Topography of the Interaction of HPr(Ser) Kinase with HPr

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ABSTRACT: The phosphocarrier protein, HPr, from Gram-positive organisms and mycoplasmas is a substrate for an ATP-dependent kinase that phosphorylates serine 46. In Gram-negative organisms, the corresponding HPr is not phosphorylated on serine 46 and the ATP-dependent kinase is absent. To determine the specificity requirements for phosphorylation of *Mycoplasma capricolum* HPr, a chimera in which residues 43−57 were replaced by the *Escherichia coli* sequence was constructed. The chimeric protein folded properly, but was not phosphorylated on either serine 46 or histidine 15. A dissection of the region required for phosphorylation specificity was carried out by further mutagenesis. The deficiency in phosphorylation at histidine 15 was localized primarily to the region including residues 51−57. Activity studies revealed that residues 48, 49, and 51−53 are important for recognition of *M. capricolum* HPr by its cognate HPr(Ser) kinase. The characteristics of this region suggest that the kinase−HPr interaction occurs mainly through a hydrophobic region. Molecular modeling comparisons of *M. capricolum* HPr and the chimeric construct provided a basis for interpreting the results of the activity assays.

An important pathway for the transport of numerous sugars by bacteria is the phosphoenolpyruvate:sugar phosphotransferase system (PTS[1]). The PTS (*1*) effects the concomitant transport and phosphorylation, from PEP, of numerous sugars. Two cytoplasmic proteins, designated enzyme I and HPr, are the first proteins that interact with PEP. Enzyme I is autophosphorylated by PEP, and P-EI transfers a phosphoryl group to HPr. The site of phosphorylation on both proteins is a histidyl residue. Phospho-HPr is able to pass its phosphoryl group to a family of sugar-specific proteins, designated enzymes II, which ultimately bring the sugar across the cell membrane in a phosphorylated form.

It has become apparent that the PTS fulfills other roles besides driving sugar transport. Considerable attention has been focused on the role of glucose-specific enzyme IIA (IIA^{glc}) of *Escherichia coli* as a positive regulator, in its phospho form, of adenylyl cyclase. Enzyme IIA^{glc} has also been shown, in its dephospho form, to inhibit the activity of glycerol kinase and several permeases.

Regulatory functions have also been attributed to HPr. In *E. coli*, dephospho-HPr stimulates the activity of glycogen phosphorylase (*2*). In Gram-positive bacteria, the regulatory role of HPr depends on the state of phosphorylation of Ser46. Carbon catabolite repression in Gram-positive bacteria is dependent on the catabolite control protein, designated CcpA, a repressor homologue. A complex of CcpA and Ser(P)-HPr binds to a catabolite responsive element (*cre*) and negatively regulates transcription (*3*).

The phosphorylation of Ser46 of HPr is catalyzed by an ATP-dependent kinase whose activity is stimulated by fructose bisphosphate and inhibited by inorganic phosphate (*4*). The presence of the kinase has been demonstrated in Gram-positive bacteria as well as in *Mycoplasma capricolum* and *Mycoplasma genitalium* (*5*). While the structures of HPrs from all species examined are similar, characterized as an open-faced β-sandwich with three α-helices packed against a four-stranded antiparallel β-sheet, only those HPrs from Gram-positive bacteria are substrates for HPr(Ser) kinase. The three-dimensional structures of HPrs from *M. capricolum*, *E. coli*, and Gram-positive bacteria are similar (*6*), but some aspect of the architecture in the vicinity of Ser46 is assumed to determine the ability of the HPr to be phosphorylated by HPr(Ser) kinase. Consequently, a mutagenesis approach was used to evaluate that assumption. The results of the study presented here pinpoint some residues that are important for the interaction of HPr(Ser) kinase from *M. capricolum* with its cognate HPr.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides were synthesized using a model 394 DNA/RNA synthesizer (Applied Biosystems). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Tryptophan was purchased from Bethesda Research Laboratories. [γ-^{32}P]ATP was purchased from Amersham. [^{32}P]PEP was synthesized and purified as previously described (*7*). *M. capricolum* IIA^{glc} was prepared as previously described (*5*).

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphocarrier protein of the PTS; P-HPr, HPr phosphorylated at the active site His15; Ser(P)HPr, HPr phosphorylated at the regulatory site Ser46; HPr(Ser) kinase, enzyme that catalyzes the phosphorylation of HPr at the regulatory Ser46; IIA^{glc}, enzyme IIA specific for glucose of the PTS; EI, enzyme I of the PTS; P-EI, enzyme I phosphorylated at the active site His189; IIA^{mtl}, enzyme IIA specific for mannitol of the PTS; CD, circular dichroism.

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DNA Methods

Construction of the Expression Vector for M. capricolum Enzyme I (pMC-EI). The DNA sequence encoding the *M. capricolum* EI (*pts*I) was amplified by PCR using pPZ5 (*8*) as a template. The forward primer (5′-GTAAGGATATACATATGTCAAAACAAAT-3′) which contained an engineered *Nde*I site (underlined) and the reverse primer downstream of the *Hin*dIII site in the *pts*I open reading frame were used to obtain PCR fragment 1. To change the Trp codon from TGA (a stop codon in *E. coli*) to TGG, mutagenesis was carried out as previously described (*9*). A forward primer upstream of the *Hin*dIII site in *pts*I and a reverse primer (5′-CTCCACACATACCAACCCATTTATTATGC-3′) containing an *E. coli* Trp codon (underlined) were used for PCR amplification (PCR2). PCR3 was produced by amplification using a forward primer (5′-GCATAATAAATGGGTTGGTATGTGTGGAG-3′) containing the *E. coli* Trp codon (underlined) and the reverse primer (5′-GTAGTGTCGACTAATAAGATAATTAACTAAC**TTA**TTAATC-3′) with an engineered *Sal*I site (underlined) and a stop codon (bold). PCR2 and -3 were mixed and used as the template for the fourth PCR with the primers upstream of the *Hin*dIII site containing the *Sal*I site. The product of PCR1 was purified and digested with *Nde*I and *Hin*dIII. The product of PCR4 was purified and digested with *Hin*dIII and *Sal*I. The trimmed products of PCR1 and PCR4 were ligated and then inserted into the *Nde*I and *Sal*I cloning sites of expression vector pREI (*10*). The construct was verified by DNA sequencing.

Mutagenesis of pMC-HPr. pMC-HPr (*11*) is an expression vector for *M. capricolum* HPr. To create a version of this vector that would permit replacement of the sequence corresponding to residues 43−57 of HPr, a mutagenesis was carried out in which a *Bam*HI site was created in the vicinity of residues 42 and 43 and a *Kpn*I site was created in the vicinity of residues 58 and 59. To create the *Bam*HI site, it was necessary to change the codon corresponding to Asn43 of *M. capricolum* HPr to Ser, which is found at that position in *E. coli* HPr. A two-step PCR mutagenesis method, previously described (*9*), used pMC-HPr as the initial template for two PCRs. One contained a primer upstream of the *Nde*I site of the vector and the reverse mutagenic primer encompassing codons 38−54 of *M. capricolum pts*H (5′-AGCCATTGCCATTACATTCATAATTGATTTTAA-**GGA**TCCTTGTTTTTCATT-3′) (*Bam*HI site underlined, complement of Ser43 codon in bold). The other PCR involved a reverse primer downstream of the *Xba*I site of the vector and the forward mutagenic primer encompassing codons 48−62 of *M. capricolum pts*H (5′-ATGAATGTAATGGCAATGGCTATAAAAACTGGTACCGAAATAACT-3′), where the *Kpn*I site is underlined. The products of the two PCRs were annealed and used as the template for a third PCR using the two vector primers. The PCR product (∼390 bp) was purified and digested with *Nde*I and *Xba*I and cloned into the same sites of pRE-His-Tag (*5*), a vector designed for the expression of His-tagged proteins.

Expression Vectors Encoding Chimeric Forms of M. capricolum HPr. The pRE-His-Tag vector containing the gene encoding *M. capricolum* HPr in which Asn43 was mutated to Ser and which contained strategically located *Bam*HI and *Kpn*I sites, described above, was used as the starting material for constructing expression vectors for the various *M. capricolum*−*E. coli* HPr chimeras. The vector was digested with *Bam*HI and *Kpn*I to remove the residue 43−57 region of the *M. capricolum pts*H gene. Complementary pairs of chemically synthesized oligonucleotides, described in Table 1, were mixed in a 1:1 molar ratio and placed in a H_2O bath at 94 °C. The bath was allowed to cool slowly to room temperature to allow the oligonucleotide pairs to anneal. The annealed double-stranded DNA fragments with cohesive *Bam*HI−*Kpn*I ends were then ligated into the vector. All the constructs were verified by DNA sequencing by the dideoxy method of Sanger et al. (*12*) using an Applied Biosystems automated sequencer. Plasmids were introduced into a *pts* deletion derivative of *E. coli* GI698 by electroporation as described previously (*13*).

Proteins

HPr Derivatives. Transformants in *E. coli* GI698Δ*pts* were cultured as described (*14*). The chimeric proteins were expressed and purified as previously described (*5*) using Ni−NTA−agarose (Qiagen) followed by FPLC on a MonoQ HR 10/10 column. All the purified HPr proteins were approximately 95% pure. Protein concentrations were determined by the method of Waddell (*15*).

Expression and Purification of M. capricolum Enzyme I. The construct pMC-EI (see above) was used to transform GI698Δ*pts*. The cells were grown as described (*14*) to an A_{600} of ∼0.6, and then tryptophan was added to induce the expression of EI. Induction was continued overnight. The cells were then harvested and disrupted as described (*5*). The supernatant solution after centrifugation at 35000*g* was loaded onto a column (1.4 cm × 40 cm) of DE-52 anion exchange resin equilibrated with buffer A [25 mM Tris·HCl (pH 7.5) and 10 mM DTT]. The proteins were eluted with a gradient (approximately 1 L) of buffer A from 0 to 0.5 M NaCl. Fractions enriched in EI, as judged by SDS−PAGE, were pooled and concentrated. The sample was then fractionated on an Ultrogel ACA-44 column (2.6 cm × 100 cm) equilibrated and eluted with buffer A containing 0.1 M NaCl. Ultrogel fractions enriched in EI were pooled and then chromatographed on a MonoQ HR 10/10 (FPLC, Pharmacia) column using buffer A and a gradient (approximately 100 mL) from 0.1 to 0.4 M NaCl. Fractions enriched in EI were refractionated on the MonoQ column using a salt gradient from 0.15 to 0.35 M NaCl. Fractions enriched in EI were concentrated and fractionated on a Superose 12 column (1.6 cm × 50 cm) using buffer A with 0.1 M NaCl. The final product was >95% pure as judged by SDS−PAGE. N-Terminal sequence analysis shows the protein to be an approximately equal mixture of the species with and without the N-terminal methionine residue.

Circular Dichroism

CD measurements were carried out using a 0.2 mm water-jacketed, temperature-controlled (20 °C) cylindrical cell in a Jasco-710 spectrometer. Spectra were corrected for the solvent CD signal. The proteins were diluted to 1 mg/mL in 25 mM KPO_4 (pH 7.5). Wavelength scanning was carried out at 10 nm/min. The spectra are the average of four accumulations. CD data were analyzed using Origin software (MicroCal, Inc., Northampton, MA).

Table 1: Composition of Cassettes for Mutagenesis of the Residues 43−57 Region of *M. capricolum* HPr[a]

CONSTRUCT	CASSETTE STRUCTURE AND TRANSLATION
N43S	5′-GA TCC TTA AAA TCA ATT ATG AAT GTA ATG GCA ATG GCT ATA AAA ACT GGT AC-3′ 3′-G AAT TTT AGT TAA TAC TTA CAT TAC CGT TAC CGA TAT TTT TGA C-5′ G42 S43 L44 K45 S46 I47 M48 N49 V50 M51 A52 M53 A54 I55 K56 T57 G58 T59
ec43-57	5′-GA TCC GCG AAA AGC CTG TTT AAA CTG CAG ACT CTG GGC CTG ACT CAA GGT AC-3′ 3′-G CGC TTT TCG GAC AAA TTT GAC GTC TGA GAC CCG GAC TGA GTT C-5′ G42 S43 A44 K45 S46 L47 F48 K49 L50 Q51 T52 L53 G54 L55 T56 Q57 G58 T59
ec43-50	------TCC GCG AAA AGC CTG TTT AAA CTG---------- ---G CGC TTT TCG GAC AAA TTT GAC---------- S43 A44 K45 S46 L47 F48 K49 L50
ec43-47	------TCC GCG AAA AGC CTG---------- ---G CGC TTT TCG GAC---------- S43 A44 K45 S46 L47
ec48	----------TTT---------- ----------AAA---------- F48
ec49	----------AAA---------- ----------TTT---------- K49
ec50	----------CTG---------- ----------GAC---------- L50
ec51-57	----------CAG ACT CTG GGC CTG ACT CAA--------- ----------GTC TGA GAC CCG GAC TGA GTT---- Q51 T52 L53 G54 L55 T56 Q57
ec51-53	----------CAG ACT CTG---------- ----------GTC TGA GAC---------- Q51 T52 L53
ec51	----------CAG---------- ----------GTC---------- Q51
ec52	----------ACT---------- ----------TGA---------- T52
ec53	----------CTG---------- ----------GAC---------- L53
ec54-57	----------GGC CTG ACT CAA--------- ----------CCG GAC TGA GTT---- G54 L55 T56 Q57

[a] Complementary oligonucleotide pairs, which when annealed formed termini for the restriction endonucleases *Bam*HI and *Kpn*I, were synthesized. Mutagenesis of pMC-HPr in which N43 was mutated to S and a *Bam*HI site was created in the region of G42-S43 and a *Kpn*I site was created in the region of G58-T59 is described in Experimental Procedures. The first construct (N43S) shown corresponds to the *M. capricolum* sequence in which N43 has been changed to S. The second construct corresponds to a replacement of residues 43−57 of *M. capricolum* with residues 43−57 of *E. coli*. The remaining sequences show the residues from *E. coli* which were inserted into the *M. capricolum* HPr sequence. Dashed lines correspond to the sequence shown for the N43S construct.

HPr Phosphorylation

Phosphorylation at Serine 46. Frozen cells of *M. capricolum* or freshly grown cells of *Bacillus subtilis* were suspended in 20 mM Tris·HCl (10−20% w/v) and disrupted in a French pressure cell at 10 000 psi. The suspension was centrifuged for 30 min at 14000*g*, and the supernatant solution was used as a source of the HPr(Ser) kinase. Incubated mixtures, in a volume of 10 μL, contained 20 mM Tris·HCl (pH 7.5), 5 mM NaF, 2 mM DTT, 2 mM $MgCl_2$, 5 mM fructose bisphosphate, 1 μg of the various control or chimeric HPrs, 0.125 mM [γ-^{32}P]ATP (∼1500 cpm/pmol), and the designated source of HPr(Ser) kinase (0.45 μg of protein/reaction of *M. capricolum* crude extract or 0.92 μg of protein/reaction of *B. subtilis* extract). Incubation was carried out for times between 0 and 30 min at room temperature and terminated by the addition of SDS gel running buffer. The samples were then deposited on SDS−polyacrylamide gels (4 to 20%, Tris·glycine, Novex). After electrophoresis, the gels were soaked in boiling TCA (15%) for 45 min and then stained with Gelcode Blue Stain Reagent (Pierce). Densities of the HPr regions in autoradiograms of the gels were quantitated using the NIH IMAGE program (version 1.55) developed at the National Institutes of Health by W. Rasband.

Phosphorylation at Histidine 15. Phosphotransfer from [^{32}P]PEP to HPr and IIA^{glc} was carried out in a reaction mixture (10 μL) containing 100 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.55 μM [^{32}P]-PEP (∼1300 cpm/pmol), 0.5 μg of the specified HPr, 0.1 μg of *M. capricolum* enzyme I, and 0.9 μg of *M. capricolum* IIA^{glc}. The samples were incubated at room temperature for 10 min. The mixture was then electrophoresed on a SDS−polyacrylamide gel (4 to 20% Tris·glycine, Novex). The

gel was stained with Gelcode Blue Stain Reagent and dried. Autoradiography was carried out using Kodak XAR film.

Molecular Modeling of M. capricolum HPr with E. coli Residue Replacements at Positions 43−57

The crystal structure of HPr from *M. capricolum* (*6*) was used as the parent molecule to develop a model of the mutated molecule at positions 43−57. The goal was to check whether the replacement can be accommodated without gross conformational perturbations, and how each of these changes might impact the neighboring residues. The model was built on a Silicon Graphics Indigo II workstation, using the MSI software package QUANTA. The side chain conformation of each mutated residue was chosen so that it resembled most closely the side chain conformation of the original residue and avoided steric clashes with neighboring residues. The resulting model was compared with the parent molecule and with the two crystal structures of HPr from *E. coli* (*16*, *17*) and that from *B. subtilis* (*18*).

RESULTS

Comparison of Structures of HPrs from E. coli and M. capricolum. We previously described the three-dimensional structure of HPr from *M. capricolum*, showing it to have the same general fold (an open-faced β-sandwich) as other previously characterized HPrs (*6*). Consistent with the finding that this HPr cannot only be phosphorylated at the active site His15, but also at the regulatory Ser46 (*19*), it was found that the mycoplasma HPr structure resembles that of HPrs from Gram-positive organisms more closely than that from *E. coli*; phosphorylation at the regulatory site is a characteristic of Gram-positive bacteria.

We previously pointed out that the first published crystal structure of *E. coli* HPr (*16*) deviates from all other known HPr structures in the region of the loop containing residues 50−53 (*6*). A recent structure of the mutant Ser46Asp HPr from *E. coli* (*17*) shows a closer resemblance to all other HPrs in the residues 50−53 region. Thus, the larger deviation of the wild-type structure probably arises from the presence of a sulfate ion in the vicinity of the above residues. With a eye on developing an insight into the requirements for recognition of an HPr by HPr(Ser) kinase, we created and tested a series of replacements in that region (Figure 1) for the ability to be phosphorylated.

Sequence Comparisons. The region of sequence of residues 42−60 of several HPrs was examined (Figure 2). While the HPr from *E. coli* is not phosphorylated at Ser46 by HPr(Ser) kinase, that from *M. capricolum*, *M. genitalium*, and *B. subtilis* is (*19*). Nevertheless, the Lys-Ser sequence at residues 45 and 46 is found in all HPrs. In all the Gram-positive and mycoplasma HPrs examined, residues 43 is Asn, 47 is Ile, 51 is Met, and 55 is Ile or Val; it seemed reasonable to predict that these residues might be determinants for Ser46 phosphorylation.

Construction of M. capricolum Asn43Ser HPr. A mutagenesis approach was used to evaluate the importance of residues in the region of residues 43−57 for Ser46 phosphorylation. A strategy was devised (see Experimental Procedures) in which an expression vector encoding *M. capricolum* HPr was modified to allow the region of residues 43−57 to be excised with unique restriction endonucleases. In this way, a variety of sequences, some of which encoded *E. coli* sequence, could be inserted into the vector. To make

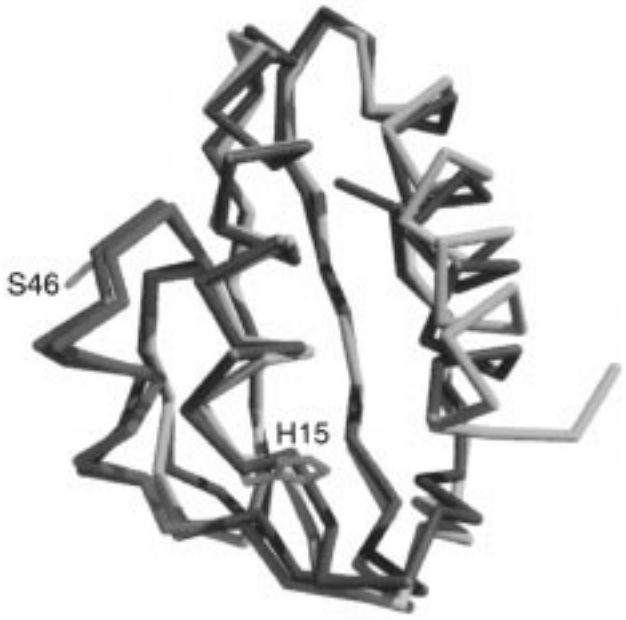

FIGURE 1: Superposition of the crystal structures of HPrs from *M. capricolum* and *E. coli*. The *E. coli* structure (1OPD) is gray. The *M. capricolum* structure (1PCH) is yellow. The region of the *M. capricolum* structure replaced by the *E. coli* sequence is red. The side chains of serine 46 (S46) and histidine 15 (H15) are magenta. Virtual bonds between Cα atoms are drawn. The superposition was calculated on Cα atom coordinates, using the program Align written by G. Cohen (*30*). The *E. coli* S46D HPr structure is shown, because the wild-type HPr crystal structure contains a sulfate ion bound close to residues 53 and 54, which perturbs the main chain conformation of the protein compared with all other HPr crystal structures.

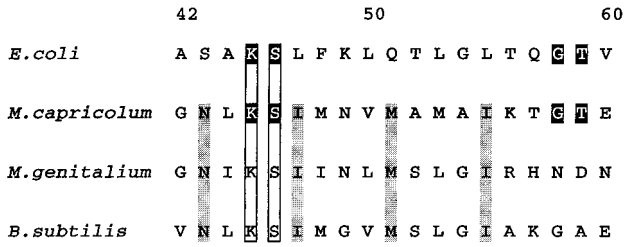

FIGURE 2: Multiple sequence alignment of HPr from diverse bacteria. Sequences from residues 42−60 were aligned and highlighted using the DNADRAW program. Boxed residues are found in all HPrs. Reverse shaded residues are identical in the HPrs from *E. coli* and *M. capricolum*. Lightly shaded residues are common to *M. capricolum*, *M. genitalium*, and *B. subtilis*, but not to *E. coli*. Accession numbers for the various HPr sequences are as follows: *E. coli*, P07006; *M. capricolum*, L22432; *M. genitalium*, L43967; and *B. subtilis*, P08877.

the construct, the sequence encoding residue 43 (Asn in *M. capricolum*) was mutated to Ser (the residue found in *E. coli*). A test of the effect of this replacement on the ability of the HPr to be phosphorylated at Ser46 by a *M. capricolum* extract indicated that the rate of phosphorylation was at least 50% as great as that of the original HPr (data not shown). Consequently, the N43S HPr (see Experimental Procedures) of *M. capricolum* was used as the reference protein for subsequent studies.

Replacement of Residues 43−57 of M. capricolum HPr by the E. coli Sequence. Oligonucleotides were prepared which, when annealed, encoded the sequence of residues 43−57 of *E. coli* HPr (see Experimental Procedures). The corresponding sequence of *M. capricolum* HPr was replaced

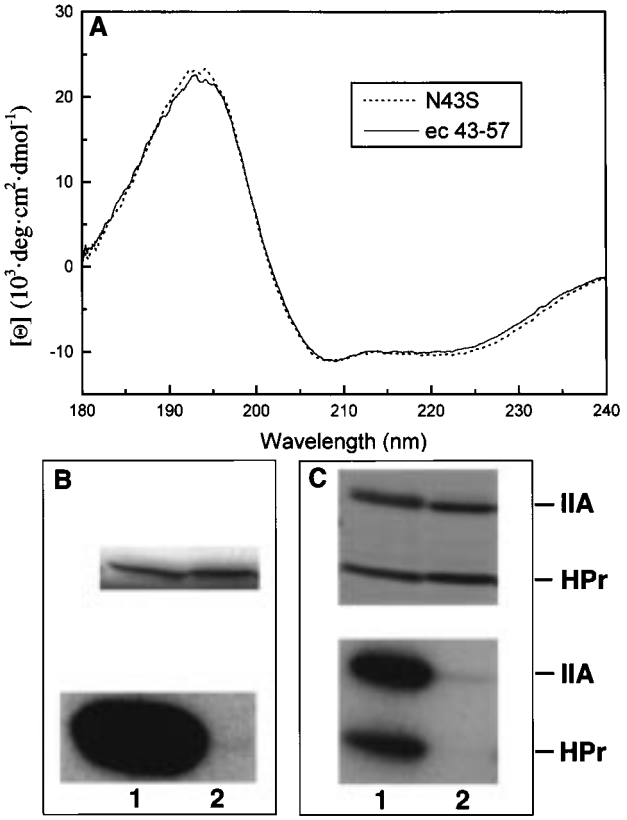

FIGURE 3: Characterization of *M. capricolum* N43S HPr and the *M. capricolum*−*E. coli* 43−57 chimera. *M. capricolum* N43S HPr and the *M. capricolum*−*E. coli* chimera in which residues 43−57 (labeled ec 43−57) of *M. capricolum* HPr were replaced by the *E. coli* sequence were created and purified as described in Experimental Procedures. (A) Circular dichroism. The experiment was carried out as described in Experimental Procedures. (B) Phosphorylation at serine 46. The experiment was carried out as described in Experimental Procedures: upper panel, stained gel; lower panel, autoradiogram; lane 1, N43S; and lane 2, ec 43−57. (C) Phosphorylation at histidine 15. The experiment was carried out as described in Experimental Procedures: upper panel, stained gel; lower panel, autoradiogram; lane 1, N43S; and lane 2, ec 43−57. The migration positions of IIA^{glc} (labeled IIA) and HPr on the gels and autoradiograms are indicated.

with the cassette from *E. coli*. Figure 1 presents a superposition of the structures of the HPrs from *E. coli* and *M. capricolum*, highlighting in red the region of residues 43−57 that were replaced by the *E. coli* sequence. It is clear that the fold of both proteins in this region is quite similar. If the sequence of residues 43−57 is a determinant for phosphorylation at Ser46, it would be expected that the chimera would not be phosphorylated. The expression vector encoding the chimeric HPr was used for the expression and purification of the protein as described in Experimental Procedures. The purified protein was essentially homogeneous.

A comparison of the *M. capricolum*(ec 43−57) HPr chimera with *M. capricolum*(N43S) HPr by circular dichroism (Figure 3A) showed essentially identical spectra, a further indication that replacement of residues 43−57 by the *E. coli* sequence did not affect the folding of the protein. The spectra were similar to that previously reported for *E. coli* HPr (*20*).

A comparison of the two proteins for their ability to be phosphorylated at Ser46 by extracts of *M. capricolum* containing HPr(Ser) kinase (Figure 3B) showed a significant difference. While the *M. capricolum*(N43S) HPr showed good phosphorylation at Ser46, the *M. capricolum*(ec 43−57) HPr chimera was inactive as a substrate for the kinase. This experiment established that the region encoding residues 43−57 of *M. capricolum* HPr is important for recognition by the kinase.

The two proteins were also tested for their ability to be phosphorylated at the active site His15 (Figure 3C). *M. capricolum*(N43S) HPr was not defective in PEP-dependent phosphorylation mediated by *M. capricolum* enzyme I. The *M. capricolum*(ec 43−57) HPr chimera was not phosphorylated at the active site. This is consistent with a model for the interaction between HPr and its partner proteins that suggests an interface of the complexes encompassing a patch of HPr containing residues within the region of residues 43−57 (*21*, *22*).

Determination of Residues Important for Ser46 Phosphorylation. Having established that replacement of residues 43−57 of *M. capricolum* HPr by the *E. coli* sequence resulted in loss of phosphorylation activity at Ser46, we undertook a dissection of the residues 43−57 region (see Figure 4A). When cassettes containing either *E. coli* residues 43−50 or 51−57 were inserted into the *M. capricolum* sequence, the resulting chimeras were not phosphorylated by an extract from *M. capricolum*; this result indicated that residues in both the residues 43−50 and the 51−57 regions are important for Ser46 phosphorylation.

The residues 43−50 region was examined more closely. Substitution of residues 43−47 from *E. coli* resulted in retention of 62% of the control activity. Replacement of residue 50 was associated with no loss of activity, while replacement of residues 48 and 49 led to complete loss of activity.

A similar analysis was carried out for the residues 51−57 region. While replacement of residues 54−57 had no effect on the activity, replacement of residues 51−53 led to complete loss of the activity. The change of residue 51 resulted in retention of 42% of control activity, while replacement of residues 52 and 53 led to complete loss of activity. This study established that the rate of phosphorylation of Ser46 by the kinase from *M. capricolum* is influenced partially by residue 51 and is totally dependent on residues 48, 49, 52, and 53 for activity.

The sequences of *M. capricolum* and *B. subtilis* HPrs differ in some of the sites determined to be essential for phosphorylation of *M. capricolum* HPr by the kinase from *M. capricolum* (Figure 2). While residue 48 is Met in both HPrs, residue 49 is Asn in *M. capricolum* and Gly in *B. subtilis*. Position 52 is Ala in *M. capricolum* and Ser in *B. subtilis*. Residue 53 is Met in *M. capricolum* and Leu in *B. subtilis* (as well as in *E. coli*). It was therefore of interest to test the collection of chimeric HPrs for phosphorylation by an extract from *B. subtilis* (Figure 4B). The results indicated some differences in the specificity of the two kinases. Notably, while residue 50 is Val in both *M. capricolum* and *B. subtilis* HPrs (Figure 2), the kinase from *M. capricolum* tolerates replacement of this residue by Leu while the enzyme from *B. subtilis* shows a reduction of 72% in kinase activity. Another interesting finding is that the *M. capricolum* kinase was equally active with the control HPr and the form in which residues 54−57 were replaced by the *E. coli* sequence,

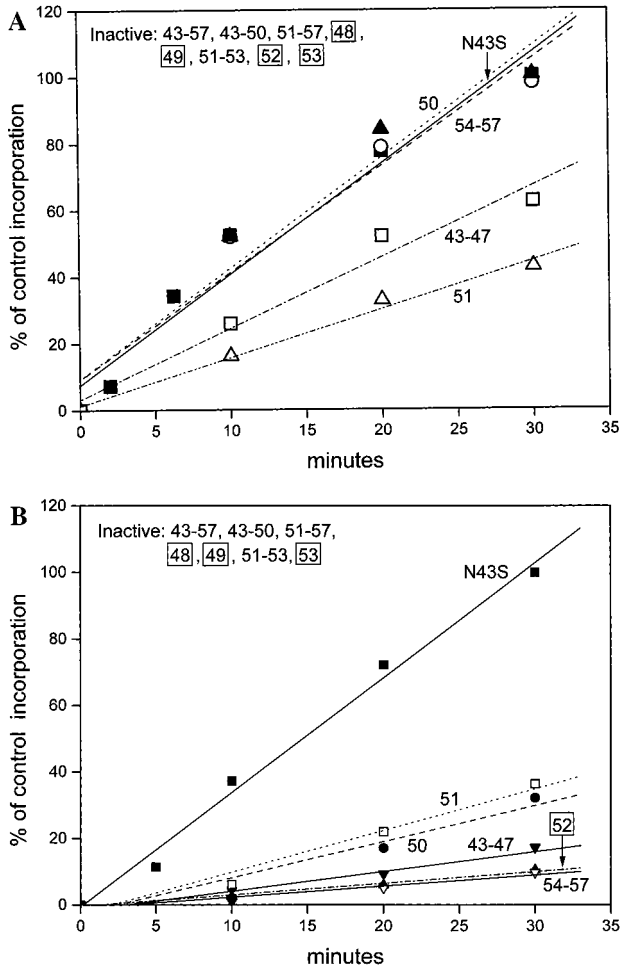

FIGURE 4: Survey of chimeric HPrs for phosphorylation at serine 46. The collection of chimeric HPrs were prepared and tested for phosphorylation at serine 46 as described in Experimental Procedures. The incorporation of ^{32}P by the N43S HPr at 30 min is taken as 100% incorporation. N43S represents *M. capricolum* HPr in which Asn43 is replaced by Ser, the residue found in *E. coli*. The numbers on each construct correspond to the residues in the *M. capricolum* HPr which have been replaced by the *E. coli* sequence. (A) Phosphorylation using the extract from *M. capricolum*. (B) Phosphorylation using the extract from *B. subtilis*. The constructs labeled "inactive" were tested under the same conditions as those showing activity (four time points), and there was no detectable incorporation of radioactivity. For simplicity, therefore, the data points are not shown.

but the enzyme from *B. subtilis* was only 9% as active with the substitution of residues 54−57. The *B. subtilis* kinase was also more sensitive than that from *M. capricolum* to replacement of residues 43−47 (62% of control activity for *M. capricolum* and 15% of control for *B. subtilis*). The one case in which the enzyme from *M. capricolum* appears to be more specific than that from *B. subtilis* involves residue 52 (Thr in *E. coli*, Ala in *M. capricolum*, and Ser in *B. subtilis*). While the Ala → Thr replacement leads to total loss of activity for the *M. capricolum* enzyme, 9% of the control activity is retained by the *B. subtilis* enzyme. This result is reasonable since Thr is more closely related to Ser than to Ala.

Phosphoryl Acceptance and Transfer at the Active Site His15. The data of Figure 3C established that replacement of residues 43−57 of *M. capricolum* HPr by the *E. coli* sequence resulted in inactivation of the HPr with respect to phosphoryl acceptance from enzyme I. This chimeric HPr

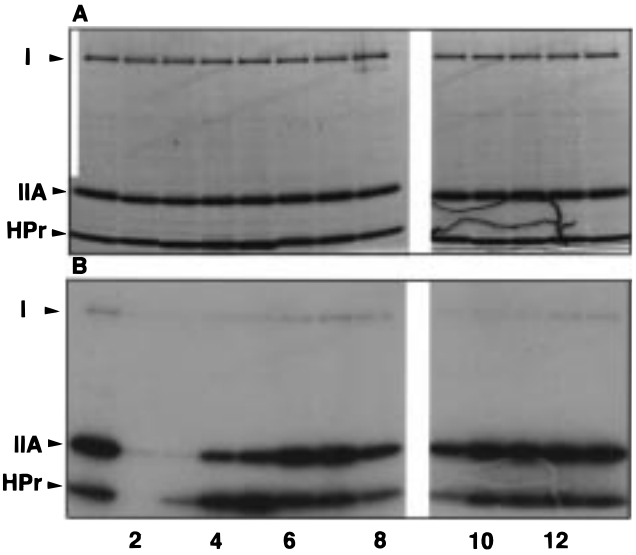

FIGURE 5: Survey of chimeric HPrs for phosphorylation at histidine 15. The collection of chimeric HPrs were prepared and tested for phosphorylation at histidine 15 as described in Experimental Procedures: lane 1, N43S; lane 2, ec 43−57; lane 3, ec 51−57; lane 4, ec 51−53; lane 5, ec 54−57; lane 6, ec 51; lane 7, ec 52; lane 8, ec 53; lane 9, ec 43−50; lane 10, ec 43−47; lane 11, ec 48; lane 12, ec 49; and lane 13, ec 50. Upper panel (A), stained gel; lower panel (B), autoradiogram. Protein markers indicated are as follows: I, EI; IIA, IIA^{glc}; and HPr.

could also not be phosphorylated by *E. coli* enzyme I (data not shown). The availability of the collection of partial replacement chimeric HPrs made it possible to determine whether specific residues in the residues 43−57 region were important for phosphorylation at the active site. Figure 5 depicts the results of an experiment in which the various HPr proteins were incubated with [^{32}P]PEP, enzyme I, and IIA^{glc} from *M. capricolum*. With respect to phosphoryl acceptance from enzyme I, none of the other constructs with partial replacements by the *E. coli* sequence was totally inactive. The replacement of residues 51−57 (lane 3) showed a substantially decreased phosphoryl acceptance. However, replacements of residues 51−53 (lane 4), 54−57 (lane 5), 51 (lane 6), 52 (lane 7), and 53 (lane 8) were all active in phosphoryl acceptance. Similarly, the replacement of residues 43−50 (lane 9) and all the partial replacements in that region (lanes 10−13) were active in phosphoryl acceptance. The data suggest that some combination of residues in the residues 43−57 region is important for the interaction of enzyme I with HPr.

The gels in Figure 5 also reveal the ability of the various HPrs to transfer a phosphoryl group to IIA^{glc}. The replacement of residues 43−57 (lane 2) is inactive in phosphoryl acceptance and thus cannot be tested for phosphoryl transfer activity in this test system. Interestingly, the replacement of residues 51−57 (lane 3) is active with respect to phosphoryl acceptance but essentially inactive with respect to phosphoryl transfer. Partial replacements (residues 54−57, lane 5) or individual substitutions (residues 51−53, lanes 6−8) were all active in phosphoryl transfer. Similar to the case with phosphoryl acceptance, the data suggest that some combination of residues in the residues 51−57 region is important for effective interaction of *M. capricolum* HPr with *M. capricolum* IIA^{glc}.

Modeling of the Effect of Replacements on the M. capricolum HPr Structure. A modeling study was carried

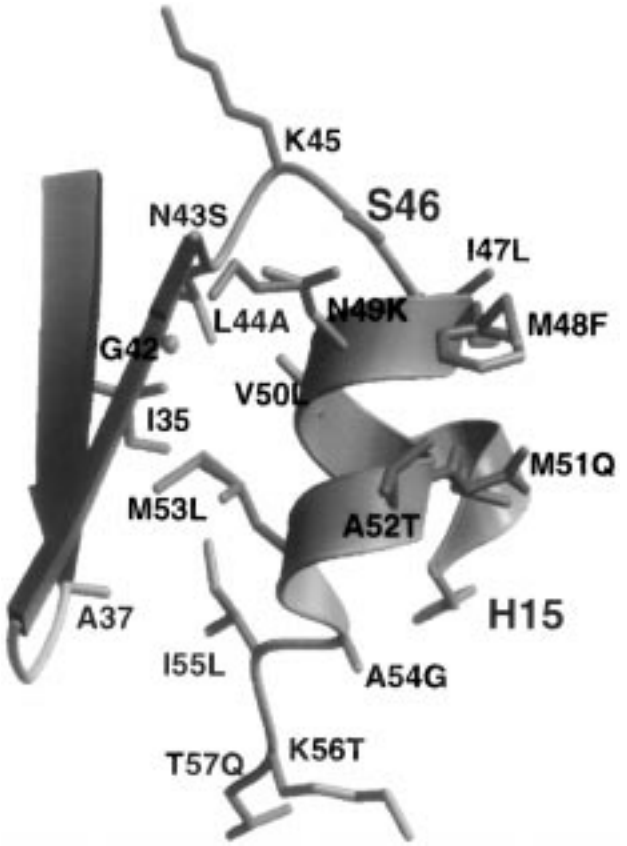

FIGURE 6: Closeup of the *M. capricolum* HPr region around serine 46. The main chain trace is gray, with secondary structure units highlighted as ribbons. Side chains of the native protein are teal-green, and modeled side chains of mutations that impaired phosphorylation of Ser46 most dramatically are red. For the sake of clarity, side chains of other mutations are not shown, but are indicated by the labels. Residues Ile35, Ala37, and Gly42 (shown as a sphere) on the β-hairpin were not mutated, but are shown because they may impact the replacement at position 53.

out to ascertain the effect on the *M. capricolum* HPr structure of replacing residues 43−57 with the corresponding residues characteristic of the *E. coli* protein (Figure 6). It should be noted that residues 45 and 46 are identical in the two proteins. Residue 43 is an Asn → Ser replacement and would not be expected to perturb the structure, since the Asn is a surface residue. Residue 44 is a Leu → Ala replacement and should create a hole in the core (unless backbone adjustment occurs) with a possible effect on the stability of the protein. Residue 47 is a conservative Ile → Leu replacement on the surface of the molecule, which might be readily accommodated. Residue 48 is a Met → Phe replacement. While it is located on the surface, it introduces a substituent with a quite different shape in the vicinity of Ser46 and therefore would be expected to exert a steric effect on the formation of the HPr−kinase complex. Residue 49 is an Asn → Lys replacement. It is a surface residue in the vicinity of Ser46 and is expected to introduce both a steric and electrostatic perturbation that would interfere with complex formation. Residue 50, located in the core, is a Val → Leu replacement. It might compensate for the hole resulting from the Leu44Ala replacement in the 43−57 mutant. As a single mutation, it would be predicted to not be important for binding to the HPr(Ser) kinase. Replacement of Met51, located on the surface, by Gln introduces a polar residue that would be expected to influence the formation of the complex. Residue 52 is an Ala → Thr replacement on the surface. It would be predicted to influence the kinase−HPr interaction by a combination of steric and polar effects. Residue 53, located in the core, is a Met → Leu change. Because the side chain of Leu is smaller than that of Met, either a hole would be formed in the core or the backbone would have to change slightly to recover efficient packing. The phosphorylation assay supports the latter effect. Residue 54, located on the surface, is an Ala → Gly replacement and should easily be accommodated in the structure. Residue 55, an Ile → Leu replacement, is in the core and should not significantly affect the structure. Residue 56, located on the surface, is a significant change from Lys → Thr but is too far away from Ser46 to affect the kinase−HPr complex. Finally, residue 57 is also a surface residue remote from Ser46; while the Thr → Gln change is significant, it would not be expected to disrupt complex formation between the kinase and HPr. The conclusion from the modeling study is that all the replacements are compatible with conservation of the folded protein. Further, the changes that are predicted to have a major impact on the interaction of the kinase with HPr are the replacements at residues 48, 49, 51, and 52. These predictions are generally, but not totally, consistent with the findings from the activity assays of the chimeric proteins (Figure 4A,B).

DISCUSSION

The studies presented here have helped to define the determinants on HPr for the interaction with the ATP-dependent kinase that phosphorylates Ser46. It has been pointed out repeatedly (*6*, *18*, *23*) that, since the general fold of all HPrs is similar, some peculiarities of the region around Ser46 must be reponsible for the specificity of the kinase which phosphorylates Gram-positive but not *E. coli* HPrs. The present mutagenesis experiments were designed to identify those residues in the region of Ser46 of *M. capricolum* HPr that are important recognition sites for the kinase. The mutagenesis strategy was to replace residues in the Ser46 region of the active HPr (*M. capricolum*) by residues found in the inactive HPr (*E. coli*). The concern that construction of the chimera in whch residues 43−57 of *M. capricolum* HPr were replaced with the *E. coli* sequence would lead to a major disruption of the structure was diminished by the demonstration that the CD spectra of the 43−57 chimera and wild-type *M. capricolum* HPr were indistinguishable. Clearly however, since the 43−57 chimera is not a substrate for the kinase, it must be structurally different from the wild-type HPr. Modeling examination of the probable three-dimensional structure of the 43−57 chimera was carried out to evaluate the structural consequences of individual amino acid replacements.

Residues 43−47 comprise the loop between β-strand 3 and helix 2. Replacement of the conserved Asn43 by Ser (see Figure 2) results in an approximately 50% decrease in the phosphorylation rate. Additional replacement of residues 44−47 leads to a further moderate drop of about 40% in reaction rate (Figure 4A). Only positions 44 and 47 can be responsible for this effect. The replacement of the surface residue Ile47 by Leu is rather conservative; the replacement of the core residue Leu44 by Ala may be accompanied by backbone adjustments to avoid an internal hole, and this in turn may affect the interaction with the kinase. Thus, a

determinant for the kinase interaction involves the loop preceding Ser46, requiring Asn43 and Leu- and/or Ile44.

There is additional evidence that does not support an important role for residue 47 in the recognition of HPr by HPr(Ser) kinase. That position is Ile in Gram-positive organisms and Leu in *E. coli* (Figure 2). It has been observed (*24*) that a mutation of HPr from *Streptococcus salivarius* in which Ile47 is replaced by Thr did not prevent the phosphorylation on Ser46.

Surface residues within helix 2 are crucial for appropriate interaction of HPr with the kinase. Gram-positive HPrs contain Met or Ile at position 48, which is replaced by the aromatic group of Phe in *E. coli*. It was previously predicted (*6*, *18*, *23*) that this residue might interfere with the protein−protein interaction. The data in Figure 4 provide compelling evidence for the importance of this residue.

Residue 49 is Asn or Gly in phosphorylatable HPrs but Lys in *E. coli* (Figure 2). As is the case with position 48, Lys is bulkier than Asn or Gly, but very importantly, it might prevent phosphorylation by perturbing the electrostatics of the HPr−kinase interface. The data in Figure 4 substantiate the importance of this position.

Residue 50 has similar side chains (Leu or Val) in all HPrs. Replacement of the Val found in *M. capricolum* by the Leu characteristic of *E. coli* HPr would not be expected to affect phosphorylation. The data with *M. capricolum* extracts (Figure 4A) support this anticipation. Interestingly, however, that is not the case with extracts from *B. subtilis* (Figure 4B). HPr in which Val50 has been replaced with Leu is only about 30% as active with the kinase from *B. subtilis*.

The next solvent-exposed position, 51, affects phosphorylation at Ser46, and this is rationalized structurally by the change of the side chain from hydrophobic (Met) to hydrophilic (Gln) (Figure 2). The replacement by Gln results in 42 and 30% residual phosphorylation activity by the enzymes from *M. capricolum* and *B. subtilis*, respectively (Figure 4A,B).

Residue 52 has a relatively small (Ala or Ser) side chain in phosphorylatable HPrs (Figure 2) which is replaced by Thr in *E. coli*. Despite the relatively conservative mutation to Thr, phosphorylation activity by the *M. capricolum* enzyme is completely eliminated by this replacement (Figure 4A). Interestingly, the *B. subtilis* enzyme retains approximately 10% of control activity with this substitution (Figure 4B). This difference might be rationalized by the observation (Figure 2) that the replacement for *M. capricolum* is Ala to Thr, while that for *B. subtilis* is Ser to Thr, a less significant side chain change.

Perhaps the most surprising finding in this study concerns residue 53, for which replacement eliminates phosphorylation at Ser46 by both kinase enzymes from *M. capricolum* and *B. subtilis*. This position is occupied by Leu in *E. coli*, *B. subtilis*, and *M. genitalium* but by Met in *M. capricolum* and is buried in the protein core. Structurally, this residue would not be expected to have such a dramatic effect on the phosphorylation reactivity. This finding implies that residue 53 may be either Met or Leu but that it must be placed in a particular context in the sequence. Further examination of the environment of Met53 reveals that it interacts with Ile35, Ala37, and Gly42 on a β-hairpin loop (Figure 6). The equivalent residues in *E. coli* HPr are Val35, Ser37, and Ala42, which may impose different spatial constraints on Leu53 of *E. coli* HPr. Mutating Met53 of *M. capricolum* HPr without the introduction of these correlated mutations may have led to unforeseen changes in the structure which eliminated phosphorylation of Ser46.

There is an important difference in the response of the kinases from *M. capricolum* and *B. subtilis* to replacement of residues 54−57. When the Ala-Ile-Lys-Thr sequence of *M. capricolum* HPr is replaced by the *E. coli* sequence (Gly-Leu-Thr-Gln), the chimeric HPr is fully active with the kinase from *M. capricolum*, but retains only ∼10% of the activity with the *B. subtilis* kinase (Figure 4). The reason for this specificity difference is not clear, but it is noteworthy that the sequence in *B. subtilis* HPr is Gly-Ile-Ala-Lys. Since residue 54 is Gly in both the *B. subtilis* and *E. coli* sequences, that residue cannot be the determinant of substrate specificity. The recent report (*25*) of the purification and characterization of the HPr(Ser) kinase from *B. subtilis* showed that some of the HPrs that are kinase substrates (e.g., that from *Enterococcus faecalis*) have a Gln residue at position 57, although the rate of phosphorylation of various HPrs was not quantitated. Therefore, that position is also excluded as the sole determinant for excluding *E. coli* HPr as a substrate. Consequently, it appears that the *B. subtilis* enzyme utilizes the sequence Ile-Lys-Thr at positions 55−57 characteristic of *M. capricolum* HPr as a substrate in a manner superior to that with Leu-Thr-Gln, found in *E. coli* HPr. In constrast, the kinase from *M. capricolum* does not exhibit a significant velocity difference with these two sequences.

While the residues 43−57 region may not be the only key to the specificity of HPr(Ser) kinase, these studies establish that it plays an important role in determining the kinase activity. Figure 6 shows the three-dimensional model of the *M. capricolum* HPr region that has been probed by site-directed mutagenesis, and highlights in red the side chains that correspond to those *E. coli* residues shown in Figure 4 to result in a major loss of phosphorylation activity. The interaction of the HPr(Ser) kinase is expected to be with surface residues of HPr. This involves the polar Asn43, the conserved charged Lys45, and hydrophobic interactions with Ile47, Met - or Ile48, and Met51. Taken together, the Ser46 phosphorylation data and the structural analysis suggest that the loss of activity associated with the replacement at residue 48 is due to a steric effect. Activity losses associated with replacements at residues 44 and 53 may be due to backbone perturbations, and those associated with replacements at residues 49, 51, and 52 may be associated with electrostatic effects.

While this work was under review, a report describing the purification of HPr(Ser) kinase from *B. subtilis* was published (*25*). The availability of that enzyme opens the possibility for performing NMR studies with [^{15}N]HPr to determine the chemical shifts associated with the kinase−HPr interaction. The mutagenesis study presented here constitutes a complementary approach to the same question but more specifically addresses the issue of why kinases from *B. subtilis* and *M. capricolum* cannot utilize HPr from *E. coli* as a substrate.

It is worth noting that *E. coli* HPr can be phosphorylated on His15 by enzyme I from *B. subtilis* at 5% of the rate of the *E. coli* enzyme I-catalyzed reaction (*26*). When we used the sensitive radiochemical assay for measuring phosphotransfer from [^{32}P]PEP to *E. coli* HPr mediated by various

enzymes I (see Figure 5), we found that *E. coli* enzyme I was active but *M. capricolum* enzyme I was totally inactive (data not shown). It was of interest that the chimera in which residues 43−57 of *E. coli* HPr were placed into *M. capricolum* HPr was incapable of being phosphorylated by enzymes I from either *E. coli* or *M. capricolum.* The analysis of the complement of constructs used here shows that some combination of residues in the residues 51−57 region play a role in the determination of the optimal interaction with EI or IIA^{glc}. It is worth pointing out that previous studies (*27*) showed that some mutations in *E. coli* HPr (Ser43Asn, Ser46Cys, Phe48Met/Lys49Gly, and Gln57Glu) resulted in little or no effect on PTS activity.

HPr is a central protein in the PTS. In its role as a phosphocarrier protein, it accepts a phosphoryl group from P-EI and can affect phosphoryl transfer to a family of enzymes II. A comparison of the regions on the surface of HPr involved in the binding to these proteins with that to the HPr(Ser) kinase is warranted.

Chen et al. (*22*) and Van Nuland et al. (*28*) showed by determining NMR chemical shifts that the greatest effects of the interactions of IIA^{glc} and EI with HPr were on residues on either side of the active site His15 and in the region containing residues 43−56. These two regions of HPr also appear to be involved in the IIA^{mtl}−HPr interaction. Therefore, there is an overlapping surface for the interaction of HPr with HPr(Ser) kinase and enzymes I and II of the PTS, at least in the vicinity of helix 2.

E. coli HPr participates in a high-affinity, species-specific interaction with *E. coli* glycogen phosphorylase (*2*). A survey of a collection of HPrs containing single amino acid replacements with respect to binding and activation of glycogen phosphorylase led to a model for important binding sites. Residues Arg17, Lys24, Lys27, Lys40, Ser46, Gln51, and Lys72 were established to be important. It is noteworthy that the region including Ser46 and Gln51 appears to be common to the glycogen phosphorylase, EI, and EIIA binding sites.

In *B. subtilis*, binding of the catabolite repressor protein, CcpA, to its target is regulated by the state of phosphorylation of HPr (*29*). The surface of Ser(P)HPr that binds to CcpA has been investigated by NMR. Residues concluded to be important for the interaction are 14−17, 21−27, 43, 44, 46−56, 78, 81, and 82. Again, the involvement of the region encompassing residues 46−56 parallels the requirement for binding to other proteins.

In summary, the region encompassing residues 43−57 of *M. capricolum* HPr is implicated in the recognition site for the ATP-dependent kinase that phosphorylates Ser46. This region appears to be a common docking area for all proteins that interact with HPr.

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