

Identification of a Site in the Phosphocarrier Protein, HPr, Which Influences Its Interactions with Sugar Permeases of the Bacterial Phosphotransferase System: Kinetic Analyses Employing Site-Specific Mutants

SUSANNE KOCH,¹ SARAH L. SUTRINA,¹ LONG-FEI WU,¹ JONATHAN REIZER,¹ KARIN SCHNETZ,² BODO RAK,² AND MILTON H. SAIER, JR.^{1*}

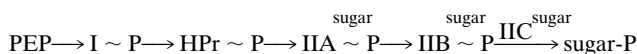
Department of Biology, University of California at San Diego, La Jolla, California 92093-0116,¹ and Institut für Biologie III, Universität Freiburg, D-7800 Freiburg, Germany²

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The permeases of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system (PTS), the sugar-specific enzymes II, are energized by sequential phosphoryl transfer from phosphoenolpyruvate to (i) enzyme I, (ii) the phosphocarrier protein HPr, (iii) the enzyme IIA domains of the permeases, and (iv) the enzyme IIB domains of the permeases which transport and phosphorylate their sugar substrates. A number of site-specific mutants of HPr were examined by using kinetic approaches. Most of the mutations exerted minimal effects on the kinetic parameters characterizing reactions involving phosphoryl transfer from phospho-HPr to various sugars. However, when the well-conserved aspartyl 69 residue in HPr was changed to a glutamyl residue, the affinities for phospho-HPr of the enzymes II specific for mannitol, *N*-acetylglucosamine, and β -glucosides decreased markedly without changing the maximal reaction rates. The same mutation reduced the spontaneous rate of phosphohistidyl HPr hydrolysis but did not appear to alter the rate of phosphoryl transfer from phospho-enzyme I to HPr. When the adjacent glutamyl residue 70 in HPr was changed to a lysyl residue, the V_{\max} values of the reactions catalyzed by the enzymes II were reduced, but the K_m values remained unaltered. Changing this residue to alanine exerted little effect. Site-specific alterations in the C terminus of the β -glucoside enzyme II which reduced the maximal reaction rate of phosphoryl transfer about 20-fold did not alter the relative kinetic parameters because of the aforementioned mutations in HPr. Published three-dimensional structural analyses of HPr and the complex of HPr with the glucose-specific enzyme IIA (IIA^{Glc}) (homologous to the β -glucoside and *N*-acetylglucosamine enzyme IIA domains) have revealed that residues 69 and 70 in HPr are distant from the active phosphorylation site and the IIA^{Glc} binding interface in HPr. The results reported therefore suggest that residues D-69 and E-70 in HPr play important roles in controlling conformational aspects of HPr that influence (i) autophosphohydrolysis, (ii) the interaction of this protein with the sugar permeases of the bacterial phosphotransferase system, and (iii) catalysis of phosphoryl transfer to the IIA domains in these permeases.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) catalyzes the concomitant chemoreception, transport, and phosphorylation of its numerous sugar substrates (19, 27, 32). It also regulates a variety of physiological processes in both gram-negative and gram-positive bacteria (31, 34). These processes include (i) catabolite repression, (ii) carbohydrate transport, (iii) carbon and energy metabolism, (iv) carbon storage, and (v) the coordination of carbon and nitrogen metabolism (20, 36). The regulatory functions of the PTS depend on its ability to serve as a protein kinase system. It phosphorylates various PTS and non-PTS proteins, thereby controlling their interactions with other macromolecules that influence catalysis or transcription. As some of these interactions apparently involve recognition of tertiary structure rather than primary structure (30), they can be influenced by alterations in residues distant from the sites of interaction (11, 12, 25, 26, 48, 49).

The generalized scheme for the PTS phosphoryl transfer chain is as follows (35):



* Corresponding author. Phone: (619) 534-4084. Fax: (619) 534-7108. Electronic mail address: msai@ucsd.edu.

Several of these PTS proteins can transfer their phosphoryl moieties to non-PTS proteins (34). Central in this scheme is the small, non-sugar-specific phosphoryl transfer protein, HPr. It interacts with enzyme I, a variety of sugar-specific IIA protein constituents of the enzyme II complexes, and with non-PTS phosphorylation targets, including transcriptional antiterminators, non-PTS transport proteins, and enzymes (28, 29, 31, 34).

HPr of *Escherichia coli* is an 85-residue, heat-stable, single-domain, phosphoryl carrier protein (19, 27). The amino acyl sequences of this protein and its homologs in various bacteria have been determined. Its homologs include fructose-inducible HPr-like protein domains, termed FPr (7, 50), the nitrogen-related, regulatory HPr-like protein, termed NPr, encoded within the *ipoN* operon of *E. coli* (20), and the regulator of poly(β -hydroxybutyrate) accumulation, PPr (21). The HPr, FPr, NPr, and PPr proteins show fully conserved residues at five positions (Fig. 1). Three of these (G-13, H-15, and R-17) are at the active site of the protein. The remaining two residues (S-46 and G-67) are important for regulation and structure, respectively (12, 24). G-67 precedes a well-conserved aspartyl residue (D-69) which is conserved in all HPr homologs except NPr, and this residue is followed by a well-conserved glutamyl residue (E-70) found in all HPr homologs except FPrs and some gram-positive HPrs. All HPr homologs lacking an E at position 70 have an A at this position (Fig. 1).

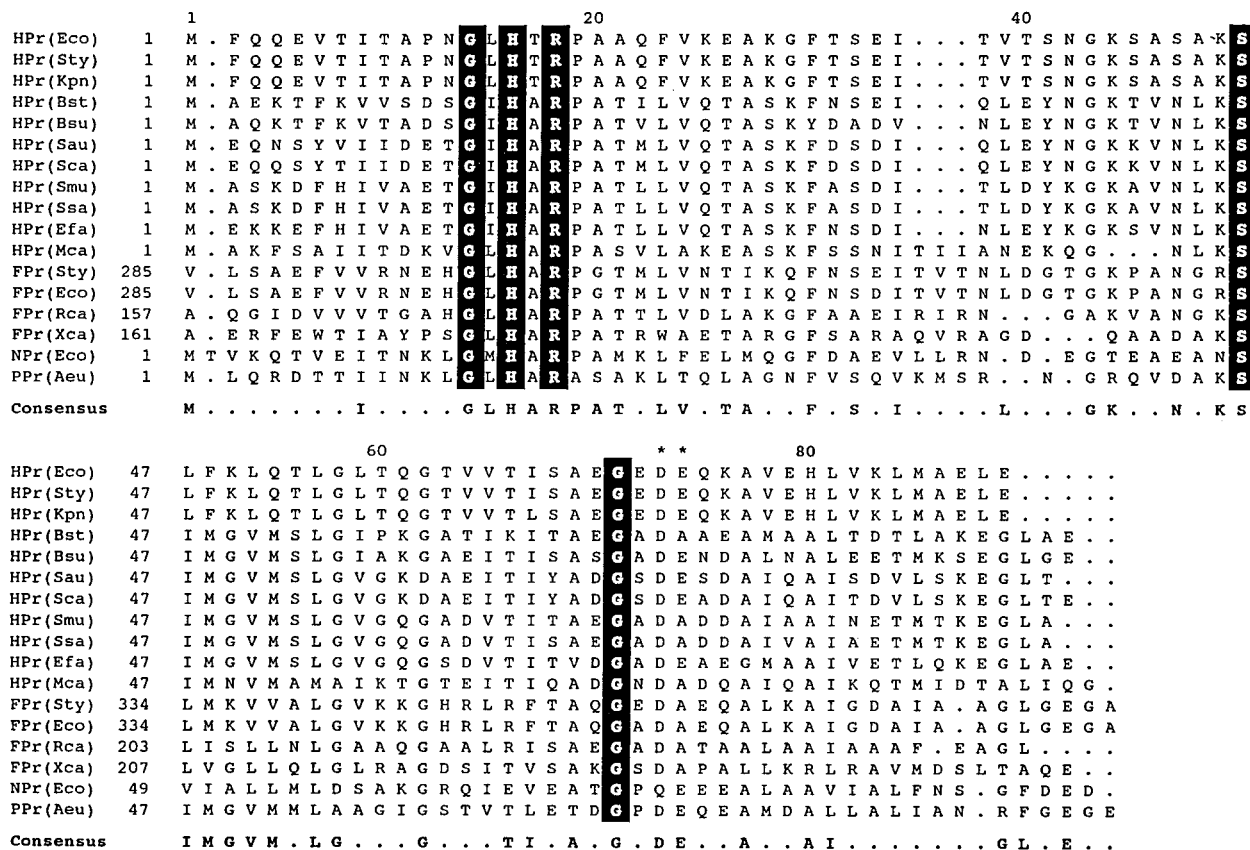


FIG. 1. Multiple alignment of 17 sequenced HPr homologs. Alignment positions are provided above the multiple alignment, and residue numbers are given at the beginning of the lines. Fully conserved residues are highlighted by white on black, while residues which predominate at any one position are included in the consensus sequence at the bottom. The PREALIGN program of Feng and Doolittle (6) was used to generate the multiple alignment. Protein abbreviations: HPr, heat-stable phosphoryl carrier protein of the PTS; FPr, fructose-inducible HPr-like domain in a di- or triphosphoryl transfer protein of a fructose PTS (50); NPr, nitrogen-related HPr homolog encoded within the *rpoN* operon of *E. coli* (20); PPr, poly(β -hydroxybutyrate) accumulation regulatory protein of *Alcaligenes eutrophus* (21). Species abbreviations: Eco, *E. coli*; Sty, *Salmonella typhimurium*; Kpn, *Klebsiella pneumoniae*; Bst, *Bacillus stearotherophilus*; Bsu, *B. subtilis*; Sau, *Staphylococcus aureus*; Sca, *Staphylococcus carnosus*; Smu, *Streptococcus mutans*; Ssa, *Streptococcus salivarius*; Efa, *Enterococcus faecalis*; Mca, *Mycoplasma capricolum*; Rca, *Rhodobacter capsulatus*; Xca, *Xanthomonas campestris*; Aeu, *Alcaligenes eutrophus*.

The catalytic significance of D-69 and E-70 in the *E. coli* HPr is the primary topic of this report. We show that a conservative change (D-69 to E [D69E]) alters the interaction of HPr with any of three PTS permeases, those specific for *N*-acetylglucosamine (enzyme II^{Nag} or II^{Nag}), mannitol (enzyme II^{Mtl}), and β -glucosides (enzyme II^{Bgl}). Additionally, this mutation decreases the spontaneous hydrolysis rate of phosphohistidyl HPr [HPr(his-P)]. Changing the adjacent residue (E-70) to lysine (E70K) also resulted in a dramatic change in the kinetic behavior. However, in contrast to the D69E mutation, which results in a 3- to 11-fold decrease in affinity of the various enzymes II for HPr, the E70K mutation results in decreases in the maximal rates of reaction, with little change in the *K_m* values. Several other alterations in HPr, including the E70A mutation, had little or no effect on the kinetic parameters examined, in agreement with the observation that several gram-positive HPrs and all FPrs have an A at position 70 (Fig. 1).

Three-dimensional analyses of HPr (9–12, 22, 48, 49) have revealed that D-69 and E-70 are distant from the active histidyl phosphorylation site in HPr, and nuclear magnetic resonance (NMR) analyses showed that they are also distant from the face of HPr that binds the glucose-specific enzyme IIA (IIA^{Glc} [4]). The results presented here therefore suggested that D-69

and E-70 may indirectly control interaction of HPr with the various permeases by influencing its conformation. Indeed, careful examination of the three-dimensional structure of HPr reveals that Asp-69 is an important structural residue (9). It is buried within the protein and part of a strong hydrogen bonding network. The oxygens in the carboxyl group of Asp-69 are probably hydrogen bonded to the backbone amide nitrogens of Thr-30 and Ser-31 as well as to the side chain hydroxyl of Ser-31 in the *E. coli* protein. Glu-70, by contrast, is probably localized to the surface of the protein (9). The effects of site-specific mutations altering these two residues on the catalytic properties of HPr have not been reported previously.

MATERIALS AND METHODS

Plasmids. Plasmid pFDX500, a derivative of pACYC177 (3), carries the *lac* repressor gene (41). Plasmid pFDX841 (42) contains the *bglF* structural gene under the control of the *lac* operator-promoter in plasmid vector pUC12 (17). Plasmids pFDX841-D551A, pFDX841-R625D, and pFDX841-R625(stop) contain site-specific mutants of the *bglF* structural gene in which the aspartyl residue 551 in II^{Bgl} was changed to Ala, the C-terminal arginyl residue 625 was changed to Asp, and the C-terminal arginyl residue 625 was removed, respectively (41).

Bacterial strains. An HPr-negative strain, LBG1650 (2, 45), was used as the source of II^{Mtl}, II^{Nag}, and crude preparations of enzyme I. Mutant and wild-type II^{Bgl} proteins were prepared from *E. coli* JLV-86 [F⁻ *thi-1 argG6 metB1 hisG1 lacY1 galT6 xyl-7 apsL104 ΔphoA8 supE44 galA50 pmi manA162 (ptsM162)*

nagE1 cr] which had been transformed first with plasmid pFDX500 and then with plasmid pFDX841 carrying the wild-type *aglF* gene or with one of the mutant plasmids pFDX841-D551A, pFDX841-R625D, and pFDX841-R625(stop). *E. coli* ES7 *recA* (45) was used for the production of mutant HPrs.

Site-specific mutagenesis and in vivo characterization of mutants. A 0.5-kb *SmaI-ClaI* fragment containing the entire *ptsH* gene was derived from pAB65 (16), generously provided by H. L. Kornberg. It was cloned into phage M13mp19 after digestion of the phage with restriction enzymes *SmaI* and *AccI*. The construct was then used for preparation of a single-stranded uracil-containing template in *E. coli* CJ236 [*dut ung thi relA*; pCJ105(Cm^r)]. The Biorad Muta-Gene M13 in vitro mutagenesis kit was used. Oligonucleotide-directed in vitro mutagenesis was carried out as described by the manufacturer. The oligonucleotide used to generate the D69Y mutant HPr was designed in such a way that the nucleotide substitution destroyed the *MboII* site (GAAGA). Mutants were screened by *MboII* digestion and then confirmed by sequencing. Similar procedures were used for generation of the E70K mutant and the double mutant, D69Y/E70K, but no restriction site was destroyed or created in construction of the E70K mutant. About 70% of the plaques corresponded to the desired mutants. The mutated DNA was then cloned back into plasmid pT7-4 and characterized in the *ptsH* mutant LBG1650 after transformation.

Relative fermentation responses were recorded on eosin-methylene blue plates lacking lactose (Difco) but containing 0.5% the indicated sugar. Relative growth rates were recorded on minimal plates containing medium 63, 15 g of Bacto Agar per liter, 20 µg of Trp, Ade, and Thr per ml, and 0.2% the sugar indicated or in liquid medium of the same composition lacking agar. For quantitative growth rate measurements, cells were grown in a 25-ml volume at 37°C in 125-ml Erlenmeyer sidearm flasks, with a rotation rate of 200 rpm, and the increase in cell density was monitored for 10 h. In all cases, the relative fermentation responses correlated with the relative growth responses (Table 1).

HPr mutants F2Y, ESQ, E5D, T7N, T62N, D69E, E70K, and E70A were produced by the method of Zoller and Smith (51) as modified by Kunkel (15). The mutated genes in M13mp11 were transferred to pUC13 for expression of HPr in *E. coli* ES7 *recA*.

Transport assays. Cells of mutant LBG1650 (*ptsH*) (2, 45) bearing the wild-type or mutant forms of the *E. coli ptsH* gene in plasmid pT7-4 (43) were grown with shaking at 30°C in LB medium (18) containing ampicillin (50 µg/ml) and the indicated sugar (1%). Cells were harvested by centrifugation, washed twice with medium 63, and resuspended in the same medium to an absorbance of 20 Klett units (filter 66) (0.08 mg [dry weight] of cells per ml). Cell suspensions in medium 63 were prewarmed to 37°C for 5 min before addition of ¹⁴C-labeled sugar to a final concentration of 10 µM (5 µCi/µmol). The transport reactions were terminated by withdrawing 1-ml samples at intervals and collecting the cells on membrane filters (0.45-µm pore size). Radioactivity was determined as described previously (39).

Growth of bacteria and preparation of membranes. For preparation of II^{Mtl}, II^{Nag}, or crude enzyme I, LBG1650 was grown to stationary phase at 37°C with shaking in LB medium containing mannitol or *N*-acetylglucosamine (0.2%). For preparation of mutant or wild-type II^{Bgl}, the transformed strains described above were incubated at 37°C with shaking in LB medium containing ampicillin (50 µg/ml), kanamycin (50 µg/ml), and isopropylthiogalactopyranoside (IPTG; 4 mM) and harvested during mid-logarithmic growth (100 to 200 Klett units).

Bacteria were harvested by centrifugation and washed twice with medium 63 and once with 50 mM Tris-HCl (pH 7.2)–1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (buffer T). They were resuspended in buffer T. Cells were disrupted by passage through a French pressure cell at 10,000 lb/in² at 4°C. Unbroken cells and cell debris were removed by low-speed centrifugation (10,000 × g, 10 min, 4°C). Membranes were prepared by high-speed centrifugation of the resulting crude extracts (100,000 × g, 2 h, 4°C). The membrane pellets were washed once with and resuspended in buffer T. For II^{Bgl}, which is unstable to butanol-urea extraction, washed membranes were used for kinetic analyses. For II^{Nag} and II^{Mtl}, membranes were extracted with butanol and urea to remove peripheral membrane proteins as previously described (33). The extracted membranes were used for kinetic analyses. For some studies, the cytoplasmic fraction from LBG1650 was subjected to a second high-speed centrifugation to remove residual membranes, and the resulting supernatant was used as a source of enzyme I.

In vitro enzyme assays and kinetic analyses of the interactions between HPr and the enzymes II. Enzymes of the PTS were assayed by complementation as described previously (8, 39). Conditions were adjusted such that enzyme II was limiting and enzyme I and ¹⁴C-sugar were present in excess. Sugar phosphorylation as a function of HPr concentration was then measured.

For kinetic analyses of II^{Bgl}-catalyzed reactions, assay mixtures contained [¹⁴C]thioethyl-β-glucoside (¹⁴C-TEG; 500 µM, 0.12 µCi/µmol), phosphoenolpyruvate (5 mM), purified enzyme I (2 µg), wild-type or mutant II^{Bgl} (washed membranes), and the indicated amounts of purified wild-type or mutant HPr in a total volume of 100 µl. For studies involving II^{Nag} or II^{Mtl}, assay mixtures contained [¹⁴C]mannitol or [¹⁴C]*N*-acetylglucosamine (20 µM, 5 µCi/µmol), 5 mM phosphoenolpyruvate, the cytoplasmic fraction from LBG1650 as a source of enzyme I, II^{Nag}, or II^{Mtl} (butanol-urea-extracted membranes derived from cells grown on *N*-acetylglucosamine or mannitol, respectively), and HPr in a total volume of 200 µl. In all cases, potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (10 mM), KF (20 mM), and dithiothreitol (2 mM) were present. After an

TABLE 1. Utilization of sugars by a strain synthesizing plasmid-encoded wild-type or site-specific mutant forms of HPr^a

HPr derivative	Growth or fermentation response with:			
	Glucose	Mannitol	<i>N</i> -acetylglucosamine	Fructose
Wild type	++	++	++	++
D69Y	±	±	±	++
E70K	+	+	+	++
D69Y/E70K	–	–	–	++

^a Plasmid pT7-4, carrying the wild-type *ptsH* gene of *E. coli*, the singly mutated gene (D69Y or E70K), or the doubly mutated gene (D69Y/E70K), was transformed into strain LBG1650 (45), and growth or fermentation responses were recorded as described in Materials and Methods. ++, strong response; +, intermediate response; ±, weak response; –, no response. Generation times of the four strains in liquid minimal mannitol medium (see Materials and Methods) were as follows: wild type, 1.4 h; D69Y, 3.9 h; E70K, 2.7 h; D69Y/E70K, >10 h.

incubation period of 20 min at 37°C (II^{Mtl} or II^{Nag}) or of 35 min (II^{Bgl}), the assay mixtures were analyzed for phosphorylated ¹⁴C-sugar, using ion-exchange chromatography to separate phosphorylated from free sugar (14).

HPr hydrolysis. The standard assay mixture (37) (100 µl, final volume) consisted of 50 mM Tris-HCl buffer (pH 7.5) 10 mM MgCl₂, 20 mM KF, 1 mM ¹⁴C-phosphoenolpyruvate (specific activity, 0.5 µCi/µmol), and enzymes. The assay was conducted either with excess enzyme I (as in Fig. 5A) or with limiting enzyme I (as in Fig. 5B). Conditions were adjusted such that less than 30% of the ¹⁴C-phosphoenolpyruvate was consumed. Control samples lacked either HPr, enzyme I, or both. After an incubation period of 1 h at 37°C with shaking, 0.4 ml of Sigma color reagent (20 mg of 2,4-dinitrophenylhydrazine per 100 ml in 1 N HCl; catalog no. 505-2) was added to stop the reaction. The mixture was then vortexed and incubated for 10 min at 37°C. One milliliter of ethyl acetate was then added and mixed by vigorous vortexing. The two-phase solutions were transferred to Eppendorf tubes and centrifuged briefly to separate the phases and remove coagulated protein from the organic (top) phase. An aliquot of 600 µl was then removed from the organic phase for determination of radioactivity by scintillation counting (37).

PTS proteins. Wild-type *E. coli* HPr was purified to homogeneity by using an overproducing plasmid as previously described (25). *E. coli* enzyme I was purified to near homogeneity from a similar plasmid by using an unpublished procedure that utilizes chromatography on Q-Sepharose followed by gel filtration on Sephacryl S-100. HPr mutant proteins were purified from appropriately transformed *E. coli* ES7 *recA* which had been grown in LB broth (18) with 100 µg of ampicillin per ml. The purification involved a reordering of the steps used in a previous HPr purification method (47). Membrane-free supernatants (about 120 ml) from about 30 g (wet weight) of cells were chromatographed on an Ultralgel AcA54 (10 by 100 cm) column equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Fractions (500 ml) were collected, and fractions 7 to 9 were pooled and loaded onto a 150-ml DEAE-cellulose column equilibrated with the same buffer. Normal elution involved a 500-ml 0 to 0.2 M KCl gradient. The elution position of HPr changed if the mutation resulted in a charge difference. HPr-containing fractions were pooled, exhaustively dialyzed against 5 mM sodium acetate buffer (pH 5.5), loaded onto a 150-ml carboxymethyl cellulose column, and eluted with a 0 to 0.2 M NaCl gradient (500 ml). This gradient and the elution position were changed appropriately for those mutants with charge differences. In some cases, this ion-exchange step produced homogeneous protein, as judged by isoelectric focusing. In other cases, chromatography on Q-Sepharose and rarely on Sephadex G-40 as well was needed. Purified HPr proteins were obtained in 30- to 400-mg quantities.

RESULTS

In vivo characterization of site-specific mutants. Site-specific mutants of the *E. coli ptsH* gene in which HPr had been altered at positions 69 and/or 70, the two adjacent, well-conserved, negatively charged residues in various HPr proteins (Fig. 1), were constructed and inserted into the low-expression vector, plasmid pT7-4 (43). An HPr-negative *E. coli* strain, LBG1650 (2, 45), was then transformed with these plasmids, and the fermentation responses were recorded (Table 1). Replacement of Asp-69 with Tyr (D69Y) resulted in a marked decrease in growth rate and fermentation response on plates containing glucose, mannitol, *N*-acetylglucosamine, or any one of a number of other sugar substrates of the PTS. The same relative growth rates were recorded quantitatively in liquid

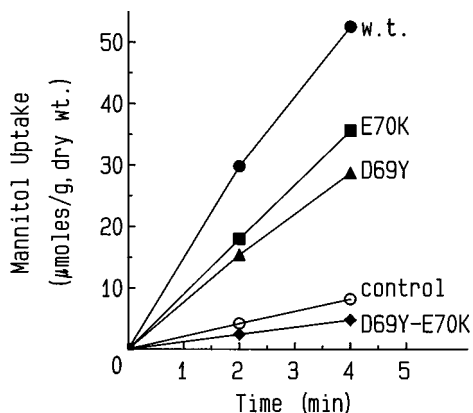


FIG. 2. Uptake of [^{14}C]mannitol in an *E. coli ptsH* mutant (LBG1650) carrying plasmids bearing wild-type or mutated *ptsH* genes. The uptake experiment was conducted as described in Materials and Methods with cells synthesizing wild-type (w.t.) HPr, the indicated mutant, or no HPr (control).

medium with mannitol as the growth substrate. However, there was no decrease in fermentation or growth rate on plates containing fructose (Table 1). The fructose phosphotransferase possesses its own fructose-inducible HPr-like domain, FPr (7, 38, 39, 50), and consequently is not dependent on functional HPr. Mutation of Glu-70 to Lys (E70K) similarly resulted in diminished growth and fermentation, although the effect was less pronounced. The double mutant (D69Y/E70K) exhibited negative growth and fermentation phenotypes (Table 1). Strains carrying the other mutant *ptsH* genes examined (see below) did not exhibit diminished fermentation responses relative to the wild-type strain when examined in a similar fashion.

The growth and fermentation results reported in Table 1 were confirmed by conducting ^{14}C -sugar uptake assays. Representative results are shown in Fig. 2. When cells were grown in medium containing glucose so that the enzymes II specific for other sugars were not induced to high levels and these enzymes II were rate limiting for sugar uptake, the rates of uptake of these sugars reflected the relative degrees of impairment noted for fermentation. Thus, as shown in Fig. 2, [^{14}C]mannitol uptake was depressed more in the D69Y mutant than in the E70K mutant, but the double mutant showed uptake rates that were comparable to those observed for the control *ptsH* mutant. Induction of the synthesis of II^{Mtl} to high levels, so that this enzyme was present in excess, largely overcame the effects of the single D69Y and E70K mutations but not that of the genetic defects in the double mutant (data not shown). Although this last observation contrasts with the defective growth and fermentation responses recorded in Table 1, it should be noted that the transport experiments were conducted with $10\ \mu\text{M}$ [^{14}C]mannitol whereas the growth and fermentation results were for media containing 10 and 25 mM mannitol, respectively. The concentration difference presumably accounts for the different results obtained by these two methods.

Interactions of purified D69E HPr with II^{Nag} , II^{Mtl} , and II^{Bgl} . Salt-washed or butanol-urea-extracted membranes derived from bacteria which had specifically been induced for synthesis of the enzyme II of interest served as a source of the various enzymes II. These were used in conjunction with purified *E. coli* enzyme I and the purified wild-type or D69E mutant HPrs in order to determine the kinetic parameters altered by this specific mutation. Results of analyses conducted

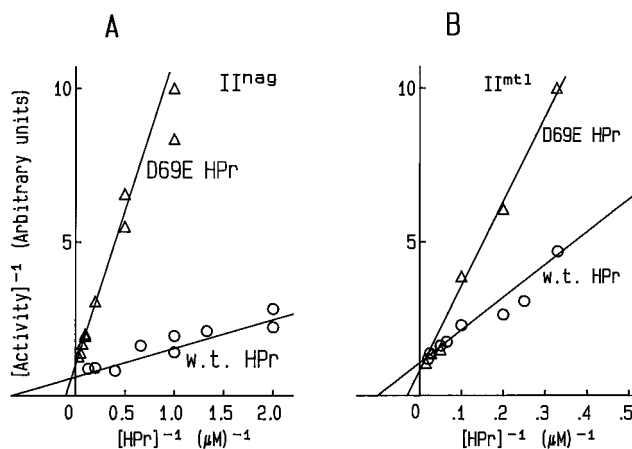


FIG. 3. Kinetic analysis of the interaction of wild-type (w.t.) HPr and the D69E mutant HPr with II^{Nag} (A) or II^{Mtl} (B). PTS assays were conducted with excess [^{14}C]N-acetylglucosamine (A) or [^{14}C]mannitol (B), excess enzyme I, and variable amounts of HPr as described in Materials and Methods. In panel A, the value of 10 on the y axis corresponds to $(0.9\ \mu\text{mol of phosphorylated sugar formed per min per g of membrane protein})^{-1}$. In panel B, the value of 10 on the y axis corresponds to $(2.3\ \mu\text{mol of phosphorylated sugar formed per min per g of membrane protein})^{-1}$.

with the N-acetylglucosamine enzyme II (II^{Nag}) and the mannitol enzyme II (II^{Mtl}) are shown in Fig. 3. In both cases, the D69E mutation decreased the affinities of the enzymes II for HPr (11- and 3.3-fold, respectively) without appreciably altering the V_{max} values (Fig. 3 and Table 2).

The results obtained with the β -glucoside enzyme II and a mutant form of II^{Bgl} in which the C-terminal arginyl residue (40) was replaced by an aspartyl residue (R625D) are shown in Fig. 4. Although the mutant showed only about 5% of the activity of the wild-type protein (41), the kinetic responses of these two enzymes II to the D69E mutant HPr were similar. Thus, the kinetic analyses indicated that the wild-type II^{Bgl} recognized the D69E mutant HPr with 1/10 the affinity of the wild-type protein and catalyzed phosphoryl transfer at about 80% of the maximal rate observed with wild-type HPr. The R625D mutant II^{Bgl} similarly showed a 10-fold reduction in apparent affinity with no reduction in maximal rate when the D69E mutant HPr instead of the wild-type protein was used

TABLE 2. Relative kinetic constants for the interaction of wild-type and mutant HPrs with various enzymes II^a

Enzyme II	K_m value (μM) obtained with:			K_m (mutant)/ K_m (wt)	V_{max} (mutant)/ V_{max} (wt)
	wt HPr	D69E HPr	E70K HPr		
II^{Nag}	1.1	12.5		11	1.0
II^{Mtl}	10	33		3.3	1.1
II^{Bgl} (wt)	40	>400		10	0.8
II^{Bgl} R625D	37	>400		11	1.0
II^{Bgl} [R625(stop)]	110	>400		3.9	0.4
II^{Bgl} D551A	29	120		5	0.7
II^{Nag}	2.5		2.5	1.0	0.4
II^{Bgl} (wt)	71		59	0.8	0.25
II^{Bgl} (R625D)	70		47	0.7	0.2

^a Kinetic analyses were conducted as described in Materials and Methods and in the legends to Fig. 3, 4, 5, and 7. Individual values may possess substantial error as revealed by the data presented in the figures. In several cases, the kinetic constants were determined in triplicate or quadruplicate, and values were generally in agreement $\pm 40\%$. wt, wild type.

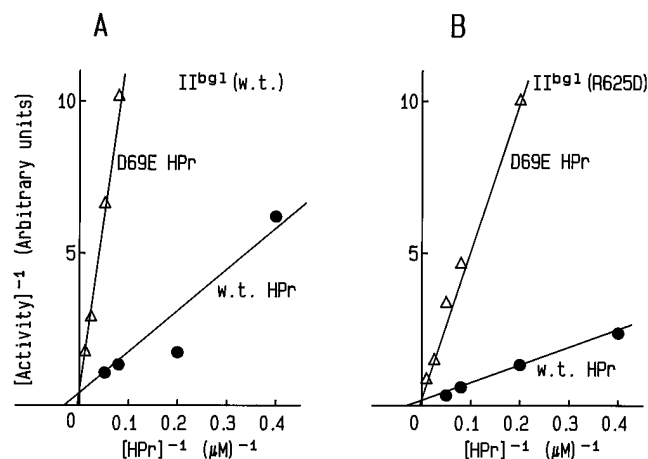


FIG. 4. Kinetic analysis of the interaction of wild-type (w.t.) II^{Bgl} (A) or mutant R625D II^{Bgl} (B) with wild-type HPr or with mutant D69E HPr. Assays were conducted with excess [^{14}C]TEG and enzyme I, limiting amounts of II^{Bgl} (washed membranes), and variable amounts of HPr as described in Materials and Methods. Double-reciprocal plots are shown. In panel A, the value of 10 on the y axis corresponds to $(1.7 \mu\text{mol of phosphorylated TEG formed per min per g of membrane protein})^{-1}$. In panel B, the value of 10 on the y axis corresponds to $(42 \text{ nmol of phosphorylated TEG formed per min per g of membrane protein})^{-1}$.

(Fig. 4 and Table 2). Two other II^{Bgl} mutants [R625(stop) and D551A (41)] similarly showed drastically increased K_m values for the D69E mutant HPr, with only moderate (30 to 60%) reductions in the V_{max} values relative to wild-type HPr (Table 2). It is therefore apparent that the D69E mutation in HPr affects primarily the affinities of the three enzymes II for the phosphoryl carrier protein HPr and that the conserved, C-terminal arginyl residue in II^{Bgl} is not involved in HPr recognition.

Spontaneous hydrolysis of D69E HPr(his-P) and interaction of the mutant protein with enzyme I. As shown previously (37), HPr(his-P) spontaneously hydrolyzes, and appropriate utilization of the assay for this reaction can allow estimation of the efficiency of phosphoryl transfer from enzyme I(his-P) to HPr. By using this assay under standard conditions with enzyme I and phosphoenolpyruvate present in excess, the D69E mutant HPr was found to undergo spontaneous hydrolysis at half the rate of the wild-type protein (Fig. 5A). Reduction in the enzyme I concentration so that phosphoryl transfer between phospho-enzyme I and HPr became limiting for hydrolysis did not alter the relative rates of hydrolysis (Fig. 5B). Thus, phosphoryl transfer from enzyme I to HPr did not seem to be appreciably impaired by the D69E mutation.

Interaction of purified E70K and E70A mutant proteins with enzymes II. As shown in Table 1, the E70K mutant HPr showed decreased activity *in vivo*. The kinetic analysis shown in Fig. 6 revealed that this mutation caused a large decrease in the activity of phosphoryl transfer from HPr to II^{Nag} , but in contrast to the D69E mutant, the maximal rate of phosphoryl transfer rather than the apparent affinity of II^{Nag} for HPr was altered. Figure 7 shows corresponding data for wild-type II^{Bgl} and the R625D mutant II^{Bgl} . Just as for the II^{Nag} -catalyzed reactions, the reactions catalyzed by these enzymes exhibited altered V_{max} values rather than K_m values in response to the E70K mutation in HPr (Table 2).

The E70A mutant HPr was similarly examined. This mutant showed a much less dramatic decrease in the V_{max} value than did the E70K mutant when the II^{Nag} - or II^{Bgl} -catalyzed reac-

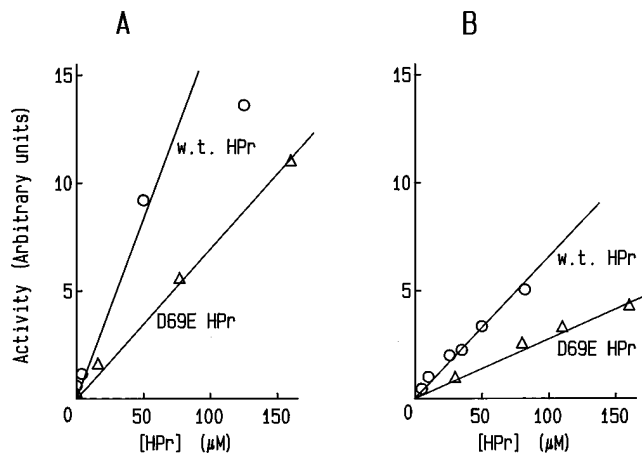


FIG. 5. Comparison of the rates of hydrolysis of wild-type (w.t.) HPr(his-P) with those of the D69E mutant HPr(his-P) under conditions of excess phosphoenolpyruvate and enzyme I (A) or under conditions such that enzyme I was rate limiting (B). The HPr concentration was varied, and the experiment was conducted as described in Materials and Methods.

tion was studied (data not shown). Apparently, introduction of the positively charged lysyl residue at position 70 was much more deleterious to the function of HPr than loss of the negatively charged glutamyl residue at this position.

Spontaneous hydrolysis of E70A and E70K HPr(his-P) and interaction of the mutant proteins with enzyme I. Phosphorylated wild-type and E70K and E70A mutant HPr proteins were examined for their spontaneous hydrolysis rates with enzyme I present in excess, essentially as described for Fig. 5A. The phosphorylated E70K mutant protein exhibited the same rate of hydrolysis as the wild-type protein $\pm 10\%$ over the concentration range of 20 to 200 μM . Phospho-E70A HPr exhibited a 35% increased rate of hydrolysis over this same concentration range (data not shown). When the experiment was conducted as described for Fig. 5B with enzyme I present in limiting amounts, the phosphorylated E70K mutant HPr showed a de-

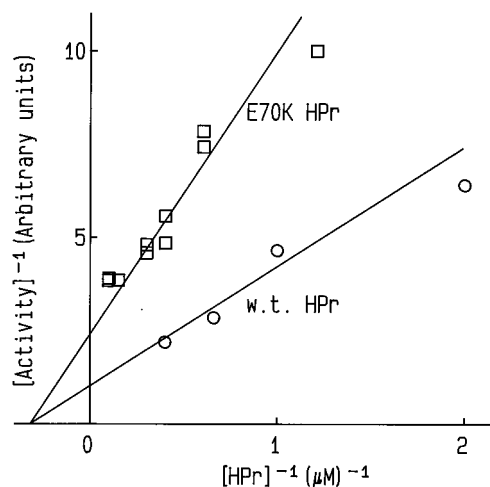


FIG. 6. Kinetic analysis of the interaction of wild-type (w.t.) HPr or E70K mutant HPr with wild-type II^{Nag} . PTS assays were conducted with excess [^{14}C]N-acetylglucosamine, excess enzyme I, limiting II^{Nag} , and variable amounts of HPr as described in Materials and Methods. The value of 10 on the y axis corresponds to $(2.8 \mu\text{mol of phosphorylated sugar formed per min per g of membrane protein})^{-1}$.

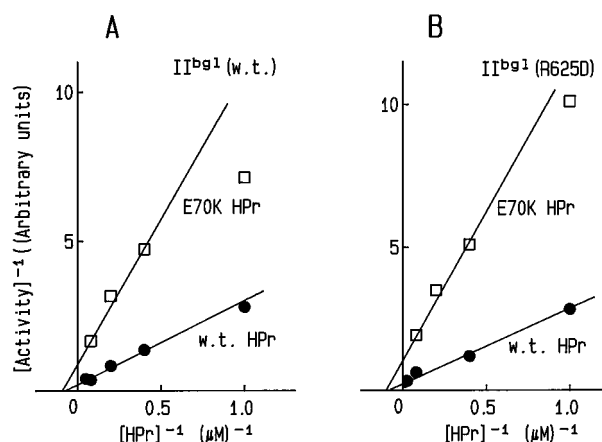


FIG. 7. Kinetic analysis of the interaction of wild-type (w.t.) II^{Bgl} (A) or mutant R625D II^{Bgl} with wild-type (w.t.) HPr or mutant E70K HPr. Assays were conducted as described in the legend to Fig. 4. In panel A, the value of 10 on the y axis corresponds to (1.1 μmol of phosphorylated TEG formed per min per g of membrane protein)⁻¹. In panel B, the value of 10 on the y axis corresponds to (85 nmol of phosphorylated TEG formed per min per g of membrane protein)⁻¹.

creased rate of hydrolysis at high HPr concentrations as a result of an apparent twofold decrease in V_{\max} and a partially compensatory twofold decrease in K_m , characterizing the enzyme I-catalyzed HPr phosphorylation reaction. The E70A mutant protein exhibited a 25% increase in V_{\max} relative to the wild-type protein, with no significant change in the K_m value (data not shown). These results suggest that neither the E70A nor the E70K mutation had a pronounced effect on the spontaneous hydrolysis rates of the phosphorylated proteins and that only the E70K mutant protein exhibited an altered interaction with enzyme I.

Properties of other site-specific mutants of HPr. Several additional purified site-specific mutant HPr proteins were examined as a function of HPr concentration as shown in Fig. 3 to determine if the mutations altered the kinetic behavior of HPr with the mannitol or *N*-acetylglucosamine enzymes II (II^{Mtl} or II^{Nag}). These mutant HPr proteins were F2Y, E5Q, E5D, T7N, and T62N. None of these mutations showed an appreciable effect on the kinetic parameters of the reactions studied (less than 25%).

DISCUSSION

In this report, we describe the kinetic properties of a number of site-specific mutants of HPr with regard to phosphoryl transfer to several enzymes II of the PTS. Our previous studies revealed that most sequenced PTS permease enzyme IIA domains possess C termini with a characteristic three-residue pattern, most frequently hydrophobic-charge-charge, where the two terminal residues are usually basic (40). We suggested that these terminal sequences might be important for interaction of the PTS permeases with HPr (40). In II^{Bgl}, the terminal basic residue is arginyl residue R-625. To determine the function of this residue, it was removed [R625(stop)] or converted to an aspartyl residue (R625D) by site-specific mutagenesis (41). In either case, a 20-fold decrease in reaction rate was observed, but the mechanistic basis for this decrease could not be determined.

Examination of the sequenced HPr proteins and their homologs revealed that two adjacent negatively charged residues in HPr, D-69 and E-70, are largely conserved, although the glutamyl residue at position 70 is sometimes replaced by an

alanine residue (Fig. 1). The *in vivo* studies revealed that both the D69Y and the E70K mutations gave rise to decreased growth rates on and fermentation of PTS sugars (Table 1), and [¹⁴C]mannitol uptake studies (Fig. 2) confirmed the conclusion that these residue positions are important for HPr function. Interestingly, the two mutations together appeared to have a synergistic effect on PTS function, as the double mutant (D69Y/E70K) was totally lacking in activity. Although detailed three-dimensional data were not available to provide an explanation for these findings when these studies were first conducted, at least the deleterious effects of the D69Y and D69E mutations can now be understood (see the introduction and reference 9).

In vitro analysis with the single mutants, D69E and E70K, confirmed the *in vivo* conclusions and allowed us to determine the effects of the mutations on the relative kinetic constants which define the interactions between HPr and the individual permeases. The D69E mutation always gave rise to augmented K_m values (decreased apparent affinities) without appreciably changing the V_{\max} values, while the E70K mutation always gave rise to decreased V_{\max} values without appreciably altering the K_m values. The E70A mutant showed nearly normal kinetic behavior, suggesting that it was the introduction of the positively charged alkyl amine side chain of the lysyl residue rather than the removal of the negatively charged carboxymethyl side chain of the native glutamyl residue which gave rise to the kinetic alterations. The fact that the D69E mutant was drastically altered with respect to its kinetic behavior despite the fact that a negatively charged glutamyl residue replaced the negatively charged aspartyl residue led to the possibility that the position of this acidic side chain in the protein is important to function.

The latter substitution apparently influenced the rate of spontaneous hydrolysis of HPr(his-P) and the apparent affinities of the permeases for HPr, but it did not appreciably affect the affinity of enzyme I for HPr. It therefore appeared that the substitution might have selectively altered the conformation of some parts of HPr without altering that of other parts. This fact is of considerable interest, as Asp-69 has been shown to be an important structural residue in HPr, and the D69E mutant has been found to exhibit decreased stability relative to the wild-type protein (9, 12a).

A similar effect has recently been demonstrated for the regulatory seryl residue 46 (31). By conducting multidimensional NMR analyses of the *Bacillus subtilis* HPr and its derivatives in which seryl residue 46 is either phosphorylated or changed to another residue (22, 48, 49), the residue at position 46 was shown to influence the local (but not the global) conformation of HPr. Thus, conversion of seryl residue 46 to either phosphoserine or aspartate results in a conformational change in the protein which manifests itself in the extension and stabilization of helix B (22). It is therefore possible that the well-conserved D-69 and E-70 residues (Fig. 1) are critical for interaction of HPr with the PTS permeases as a result of an effect on some specific and restricted aspect of the HPr protein conformation. In fact, the functional consequences of the mutations in HPr on interactions with the permeases may be due to induced conformational effects. It is important to note that the changes induced by the D69E mutation are highly selective, since the V_{\max} values of the phosphoryl transfer reactions catalyzed by enzyme I and the enzymes II were not appreciably altered (Table 2 and Fig. 5). This result suggests that the strong hydrogen bonding network in which Asp-69 participates is not crucial to the maintenance or the overall fold of HPr. D69E HPr may be partially unfolded, but it cannot be denatured.

The observation that mutations at residues 69 and 70 (even

to residues of opposite charge) are not complemented by elimination of the C-terminal arginyl residue in II^{Bgl}, or by its replacement with a residue of opposite charge, essentially negates our earlier hypothesis that the interaction between II^{Bgl} and HPr is mediated by electrostatic attraction between the negative charges associated with residues 69 and 70 in HPr and the C-terminal positively charged residues in the PTS permeases (40, 41). This conclusion is substantiated by the three-dimensional analyses reported for the complex of the *B. subtilis* HPr and IIA^{Glc} proteins (4).

The structures of HPr proteins from several bacterial species have been determined by both NMR and X-ray diffraction (see reference 11 for a comprehensive review). In all cases, the protein consists of an open-faced sandwich with three α -helices overlying a skewed β -sheet consisting of four β -strands. Asp-69 and Glu-70 are located on a loop between the last β -strand and the C-terminal α -helix in these structures, distant from the active-site histidyl residue. While Asp-69 is internally buried and of structural significance, E-70 is probably solvent exposed (9). It must therefore be concluded that the mutational alterations at positions 69 and 70 that alter the kinetic behavior of the phosphoryl transfer reactions probably do so by inducing conformational changes in HPr that affect the active site. This suggestion correlates with the dramatic effect that a change in seryl residue 46 to aspartate has on the catalytic activity of HPr (25, 26). In this regard, it is interesting that the active-site histidine (His-15), the regulatory serine (Ser-46), and the anion pair (Asp-69–Glu-70 analyzed here) are all on loops separating a β -strand from an α -helix. Since enzyme I, which phosphorylates His-15, and the ATP-dependent kinase, which phosphorylates Ser-46 in gram-positive bacteria, both appear to recognize tertiary rather than primary structure (30), these enzymes as well as the enzymes IIA of the PTS may be capable of sensing slight alterations in the HPr conformation.

The 1-phosphohistidine in phospho-HPr undergoes spontaneous phosphohydrolysis independently of the presence of enzyme I or an enzyme II (37). The active site of HPr includes E-85, the C-terminal α -carboxyl group. Two examples of mutant HPrs with hydrolysis rates that are 50% of the wild-type *E. coli* HPr rate have been reported. The first example is a mutant *E. coli* HPr (Term-85) that lacks E-85 and possesses L-84 at the C terminus, while the second example is the *Enterococcus faecalis* HPr, which has a C-terminal glutamine (11–13, 44–47). In the case of Term-85, determination of its structure by NMR revealed an adjustment such that E-83 contributes its carboxyl group to the active site (1, 11), presumably in a less effective arrangement. It is therefore reasonable to suggest that the D69E mutation causes a change in the orientation of the loop or turn leading to the last α -helix and that this change alters the interaction of E-85 at the active site. Such an alteration could account for the kinetic changes reported in Table 2.

The studies described in this report and in that of Schnetz et al. (41) used a site-specific mutagenic approach to attempt identification of residues that directly or indirectly influence protein-protein interactions in the phosphoryl transfer cascade of the PTS. The application of X-ray crystallography and multidimensional NMR (4, 5, 9–12, 22) to these mutant proteins is likely to further define the nature of the forces which determine macromolecular complementarity among the phosphoryl transfer proteins of the PTS. These studies should be applicable to numerous processes involving macromolecular recognition.

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