600–700 klett units (extract 66). Cell suspensions containing 0.5% bactopectone and 0.1% yeast extract were prewarmed at 37°C for 5 min before addition of ^{14}C -labeled glucose, fructose, mannitol or maltose (1 mM; 0.5 μ Ci/ μ mol) or TMG (2 mM; 0.2 μ Ci/ μ mol). The transport reactions were terminated by withdrawing samples at intervals and collecting the cells on membrane filters (0.45 μ m; Millipore Corp., Bedford, MA). Radioactivity was determined as described previously (Reizer and Panos, 1982). An intracellular volume of 3.1 μ l/mg cells (dry wt) was used to determine the concentration of substrate accumulated (Simoni and Roseman, 1973).

Phosphoamino acid analyses

Radiolabeled proteins were subjected to native-PAGE, and the appropriate bands in the wet gel were localized by autoradiography. These bands were excised, and the phosphoproteins were eluted from the gel by grinding in ammonium bicarbonate (50 mM) and precipitated with trichloroacetic acid (20%) in the cold. The precipitated protein was washed with cold ethanol and subjected to acid hydrolysis in 6 M HCl at 110°C for 60 min. The HCl was removed *in vacuo*, and phosphorylated amino acids were separated as previously described (Cooper *et al.*, 1983) on cellulose thin-layer (0.1 mm) plates by two-dimensional electrophoresis using formic acid/acetic acid/water

(25:78:897 by vol.) buffer (pH 1.9) for 20 min at 1500 V (first dimension), followed by pyridine/acetic acid/water (5:50:945 by vol.) buffer (pH 3.5) for 16 min at 1300 V (second dimension). Standard phosphoserine, phosphothreonine and phosphotyrosine were visualized with ninhydrin (0.2% in acetone), and radiolabeled phosphoamino acids were detected by autoradiography.

PEP-dependent sugar phosphorylation assays

Complementation assays for HPr were performed as described previously (Saier et al., 1976). Assay mixtures contained 2.5 mM MgCl_2 , 25 mM KF, 2.5 mM dithiothreitol, 50mM potassium phosphate or Tris-HCl buffer (pH 7.4), $10 \mu\text{M}$ [^{14}C] sugar ($5 \mu\text{Ci}/\mu\text{mol}$), 5 mM PEP and the crude extract from a *ptsH* strain of *S.aureus*, which had been induced for a specific Enzyme II/III pair by growth in the presence of an appropriate sugar if necessary (i.e. for non-constitutive systems). Reactions were started by addition of HPr at 37°C, and at intervals the reaction mixtures were assayed for [^{14}C] sugar-P production using ion exchange columns to separate phosphorylated from free sugar (Kundig and Roseman, 1971). Alternatively, the semiquantitative colorimetric assay with ONPG as substrate was used (Hengstenberg et al., 1969).

For kinetic studies, purified preparations of *S.faecalis* Enzyme I and *S.aureus* III^{lac} were used, and washed membranes from a *lac*-constitutive *ptsH* strain of *S.aureus* served as a source of Enzyme II^{lac}. Assay conditions were adjusted such that either Enzyme I or III^{lac} was rate limiting, while other PTS components (Enzyme II^{lac} and either III^{lac} or Enzyme I respectively) were present in excess. Phosphorylation of [^{14}C]TMG ($10 \mu\text{M}$) was then studied as a function of HPr concentration under conditions of limiting Enzyme I or limiting III^{lac}. Apparent K_m and V_{max} values of Enzyme I and III^{lac} for the various HPrs were obtained by extrapolation of the data on double reciprocal plots.

Acknowledgements

We thank Dr Rachel Klevit for conducting confirmatory NMR structural analyses of some of the mutant HPr proteins. Renée S.Chemelar, Joel Hoskins and Babak Oskouian provided valuable assistance in some of the experiments described. This work was supported by USPHS grants 5RO1 AI 21702 and 2RO1 AI 14176 from the National Institutes of Allergy and Infectious Diseases.

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Received on February 29, 1989

Note added in proof

Certain commercial equipment, instruments and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument, or equipment identified is necessarily the best available for the purpose.