Mutation of Serine-46 to Aspartate in the Histidine-Containing Protein of *Escherichia coli* Mimics the Inactivation by Phosphorylation of Serine-46 in HPrs from Gram-Positive Bacteria[†,#]

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ABSTRACT: Histidine-containing protein (HPr) is a phosphocarrier protein of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. HPr is phosphorylated at the active site residue, His15, by phosphoenolpyruvate-dependent enzyme I in the first enzyme reaction in the process of phosphoryl transfer to sugar. In many Gram-positive bacterial species HPr may also be phosphorylated at Ser46 by an ATP-dependent protein kinase but not in the Gram-negative *Escherichia coli* and *Salmonella typhimurium*. One effect of the phosphorylation at Ser46 is to make HPr a poor acceptor for phosphorylation at His15. In *Bacillus subtilis* HPr, the mutation Ser46Asp mimics the effects of phosphorylation. A series of mutations were made at Ser46 in *E. coli* HPr: Ala, Arg, Asn, Asp, Glu, and Gly. The two acidic replacements mimic the effects of phosphorylation of Ser46 in HPrs from Gram-positive bacteria. In particular, when mutated to Asp46, the His15 phosphoacceptor activity (enzyme I K_m/k_{cat}) decreases by about 2000-fold (enzyme I K_m, 4 mM HPr; k_{cat}, ~30%). The alanine and glycine mutations had near-wild-type properties, and the asparagine and arginine mutations yielded small changes to the K_m values. The crystallographic tertiary structure of Ser46Asp HPr has been determined at 1.5 Å resolution, and several changes have been observed which appear to be the effect of the mutation. There is a tightening of helix B, which is demonstrated by a consistent shortening of hydrogen bond lengths throughout the helix as compared to the wild-type structure. There is a repositioning of the Gly54 residue to adopt a 3_{10} helical pattern which is not present in the wild-type HPr. In addition, the higher resolution of the mutant structure allows for a more definitive placement of the carbonyl of Pro11. The consequence of this change is that there is no torsion angle strain at residue 16. This result suggests that there is no active site torsion angle strain in wild-type *E. coli* HPr. The lack of substantial change at the active center of *E. coli* HPr Ser46Asp HPr suggests that the effect of the Ser46 phosphorylation in HPrs from Gram-positive bacteria is due to an electrostatic interference with enzyme I binding.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] is found in many bacterial species. It is primarily responsible for the translocation and phosphorylation of a variety of sugars, the selection of which varies considerably with species. In addition, the PTS has significant regulatory roles which vary in detail depending upon species [see reviews by Postma et al. (1993) and Meadow et al. (1990)]. In bacteria such as *Escherichia coli* and *Salmonella typhimurium* the major regulatory protein of the PTS appears to be the phosphocarrier protein IIA^{glc} or P-IIA^{glc}, which contains an active center $N^{\epsilon 2}$-phosphohistidine. This protein is phosphorylated by the histidine-containing protein HPr, which contains an active center $N^{\delta 1}$-phosphohistidine. In many species other than *E. coli* and *S. typhimurium*, HPr can also be phosphorylated by an ATP-dependent protein kinase at Ser46 (Saier & Deutscher, 1983), and P-(Ser)HPr is proposed as the major regulatory protein (Reizer et al., 1993). P-(Ser)HPr can be found in several species which do not have functional PTS for sugar transport (Romano et al., 1987; Reizer et al., 1988, 1993), which emphasizes the regulatory role of HPr. Recently, there have been a number of reports that show that P-(Ser)HPr has several regulatory roles that probably involve binding to permeases and enzymes in a manner reminiscent of the mechanism by which IIA^{glc} exerts its effects in *E. coli* (Ye et al., 1994; Ye & Saier, 1995a,b). A major effect of

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; P-(Ser)HPr, HPr phosphorylated at residue serine-46; P-(His)-HPr, HPr phosphorylated at residue histidine 15; P-(Ser)P-(His)HPr, HPr phosphorylated at both residues; IIA^{sugar}, the first domain of the sugar-specific enzymes II; FPr, a fructose-induced protein that contains two domains, HPr and $IIA^{fructose}$; man, mannose; glc, glucose; SPRIA, solid-phase radioimmunoassay.

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phosphorylation of Ser46 of HPr from Gram-positive bacteria is to inhibit its ability to act as a phosphoacceptor substrate in the enzyme I reaction. In addition, the mutant Ser46Asp HPr had kinetic properties similar to those of P-(Ser)HPr and this mutant has been shown to have many of the physiological properties of P-(Ser)HPr (Reizer et al., 1989; Deutscher et al., 1994).

Ser46 is conserved in all 12 HPrs that have been sequenced (Reizer et al., 1993), yet no evidence has been found that HPr from either *E. coli* or *S. typhimurium* can be phosphorylated. An acid-stable phosphorylation (P-serine) is not detected in HPr when crude extracts from these species are incubated with [γ-^{32}P]ATP (Waygood et al., 1984). Moreover, *E. coli* HPr cannot be phosphorylated at Ser46 by the ATP-dependent protein kinase found in extracts of several species. However, addition of *E. coli* HPr to crude extracts of *Streptococcus salivarius* appeared to inhibit the formation of *S. salivarius* P-(Ser)HPr, suggesting that *E. coli* HPr has enough structural similarity to act as a competitive inhibitor of the ATP-dependent kinase (Waygood et al., 1986).

The structures of *E. coli*, *Streptococcus faecalis*, and *B. subtilis* HPrs have all been determined by X-ray diffraction at high resolution (Herzberg et al., 1992; Jia et al., 1993a,b, 1994b), and a detailed comparison has been made (Jia et al., 1994a). There is high structural homology for the region around Ser46, but in *E. coli* HPr there are different side chains in the vicinity, in particular, several lysine residues, Lys27 and Lys49, and the bulky Phe48 which are not found in HPrs which can be phosphorylated at Ser46. These may be the structural features that prevent the phosphorylation of Ser46 in *E. coli* HPr (Waygood et al., 1987; Jia et al., 1993a). There are also structures derived from NMR studies; Wittekind et al. (1989, 1990, 1992) and Pullen et al. (1995) have investigated the structural effects of mutation and phosphorylation of Ser46 in *B. subtilis* HPr. The NMR studies showed some modest chemical shift changes but no evidence for any large structural changes. Herzberg et al. (1992) suggested that the phosphorylation of Ser46 places a phosphoryl group in close proximity to the active site, and thus this leads to the inactivation of HPr with respect to its phosphoacceptor activity in the enzyme I reaction.

Because of the structural similarities between *E. coli* HPr and the HPrs from Gram-positive organisms, we have investigated the effect of mutation on Ser46 in *E. coli* HPr. We have mutated Ser46 to alanine, arginine, asparagine, aspartate, glutamate, and glycine. The acidic mutations yielded HPrs that have *in vitro* properties similar to P-(Ser)-HPr or Ser46Asp mutation of the HPrs from Gram-positive organisms as was presented in a preliminary report (Anderson & Waygood, 1994). Moreover, the tertiary structure of Ser46Asp HPr has been determined at 1.5 Å resolution. Additional structural investigation by NMR spectroscopy and circular dichroism is reported in the accompanying paper (Thapar et al., 1996).

MATERIALS AND METHODS

Materials. Vent polymerase was obtained from New England Biolabs Inc. Radioactive compounds, restriction enzymes, other molecular biological supplies, oligonucleotide primers, enzyme I, and sugar-specific enzymes II were obtained as described elsewhere (Anderson et al., 1993). Enzyme IIA^{glc} was prepared as described previously (Meadow & Roseman, 1982). Monoclonal antibodies specific for HPr Jel42, Jel44, and Jel323 were isolated and characterized as described previously (Waygood et al., 1987; Sharma et al., 1991).

Mutagenesis. The HPr mutants with other residues at Arg17 have been described by Anderson et al. (1993). The Ser46 mutations were obtained using the general method described by Landt et al. (1990) and the following mutagenic primers: Ser46Ala, 5′-TAAACAGGGCTTTCGCGCT-GG-3′; Ser46Asn, 5′-TAAACAGGTTTTTCGCGCT-GG-3′; Ser46Arg, 5′-TAAACAGGCGTTTCGCGCTGG-3′; Ser46Asp, 5′-TAAACAGGTCTTTCGCGCTGG-3′; Ser46Glu, 5′-TAAACAGCTCTTTCGCGCTGG-3′; Ser46Gly, 5′-TAAA-CAGGCCTTTCGCGCTGG-3′. These primers were synthesized using the standard phosphoramidate chemistry of Mateucci and Carruthers (1981). The HPr gene cloned in pUC19 (Anderson et al., 1991a) was amplified by PCR using the mutagenic primer, the M13 reverse sequencing primer, and Vent polymerase. This PCR product was isolated from low melting point agarose gels and used as a primer with the universal M13 sequencing primer and pUC19 containing the HPr gene in a second PCR reaction. The second PCR product produces a mutated HPr gene flanked by the multicloning site of pUC19, and the mutated HPr gene was transferred into pUC19 using the restriction enzymes *Hin*dIII and *Eco*RI. The method gave 100% mutagenesis. The complete HPr gene was sequenced by dsDNA sequencing by standard dideoxynucleotide methods after transfer to pUC19 to ensure no additional mutations were introduced by the PCR reaction.

Bacterial Strains. The strain used for the complementation studies was *E. coli* strain ESK120, which was formally called ES7RecA (Sharma et al., 1991). The genotype is *trp,rpsL, lacZ,ptsH465,srlC300::Tn10,recA56*. ESK108 is *E. coli* strain TP2811 F$^-$*,xyl,argH1,lacx74,aroB,ilv*A,Δ*(ptsHIcrr)*,Km^R (Levy et al., 1990).

Preparation of ^{32}P-HPr. In order to ensure the recovery of sufficient phosphorylated Ser46Asp and Ser46Glu HPr, 8 mg of protein was used in place of 1 mg in the phosphorylation procedure that has been described (Anderson et al., 1991a). Phosphohydrolysis was performed as described by Anderson et al. (1991a).

Protein Determination. The amount of HPr is usually determined by the substrate depletion assay based upon the coupled assay of enzyme I and lactate dehydrogenase (Waygood et al., 1979). Because of the poor activity of Ser46Asp and Ser46Glu HPr, this assay could not be used. Determinations of these proteins were thus carried out only by the method of Waddell (1956).

Crystallization and Data Collection. Crystals were grown by the hanging drop vapor diffusion method at 14 °C. Microcrystalline washing and seeding were used (Thaller et al., 1981). Crystals formed in 0.1 M citrate phosphate buffer, pH 4.6, and 46−50% saturated ammonium sulfate. Diffraction data were collected to 1.5 Å with a Mar image plate scanner using a wavelength of 0.927 Å from a synchroton source (X11, DORIS ring) at the EMBL Outstation, DESY, Hamburg, Germany. Rotations of 2.5° were used to obtain high-resolution data and 4.0° to collect high-intensity, low-angle diffractions which overloaded the image plate on the high-resolution scans.

Molecular Replacement and Refinement. The Amore package of molecular replacement (CCP4, 1994) was used with the wild-type HPr structure (Jia et al., 1993a) as the molecular probe. The molecular replacement rotation solu-

Table 1: Enzyme I Kinetic Parameters with HPr Ser46 Mutants

HPr	K_m (μM)	V_{max} (% wild type)	K_{cat}/K_m (relative)
wild type	6	100	16.7
Ser46Ala	15	100	6.67
Ser46Arg	40	100	2.50
Ser46Asn	65	100	1.54
Ser46Asp[a]	~4000	~30	~0.0075
Ser46Glu[a]	~1500	100	~0.067
Ser46Gly	6	100	16.7

[a] K_m and V_{max} values were determined as previously described (Anderson et al., 1991) using HPr concentrations from about $0.1K_m$ to $5K_m$. However, for Ser46Asp and Ser46Glu HPrs, the maximum concentrations achieved were about 2000 μM (18 mg/mL HPr), and thus the parameter values are an extrapolation.

tion was found using wild-type HPr even though the Asp46 electron density was evident. Then the Ser46Asp replacement was introduced, and least squares refinement was performed with X-PLOR 3.1 (Brunger, 1993). Rigid-body refinement was performed on the initial solution and subsequently after simulated annealing.

Electrostatic Calculations. Electrostatic calculations were performed with GRASP (Nicholls et al., 1992) using HPr structures without water. All basic and acidic residues were assigned the appropriate unit charges.

Other Methods. All the methods used to characterize the activities of mutant HPrs have been described elsewhere (Anderson et al., 1991a, 1993). The relative binding by monoclonal antibodies Jel42, Jel44, and Jel323 has been described by Sharma et al. (1991).

RESULTS

Protein Production. E. coli strain ESK108 or strain ESK120 when transformed and grown on rich media yielded about 200−300 mg of pure mutant HPr protein from 15−20 g wet weight of cells. In general, the mutations at Ser46 did not have a deleterious effect on the expression of HPr. The proteins were purified by standard methods (Anderson et al., 1991) and gave a single band on isoelectric focusing gels.

PTS Kinetics. HPr is a substrate of enzyme I which phosphorylates HPr at the expense of phosphoenolpyruvate. Enzyme I kinetics were determined using a spectrophotometric assay in which pyruvate production was coupled to lactate dehydrogenase (Waygood et al., 1979). Ser46Gly and Ser46Ala mutations had essentially no effect on the ability of HPr to act as a phosphoacceptor; Ser46Arg and Ser46Asn mutations had modest effects on K_m, 8-fold and 10-fold, respectively; the Ser46Asp and Ser46Glu mutations had large effects (Table 1). The K_m/k_{cat} is reduced by a factor of about 2000-fold for Ser46Asp (Table 1). Because of the high K_m values for the Ser46Asp and Ser46Glu HPrs, it was not possible to determine enzyme I initial velocity at HPr concentrations above the K_m, and thus the K_m values are extrapolations.

It has been reported (Deutscher et al., 1984) that the kinetic properties of P-(Ser)HPr can be modified by the presence of various IIA^{sugar} proteins. Inclusion of *E. coli* IIA^{glc} at 30 μM in an assay containing 1000 μM Ser46Asp HPr did not alter the initial velocity. Enzyme I assays were also carried out by coupling P-HPr production to the enzyme II^{man} complex (membranes of *S. typhimurium* strain SB2950; Waygood et al. 1979) in a glucose phosphorylation assay, and the K_m values obtained for wild-type HPr, Ser46Asp, and Ser46Gly HPrs were similar to those obtained with the spectrophotometric assay (Table 2).

Enzyme II^{sugar} kinetics for Ser46Asp and Ser46Glu HPrs could not be carried out because it was not possible to add sufficient enzyme I into the assays to meet the required condition of an assay only dependent upon the level of enzyme II (Waygood et al., 1979). The level of impairment of these two mutants made it impossible to ensure that, in enzyme II assays, the mutant HPr was all in the P-(His)HPr form.

Phosphohydrolysis. P-(His)HPr has an unstable phosphohistidine at His15, and it has been proposed that the phosphorylation at Ser46 is close enough to interfere with the formation of P-(His)HPr in *B. subtilis* HPr (Herzberg et al., 1992). The pH dependence of the phosphohydrolysis for both Ser46Asp and Ser46Arg P-(His)HPrs is shown in Figure 1. Ser46Asn and Ser46Glu HPr had similar properties. The extent of this impairment is similar to alterations to the C-terminal residue, Glu85 (Anderson et al., 1991), but not as great as the change seen in Arg17 mutations (Anderson et al., 1993). These results suggest an influence on phosphohydrolysis by mutations at residue 46 but no effect specific to the acidic replacements.[2]

Phenotype. Mutant HPrs with impaired activity that are overproduced from a pUC-derived plasmid in which the gene is still under the control of the HPr promoter usually complement a *ptsH* strain as tested by mannitol fermentation (Sharma et al., 1991). Ser46Asp HPr is impaired by ~2000-fold with respect to enzyme I activity, and the fermentation properties of cells containing the Ser46Asp HPr and several HPrs containing changes at arginine-17 were investigated. The Arg17 mutant HPrs are not as impaired, ~10−1000-fold (Anderson et al., 1993). When all of these HPrs were produced from plasmids in strain ESK120 (*ptsH*), the cells gave a fermentation-positive result for sugars such as glucose, mannose, mannitol, melibiose, and maltose. In general, the fermentation-positive phenotype was often slower in presentation, i.e., appeared at 24 h but not at 15 h. The overproduction of these mutant HPrs, however, interfered with fructose fermentation to yield a negative response. In *E. coli* there is a separate PTS dependent upon an inducible HPr domain, FPr (Postma et al., 1993). Presumably, the FPr is effectively competed by the excess HPr. It is clear that the overproduction of impaired HPrs compensates for their loss of activity, and no unusual fermentative phenotype was found for Ser46Asp HPr.

Monoclonal Antibody Binding. With such a large change in activity, there was a possibility that Ser46Asp HPr had a significant conformational change. The epitopes of three HPr-specific antibodies, Jel42, Jel44, and Jel323, have been described by site-directed mutagenesis. Ser46 is part of the epitope for Jel323 but not for Jel42 and Jel44 (Sharma et al., 1991; Prasad et al., 1993). Thus a mutation at residue 46 should have a local effect on Jel323 binding but not on the other two antibodies unless a larger conformational change had occurred. Ser46Asp does not affect either Jel42 or Jel44 binding but has a significant change for Jel323 as expected, a 1500-fold decrease. The antibody binding results

[2] Phosphohydrolysis properties of the P-His15 residue of the mutants Ser46Asp, Ser46Thr, and Ser46Ala of *B. subtilis* HPr were studied at the time other PTS proteins from *B. subtilis* were investigated (Anderson et al., 1991b), but the results were not reported. Essentially the same result was obtained: all three mutants yielded the same modest change in phosphohydrolysis properties.

Table 2: Kinetic Measurements with P-(Ser)HPr and Ser46Asp HPr

HPr (species)	EII[a] (species, domain)	enzyme I kinetic parameters			decrease in activity
		K_m (μM)	V_{max} (%)[b]	k_{cat}/K_m (relative)[c]	
		S. faecalis Enzyme I (Deutscher et al., 1984)			
S. lactis HPr					1[d]
P-(Ser)HPr					5000[d]
P-(Ser)HPr	*S. aureus* IIA^{lac}				100[d]
P-(Ser)HPr	*S. faecalis* IIA^{gluconate}				1[d]
		S. faecalis Enzyme I (Reizer et al., 1989)			
B. subtilis HPr	*S. aureus* IIA^{lac}, IIBC^{lac}	4	100	25	
Ser46Asp	*S. aureus* IIA^{lac}, IIBC^{lac}	16	10	0.63	40
		B. subtilis Enzyme I (Reizer et al., 1992)			
B. subtilis HPr	*B. subtilis* IIA^{glc}, IIBC^{glc}	23 ± 10	100	4.4	
Ser46Asp	*B. subtilis* IIA^{glc}, IIBC^{glc}	256 ± 107	20	0.078	56
P-(Ser)HPr	*B. subtilis* IIA^{glc}, IIBC^{glc}	345 ± 25	16	0.046	96
HPr	*S. aureus* IIA^{lac}, IIBC^{lac}	52 ± 16	100	1.9	
Ser46Asp	*S. aureus* IIA^{lac}, IIBC^{lac}	237 ± 132	19	0.080	23
P-(Ser)HPr	*S. aureus* IIA^{lac}, IIBC^{lac}	203 ± 60	25	0.12	16
HPr	*B. subtilis* IIABC^{glc}	20	100	5.0	
Ser46Asp	*B. subtilis* IIABC^{glc}	167	30	0.18	27
HPr	*B. subtilis* IIA^{mtl}, IIBC^{mtl}	4.5	100	22	
Ser46Asp	*B. subtilis* IIA^{mtl}, IIBC^{mtl}	48	30	0.56	39
		E. coli Enzyme I (This Work)[e]			
E. coli HPr[f]	none	6	100	17	
Ser46Asp[f]	none	4000	30	0.0075	2300
HPr	*S. typhimurium* IIAB^{man}, IICD^{man}	4	100	25	
Ser46Asp	*S. typhimurium* IIAB^{man}, IICD^{man}	3000	10	0.0033	3000

[a] Assays had the listed enzyme II components present. Except where noted, the assays were by sugar phosphorylation. [b] The V_{max} for enzyme I with the appropriate wild-type HPr is 100%. [c] The wild type k_{cat} is arbitrarily set at 100 for each comparable group. [d] These values are based upon a comparative assessment of amounts formed in a standard reaction and assessed by electrophoresis gels. [e] See Table 1 for additional data. [f] Lactate dehydrogenase coupled assay.

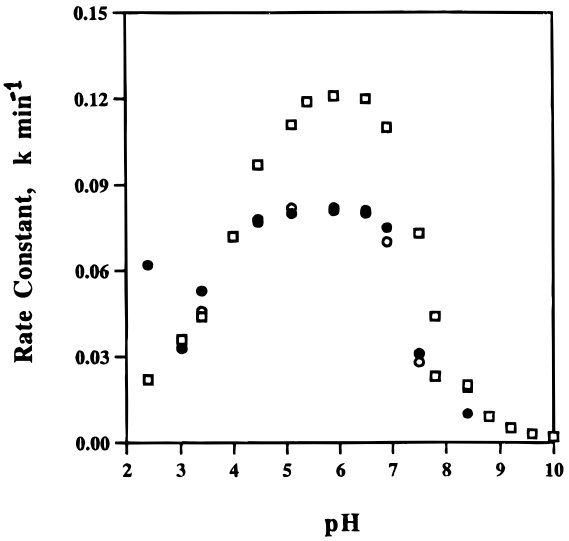

FIGURE 1: Phosphohydrolysis of P-(His)HPr. The pH dependence of spontaneous phosphohydrolysis was determined at 37 °C as described in Materials and Methods. Results are presented for wild-type HPr (□), Ser46Asp HPr (●), and Ser46Arg HPr (○).

(Table 3) are consistent with a local modification that does not yield significant conformational changes.

Determination of the Tertiary Structure of Ser46Asp HPr. Ser46Asp HPr crystals were obtained at pH 4.6 in the presence of ammonium sulfate, whereas the wild-type crystals were obtained at pH 3.7. The Ser46Asp HPr was found in a space group of *P*1 with unit cell dimensions of a = 25.85 Å, b = 27.33 Å, c = 27.60 Å, α = 66.90°, β = 75.71°, and γ = 70.00°. Data were collected to 1.5 Å, and the final model was refined against all unique data, 9806 reflections between the resolution of 8.0 and 1.5 Å with no σ cut. The final model contains one sulfate ion, at the active site, and 75 water molecules. The final *R*-factor is 0.182

Table 3: Effects of Mutation on Antibody Binding

HPr	relative binding as determined by competition SPRIA		
	Jel42	Jel44	Jel323
wild type	1	1	1
Ser46Ala	4	1	0.1
Ser46Arg	1	1	<0.001
Ser46Asn	1	1	0.002
Ser46Asp	1	1	6×10^{-4}
Ser46Glu	1	1	1×10^{-4}
Ser46Gly	1	1	0.1

with a bond length rms deviation of 0.0012 Å and a bond angle rms deviation of 2.4°. The average restrained isotropic *B*-factors are 12.0, 15.3, and 14.6 Å for the main chain, side chain, and solvent atoms, respectively. Only residue Pro11 has Φ = 66° and Ψ = 14° bond angles outside of the allowed regions of the Ramachandran plot (Ramakrishnan & Ramachandran, 1965).

Improvement of the Tertiary Structure of HPr. The structure of Ser46Asp HPr is at 1.5 Å resolution while the wild-type HPr structure was at 2.0 Å resolution (Jia et al., 1993a). In general, the two structures are very similar as seen by the plot of the rms deviations (Figure 2). There are two significant differences, one at Pro11 and the other at Gly54. The former changes the conformation of the Pro11 residue carbonyl group. In the 2.0 Å resolution structure, there was some ambiguity in the density at this residue, and the positioning of the carbonyl group was not clear in the electron density map. The conformation that was chosen was the best fit based largely on dihedral energy considerations of residue 12. The 1.5 Å resolution electron density map clearly shows that the carbonyl group is in a different conformation (Figure 3). This positioning influences residue Thr16 in the active site loop. In the 2.0 Å native structure, residue 16 had torsion angle values of $\Phi = -43°$ and Ψ =

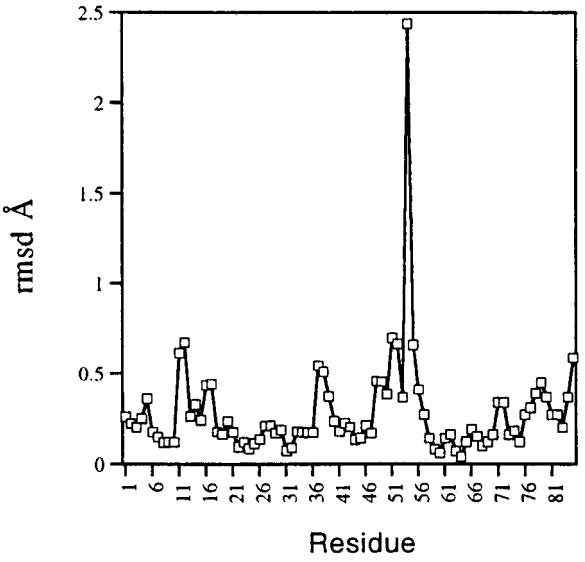

FIGURE 2: RSMD comparison of a-carbon atoms. Comparison between the α-carbon atoms of wild-type and Ser46Asp HPr.

−65°. This partial torsion angle strain was interpreted to be indicative of a semiclosed conformation. A cycle of phosphotransfer was proposed in which stored energy in the torsion angle strain at residue 16 is essential for acceptance of the phosphoryl group at His15. This proposal was based primarily on the *S. faecalis* crystallographic structure where the strain at residue 16 was most apparent in the absence of either a sulfate or phosphate anion at the active center. The *S. faecalis* HPr structure was considered representative of the unphosphorylated, open, strained conformation of HPr (Jia et al., 1992). In the *B. subtilis* crystallographic structure of HPr, a sulfate anion was present in the active center, thus resulting in the closed conformation of the active center with the absence of any torsion angle strain. The wild-type *E. coli* structure represented a mixture of the two conformers; a sulfate was present at the active center but crystallographic contacts prevented Arg17 from adopting the closed conformation. In this semiclosed conformation, partial torsion angle strain was found at residue 16. From the *E. coli* Asp46Ser HPr structure, however, it seems that this torsion angle strain in *E. coli* HPr was likely the result of misplacement of the carbonyl of Pro11. With the improved electron density, the refinement results in an alleviation of any strain at residue 16 with $\Phi = -56°$ and $\Psi = -50°$ in the Ser46Asp HPr structure. This change appears to be a consequence of better resolution in the structure determination rather than the effect of the mutation.

Alterations as a Consequence of the Mutation. The substitution Ser46Asp can been seen in the electron density (Figure 4). Ser46 N-caps the small helix B composed of residues 46−53, and the mutation of Ser46Asp alters this helix. Helix B is irregular; residues 46 and 47 conform to an α-helix conformation, residue 48 is a transition point, and residues 49−53 are in a 3_{10} helix (Figure 5). Apart from the active site, the α-carbon atoms of these residues have the greatest variability in HPr structural determinations, in particular, residues Leu53−Gly54, a pair, which is conserved in most HPrs. These residues are the C-cap region of the minor B-helix and adopt one of two possible conformations on the basis of whether the HPr is from Gram-negative or Gram-positive bacteria. The different conformations appear to be influenced by the need to accommodate the side chain of residue 37: tyrosine in HPr from *B. subtilis* and *S. faecalis* and serine in HPr from *E. coli* (Jia et al., 1994). In the Gram-positive HPrs, the Gly54 region is in helical conformation while in *E. coli* HPr, Gly54 adopts a more random, nonhelical conformation and is hydrogen bonded with the Ser37 residue. In the Ser46Asp mutation of *E. coli* HPr there is a change in the B-helix from that of the wild-type protein (Figure 5).

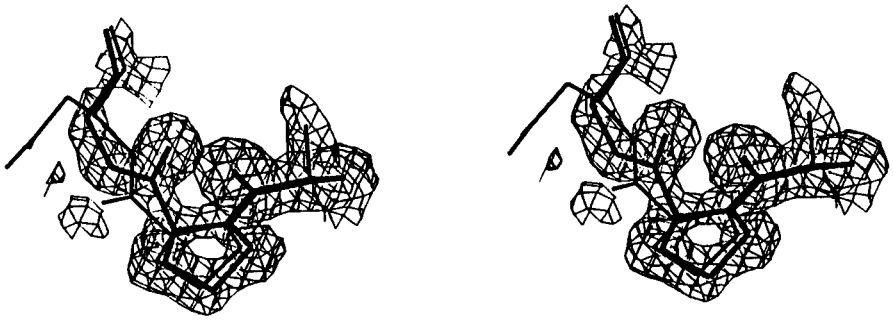

FIGURE 3: Proline-11 electron density. The electron density of Ser46Asp HPr showing the conformation of the Pro11 carbonyl group: heavy line, Ser46Asp HPr; light line, wild-type HPr structure (Jia et al., 1993a).

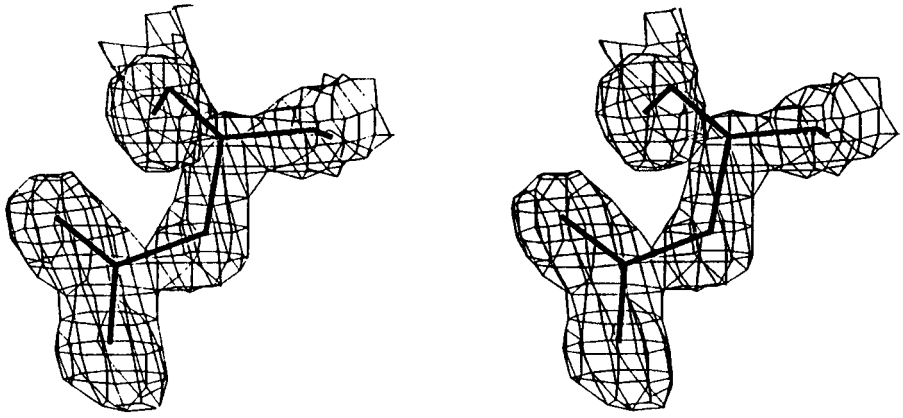

FIGURE 4: Aspartate-46 electron density. The $2F_o - F_c$ electron density map for Asp46.

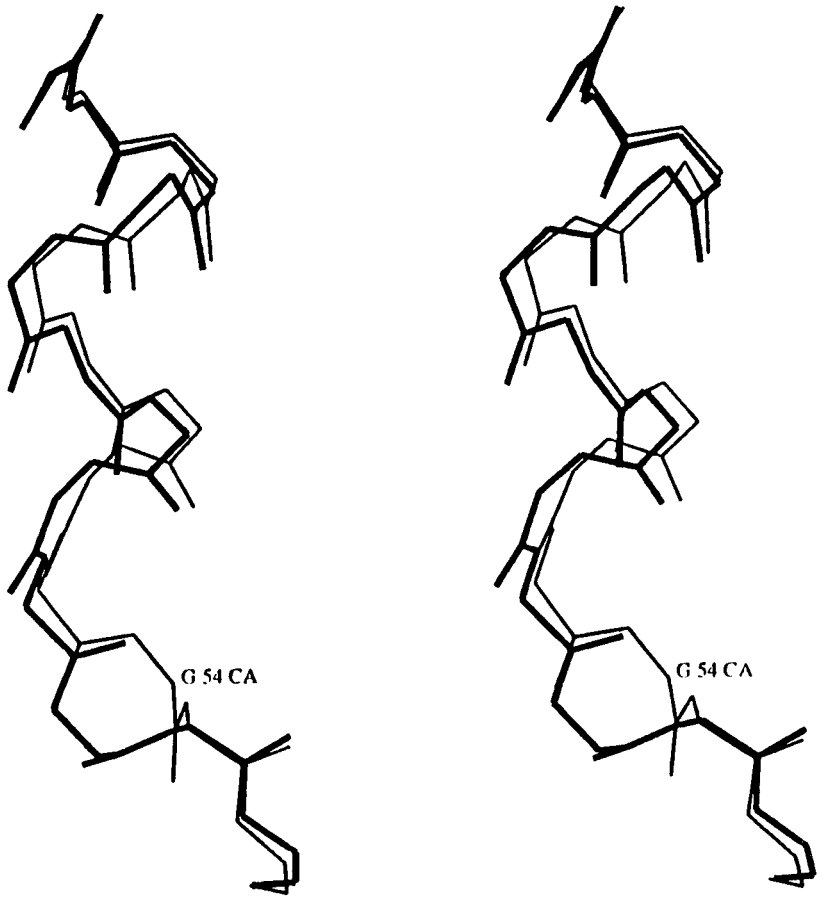

FIGURE 5: Comparison of the B-helix structure. The structures of helix B from wild-type HPr (heavy line) and Ser46Asp (light line) are compared. The helix in Ser46Asp HPr has a general tightening (Table 4), and Gly54 adopts a 3_{10} helical conformation.

Table 4: Hydrogen Bond Lengths in Helix B

possible donor	possible acceptor	distance in wild type (Å)	distance in Ser46Asp (Å)	decrease (Å)
S(D)46O	50N	3.11	2.93	0.18
L47O	Q51N	3.19	3.03	0.16
F48O[a]	Q51N	3.35	3.44	−0.09
	T52N	3.95	3.52	0.43
K49O	T52N	3.19	3.06	0.13
L50O	L53N	3.18	3.08	0.10
Q51O[b]	G54N		3.37	

[a] At residue 48 the helix changes from an α-helix to a 3_{10} helix. [b] In wild type the orientation of G54 is incompatible with a helix conformation.

These changes include both a consistent tightening of the minor helix as demonstrated by the shortened hydrogen bond lengths (Table 4) throughout the helix, resulting in a movement of the Gly54 region to adopt the same helical conformation seen in HPrs from Gram-positive bacteria.

Electrostatic Changes. The surface in the region of Ser46 can be described as a hydrophobic patch with minor areas of basic potential in *E. coli* HPr (Figure 6). This region has been previously proposed to be involved in the interaction with enzyme I. The effect of the introduction of any charged side chains at this residue is to disrupt this pattern, and in the case of the acidic residues, the ability of the HPr to interact with enzyme I is altered.

DISCUSSION

HPr, which is one of the most commonly shared proteins of the different sugar-specific PTS systems, has a very similar tertiary structure in a number of bacterial species despite sequence identities as low as 34% (Jia et al., 1994b). The active site residues of HPr, His15 and Arg17, are conserved in all HPrs sequenced to date, as are Gly13, Ser46, Leu53, Gly67, and Asp69. Gly54 is conserved in 11 out of 12 sequences, the exception being an alanine residue (Reizer et al., 1993). Ser46 can be phosphorylated by an ATP-dependent kinase in a variety of bacterial species but not in the Gram-negative bacteria *E. coli* and *S. typhimurium*. There are a number of different physiological roles for the regulation that ensues from the phosphorylation of Ser46 as described in the introduction; however, one primary *in vitro* effect is to cause P-(Ser)HPr to be a poor substrate for enzyme I. The question concerning the mechanism of this inhibition is whether there is a conformational change in P-(Ser)HPr or does the phosphorylation at Ser46 simply interfere with the binding of enzyme I? The fact that P-(His)-HPr is a poor substrate for the ATP-dependent kinase also suggests that there is some overlapping of the active sites for the two kinases (Reizer et al., 1984, 1989). Herzberg et al. (1992) have noted that Ser46 and His15 are reasonably close in *B. subtilis* HPr, and a similar circumstance is found in *E. coli* HPr (Jia et al., 1993a).

Reizer et al. (1989) produced four mutants at Ser46 in *B. subtilis* HPr. The neutral substitutions, Ser46Thr, Ser46Ala, and Ser46Tyr, removed the ability of HPr to act as a regulator, whereas the acidic mutant, Ser46Asp, had many properties similar to P-(Ser)HPr. While *E. coli* HPr cannot be phosphorylated by the ATP-dependent kinase to yield P-(Ser)HPr, it can be mutated at residue 46. It is clear from the results in this paper that the Ser46Asp and Ser46Glu mutations in *E. coli* HPr have, with respect to *in vitro* PTS activity, properties similar to either P-(Ser)HPr or the

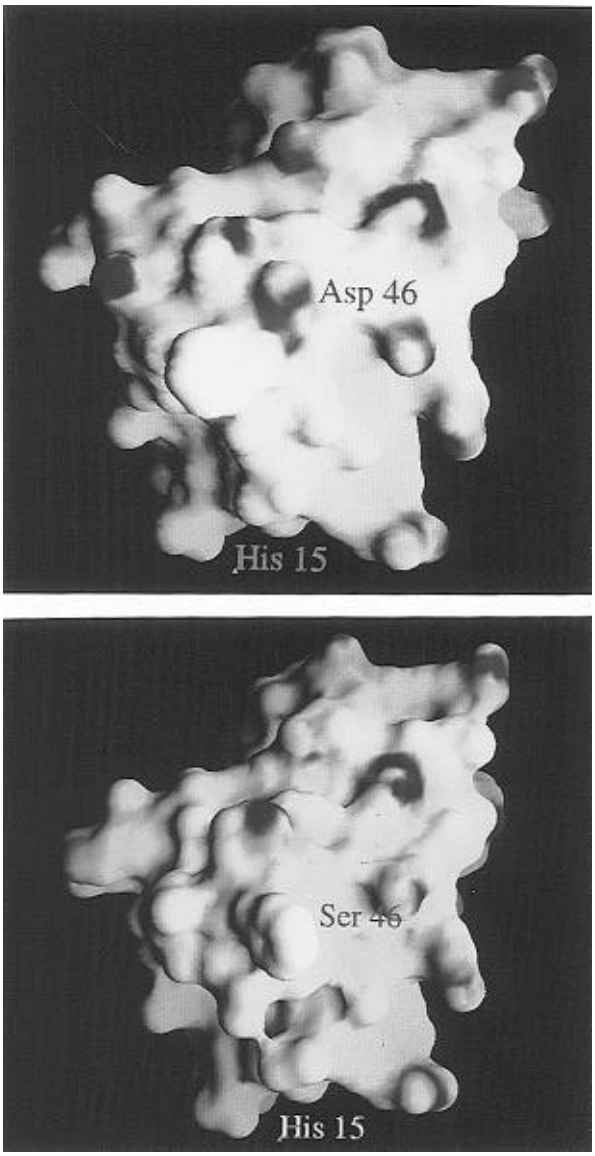

FIGURE 6: Electrostatic surface. The electrostatic surface environments of wild-type HPr (bottom) and Ser46Asp HPr (top) as calculated by the GRASP program (Nicholls et al., 1992) are presented. Electrostatic potentials are as follows: blue, positive; red, negative; white, neutral.

Ser46Asp mutation in *B. subtilis* HPr. There have been measurements of the activity of P-(Ser)HPr from various species, and these are summarized in Table 2. The variety of PTS components used in these assays makes direct comparisons difficult. Deutscher et al. (1984) showed that, in a comparative assay in which P-(His)HPr and P-(His)P-(Ser)HPr were determined by electrophoresis separation, P-(Ser)HPr from *Streptococcus lactis* was phosphorylated by *S. faecalis* enzyme I about 5000-fold slower than HPr. The addition of $IIA^{gluconate}$ from *S. faecalis* removed the inhibitory effects of Ser46 phosphorylation, while $IIA^{lactose}$ from *Staphylococcus aureus* reduced the inhibition by a factor of about 50. Reizer et al. (1989) carried out a much more extensive kinetic investigation and concluded that the P-(Ser)HPr was not involved in a discrimination between enzyme IIA domains. These results collectively suggest that the phosphorylation of Ser46 causes inhibitory effects from about 10- to 100-fold in PTS assays using components from Gram-positive bacteria.

The results presented here for *E. coli* Ser46Asp HPr find a relative magnitude of effect on the enzyme I kinetics that is similar to the report of Deutscher et al. (1984) but are in agreement with Reizer et al. (1992) that the kinetic properties of the enzyme I/Ser46Asp interaction appear not to be modified by the presence of a IIA^{sugar}. Neither the addition of IIA^{glc} to the lactate dehydrogenase coupled assay nor the use of sugar phosphorylation assays in which IIA^{man} was present altered the magnitude of the effect on enzyme I kinetics. Because of the severe impairment of the enzyme I reaction, it proved impossible to assess the effects of the mutation on the enzyme II^{sugar} interaction. Reizer et al. (1991) showed that the effect of either Ser46Asp mutation or P-(Ser)HPr on enzyme II kinetics was a K_m effect of about 5−10-fold. van Nuland et al. (1995) have presented evidence that the residues of HPr involved in the interaction with enzyme I are similar to those described for the enzyme IIA^{mtl} interaction.

Conclusions from the structural investigations reported here, as well as in the accompanying paper (Thapar et al., 1996) and by others (Wittekind et al., 1989; Liao & Herzberg, 1994; Pullen et al., 1995), all suggest that the active center of HPr is not affected by mutation or phosphorylation at Ser46, and thus the effects of these changes are to alter the ability of HPr to bind to either enzyme I or enzyme IIA. If there is no effect on the catalytic mechanism, it would be expected that the V_{max} values for the kinetics with such modified HPrs should be close to 100%. In the work reported here, the V_{max} for the Ser46Asp HPr is derived from an extrapolation from concentrations of protein all lower than the K_m value, and thus the 30% value for V_{max} must be taken with some caution. The kinetics with Ser46Glu HPr, for which there is a severe impediment to binding (a K_m effect), yield a V_{max} of 100%. This result is consistent with a change that only involves binding to the enzyme. Perhaps it might prove useful to investigate some of the enzymes I from Gram-positive bacteria using the lactate dehydrogenase coupled enzyme assay as other kinetic measurements (Table 2) have been carried out primarily by the sugar phosphorylation PTS assay.

The greater impairment for *E. coli* Ser46Asp HPr as compared to the equivalent mutation in *B. subtilis* HPr could possibly be a function of the displacement of Phe48, which is usually a methionine residue in Gram-positive bacteria. However, it would seem likely that both Arg46 and Asn46 mutations would cause a similar displacement of Phe48, and these substitutions have modest effects on enzyme I kinetics. Mutations at residue 46, particularly arginine, asparagine, and the acidic residues, had large effects on antibody binding (Table 3). The greater effect on the kinetic response (i.e., enzyme I binding) thus appears to be due to the introduction of an acidic residue or of phosphorylation, rather than the disruption of local conformation.

The effects on the phosphohydrolysis properties of P-(His)-HPr by the Ser46Asp mutation were modest. Herzberg et al. (1992) had suggested that the P-Ser46 is close to the active site, but as the negative charge at residue 46 did not further destabilize the P-(His)HPr nor yield higher phosphohydrolysis rates nor prove much different in effect than other substitutions, it would appear that the two phosphorylation sites do not interact directly.

The 1.5 Å resolution structure reported here shows that, in comparison to wild type, the Ser46Asp HPr does not undergo large conformational changes. There are some modest changes to helix B. This is in agreement with the NMR studies (Wittekind et al., 1989; Pullen et al., 1995) on

B. subtilis Ser46Asp and P-(Ser)HPr and the recent tertiary structure determined at 2.0 Å resolution (Liao & Herzberg, 1994). A similar conclusion is drawn from the NMR studies on the mutant of *E. coli* HPr in the accompanying paper (Thapar et al., 1996). The structure of the active center determined by X-ray diffraction appears not to be affected, but the active center contains a sulfate anion which is considered to produce a structure that resembles that of P-(His)HPr (Jia et al., 1993, 1994). Thus the structure presented here is an analogue of P-(Ser)P-(His)HPr, while the NMR structural investigation is on Ser46Asp HPr as an analogue of P-(Ser)HPr. The common structural change is that the helix B is tightened, and in the X-ray structure, helix B is extended to include residue Gly54. This change in structure results in this helix more closely resembling the helix found in Gram-positive structural determination. In the X-ray analysis of the P-(Ser)HPr and Ser46Asp mutant of *B. subtilis* HPr no significant structural changes as a result of the phosphorylation or mutation were found. It has been concluded that the introduction of negative charge at residue 46 disrupts the electrostatic identity of the HPr molecule, thus preventing enzyme I binding (Liao & Herzberg, 1994; Pullen et al., 1995). The results presented here demonstrate that, despite the nonphysiological nature of the Ser46 phosphorylation in *E. coli* HPr, similar structural and kinetic results emerge with the Ser46Asp mutation. This is particularly evident with the structural determination showing the alteration of the minor B helix to a more Gram-positive form as well as similar changes in the electrostatic environment which produce a similar inability to interact with enzyme I.

In conclusion, it would appear that the phosphorylation or acidic replacement of Ser46 in HPr yields modest conformational changes that do not affect the active site. The principal effect of these alterations to Ser46 is the inhibition of the binding of HPr to enzyme I.

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