Importance of the Region Around Glycine-338 for the Activity of Enzyme I of the *Escherichia coli* Phosphoenolpyruvate:Sugar Phosphotransferase System

Yeong-Jae Seok, Byeong Ryong Lee,‡ Celia Gazdar, Ingrid Svenson, Nalini Yadla, and Alan Peterkofsky*

Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, Bethesda, Maryland 20892

Received August 29, 1995; Revised Manuscript Received October 30, 1995⊗

ABSTRACT: The gene encoding enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system from an *Escherichia coli* enzyme I mutant was cloned and sequenced. The mutation was shown to be a guanine to adenine transition resulting in an altered protein in which glycine-338 was replaced by aspartic acid. The enzyme I structural gene was mutated to change glycine-338 to a variety of other amino acid residues. Fermentation tests indicated that glycine-338 could be mutated to alanine with no gross loss in phosphotransferase activity, while mutation to valine, glutamic acid, aspartic acid, arginine, histidine, or asparagine led to significant loss of activity. An expression vector for enzyme I was mutated to change glycine-338 to a variety of other amino acid residues and highly purified mutant proteins were prepared. Analysis of phosphorylation of the proteins by PEP indicated that mutation of glycine-338 to alanine had little effect on phosphorylation, mutation to valine substantially decreased phosphorylation, change to histidine or arginine drastically diminished phosphorylation, and mutation to aspartic or glutamic acids abolished phosphorylation activity. Mutation at glycine-338 influences the autophosphorylation rather than the phosphoryl transfer activity of enzyme I.

The widespread bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] (Postma et al., 1993) is capable of carrying out the coupled translocation and phosphorylation of numerous sugars. The system is composed of two general cytoplasmic proteins that are used for all sugars and, in addition, some sugar-specific components known as enzymes II which are partly embedded in the cytoplasmic membrane. One of the general proteins is the approximately 63 kDa enzyme I (EnzI) which is autophosphorylated on a histidine residue by PEP. Phosphorylated EnzI participates in a phosphotransfer reaction to the histidine-15 residue of the other general protein, HPr (approximately 9 kDa). Phosphorylated HPr can then donate its phosphoryl group to a variety of sugar-specific enzymes II, which ultimately phosphorylate specific sugars.

On the basis of proteolysis studies (LiCalsi et al., 1991; Lee et al., 1994), it has been proposed that EnzI from *Escherichia coli* and *Salmonella typhimurium* is composed of a compact amino-terminal domain containing the phosphorylatable histidine residue and a flexible carboxy-terminal domain. The amino-terminal domain of EnzI (EnzI-N) cannot be phosphorylated by PEP but can participate in a reversible phosphotransfer with HPr (LiCalsi et al., 1991; Seok et al., 1996). It has therefore been suggested that the binding site for HPr is associated with the amino-terminal domain while that for PEP is in the carboxy-terminal domain. Further, the carboxy-terminal domain of EnzI confers on the protein the capability to discriminate among various HPrs as phosphoacceptors (Seok et al., 1996).

We previously reported that an apparently leaky EnzI mutant of *E. coli* exhibited defective PTS activity both with respect to maximum activity and the affinity for PEP (Peterkofsky & Gazdar, 1978). It was anticipated that an insight into the nature of the mutation would assist in an understanding of the structural basis for EnzI activity. Therefore, the mutation was cloned, sequenced, and characterized.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. Table 1 lists plasmids and Table 2 lists bacterial strains used in this work.

Cloning and Sequencing of the ptsI Gene from Strain 1103. Chromosomal DNA from strain 1103 (Fox & Wilson, 1968), encoding a mutated form of EnzI, and its parent (strain 1100) was prepared as previously described (Shah & Peterkofsky, 1991). The DNAs were restricted with *Hin*dIII and *Bam*HI. As expected (Saffen et al., 1987), approximately 1.8 kb fragments hybridized with a probe containing a sequence in the *ptsI* gene (encoding EnzI). The 1.8 kb fragments were purified from an agarose gel and ligated to pBR322 which had been restricted with *Hin*dIII and *Bam*HI. The inserts in clones, designated pAP23 (containing the mutated sequence) and pAP18 (containing the wild-type sequence), were completely sequenced on both strands (Sanger et al., 1977).

Mutagenesis of Histidine-189 to Glycine. A PCR reaction was carried out using pPR6 (see Table 1) as template. A

* Address correspondence to this author at National Institutes of Health, Building 36, Room 4C-11, Bethesda, MD 20892. Telephone: (301) 496-2408. Fax: (301) 480-0182. E-mail: alan@codon.nih.gov.

‡ Present address: Department of Biological Education, Seo-Won University, Mochung-Dong, Chong-Ju City, Chung-Buk, South Korea.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; EnzI, enzyme I of the PTS; EnzI-N, amino terminal domain of enzyme I; mutations are described as the amino acid residue number preceded by the wild-type residue and followed by the mutated residue, e.g., Gly338Asp.

Table 1: Plasmids Used

plasmid[a]	relevant characteristics	reference
pDS20	contains genes encoding HPr, EnzI and enzyme IIA^{glc}	Saffen et al. (1987)
pRK248	cI857ts, Tet^r	Bernard and Helinski (1979)
pAP18	gene encoding EnzI derived from strain 1100 cloned into pBR322	this work
pAP23	gene encoding EnzI derived from strain 1103 cloned into pBR322	this work
pAP100	codes for EnzI gene, under control of ptsH promoter, Amp^r	this work
pAP100(G338A)	Gly338 mutated to code for Ala	this work
pAP100(G338V)	Gly338 mutated to code for Val	this work
pAP100(G338E)	Gly338 mutated to code for Glu	this work
pAP100(G338D, M561I)	Gly338 mutated to code for Asp, Met561 mutated to code for Ile	this work
pAP100(G338D)	Gly338 mutated to code for Asp	this work
pAP100(G338H)	Gly338 mutated to code for His	this work
pAP100(G338R)	Gly338 mutated to code for Arg	this work
pAP100(G338N)	Gly338 mutated to code for Asn	this work
pAP100(G337D)	Gly337 mutated to code for Asp	this work
pPR6	codes for EnzI gene, under control of the PL promoter, Amp^r	Reddy et al. (1991)
pPR6(G338A)	Gly338 mutated to code for Ala	this work
pPR6(G338V)	Gly338 mutated to code for Val	this work
pPR6(G338E)	Gly338 mutated to code for Glu	this work
pPR6(G338D)	Gly338 mutated to code for Asp	this work
pPR6(G338H)	Gly338 mutated to code for His	this work
pPR6(G338R)	Gly338 mutated to code for Arg	this work
pPR6(H189G)	His189 mutated to code for Gly	this work

[a] See Experimental Procedures for descriptions of plasmid constructions.

Table 2: *E. Coli* Strains Used

strain number[a]	relevant properties	reference
70	strain 1100, K12 derivative, constitutive for PTS activity	Fox and Wilson (1968)
72	strain 1103, derivative of strain 1100, defective in EnzI activity	Fox and Wilson (1968)
572	DS166; Δ(*ptsI-cysA*)	Saffen et al. (1987)
752	572/pAP23	this work
754	572/pAP18	this work
971	strain TP2819, Δ(*pts I*−*crr*), Kan^r	Levy et al. (1990)
1026	TP2819/pAP100	this work
1027	TP2819/pAP100(G338A)	this work
1028	TP2819/pAP100(G338V)	this work
1029	TP2819/pAP100(G338E)	this work
1030	TP2819/pAP100(G338D)	this work
1031	TP2819/pAP100(G338H)	this work
1032	TP2819/pAP100(G338R)	this work
(not saved)	TP2819/pAP100(G338N)	this work
1033	TP2819/pPR6, pRK248	this work
1034	TP2819/pPR6(G338A), pRK248	this work
1035	TP2819/pPR6(G338V), pRK248	this work
1036	TP2819/pPR6(G338E), pRK248	this work
1037	TP2819/pPR6(G338D), pRK248	this work
1038	TP2819/pPR6(G338H), pRK248	this work
1039	TP2819/pPR6(G338R), pRK248	this work
468	DC646, λ lysogen	Zuber et al. (1987)
1072	DC646/pPR6(G337D)	this work
1073	TP2819/pRK248, pPR6(G337D)	this work
1074	TP2819/pAP100(G337D)	this work
1080	TP2819/pRK248, pR6(H189G)	this work
1081	DC646/pPR6(H189G)	this work

[a] See Experimental Procedures for description of strain constructions.

mutagenic oligonucleotide (76 nucleotides in length) spanning bases 1140−1065 (numbering corresponds to the Genbank sequence for *E. coli ptsI*) containing a unique *Kpn*I site and in which the codon CAC (for His189) was changed to GGA (encoding Gly) was used. For the purposes of screening for successful mutant clones, the mutagenic primer

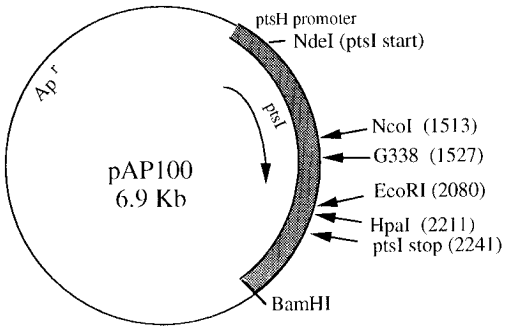

FIGURE 1: Construction of plasmid pAP100. Plasmid pDS20 (see Table 1) was used as the source of the *ptsH* promoter. A PCR reaction was carried out using one oligonucleotide primer upstream of the *Sca*I site and another primer covering the region of the *ptsH* start site. That primer modified the start sequence to create an *Nde*I site. The PCR product was digested with *Sca*I and *Nde*I and purified. This fragment was ligated to the large fragment derived from digestion of pPR6 (see Table 1) with *Sca*I and *Nde*I. The recombinant plasmid pAP100 contained the *ptsH* promoter fused to the gene (*ptsI*) encoding EnzI. The filled portion of the plasmid corresponds to the *E. coli* sequence. The remainder of the plasmid is derived from the cloning vectors.

also contained a new *Bsp*EI site in the region of Ser188−His189. The second PCR primer covered the region encoding the λP_L promoter. Both the PCR product and pPR6 were digested with *Nde*I and *Kpn*I. The large fragment from pPR6 was ligated to the PCR product and then competent cells of XL1-Blue (Stratagene) were transformed by electroporation. Positive clones were verified by *Bsp*EI digestion and then transformed into TP2819/pRK248 (Table 2).

Mutagenesis of Glycine-337 and Glycine-338. PCR reactions were carried out using pAP100 (see Figure 1 and Table 1) as template. All mutagenesis reactions contained a common primer corresponding to the sequence of pBR322 from bases 425−402 (downstream of the *Bam*HI site; numbering corresponds to the Genbank sequence for pBR322). All mutagenic oligonucleotides contained both the *Nco*I site (at nucleotide 1513 of the Genbank sequence for *ptsI*) and the mutated position (either Gly337 or Gly338). The PCR products were blunt-end ligated to *Sma*I-digested pUC9, and then transformation of *E. coli* DH5α was carried out, followed by blue-white selection for plasmids with inserts. Plasmid preparations were made from white colonies. The recombinant plasmids were digested with *Nco*I and *Hpa*I to produce fragments (about 700 bp) containing the desired mutations at Gly337 or Gly338. These fragments were ligated to the large fragment produced by digesting pAP100 (see Figure 1) with *Nco*I and *Hpa*I, followed by dephosphorylation with calf intestine phosphatase. The ligation mixtures were transformed into frozen competent cells of *E. coli* DH5α. Successful cloning was verified by digestion of plasmid preparations with *Bgl*I (clones with a single insert gave three fragments of sizes 3688, 2317, and 234 bp). Mutagenized plasmids were finally verified by sequencing. In the case of one of the mutations (Gly338Asp), there was an unexpected mutation in the region of the *Hpa*I site (M561I). This double mutant was resolved by replacing the *Eco*RI fragment containing the mutation Gly338Asp in the proper orientation into the pPR6 vector digested with *Eco*RI. There is a single *Eco*RI site in the pREI cloning vector (Reddy et al., 1989) and only one *Eco*RI site in *ptsI* (Saffen et al., 1987) between the two observed mutations.

Construction of Mutated Expression Vectors. Recombinant forms of pAP100 containing mutations at Gly337 or Gly338 were digested with *Nde*I and *Bam*HI (see Figure 1). The small fragments (approximately 2.8 kb) were purified and ligated to the large fragment recovered from pPR6 digested with *Nde*I and *Bam*HI and then dephosphorylated with calf intestine phosphatase. The ligation mixtures were transformed into strain 468 (see Table 1); plasmids were isolated and the mutations were verified by sequencing. The mutated expression vectors were then used to transform TP2819/pRK248.

Purification of Mutated Enzyme I Proteins. Cultures (500 mL) of strain TP2819/pRK248 transformed with the mutated versions of pPR6 were grown in six 2-L flasks at 30 °C in LB medium supplemented with tetracycine, ampicillin, and kanamycin. At $A_{600} = 0.4-0.5$, 500 mL of culture medium supplemented with the antibiotics preheated to 55 °C was added to each flask. Incubation with shaking was continued for 2 h at 42 °C. The cells were then collected and processed as previously described for EnzI purification, using DE-52 and AcA44 column chromatography (Reddy et al., 1991). It should be noted that the proteins in which Gly338 was mutated to either Asp or Arg eluted from the DE-52 column considerably later than did the other forms of the protein.

Preparation of the Amino-Terminal Domain of Enzyme I. A sample (approximately 10 mg) of purified EnzI was incubated with immobilized TPCK trypsin (Pierce, 30 units) at room temperature for 45 min with shaking. The immobilized trypsin was then removed by filtration. The filtrate was then fractionated on an AcA44 gel filtration column under the same conditions described for the purification of EnzI (Reddy et al., 1991). The fractions corresponding to EnzI-N were concentrated to 400 μL by pressure filtration through an Amicon PM-10 membrane. The yield of nearly homogeneous EnzI-N was 540 μg.

Autophosphorylation by PEP and Phosphoryl Transfer to HPr by Enzyme I Proteins. [^{32}P]PEP (1850 cpm/pmol) was synthesized from pyruvic acid and [γ^{32}P]ATP using pyruvate kinase (Mattoo & Waygood, 1983). Samples of EnzI proteins (1 μg) were incubated in a total volume of 10 μL with [^{32}P]PEP (20 μM) and 1 μg of HPr at room temperature in 0.1 M Tris·HCl, pH 7.5, 1.0 mM EDTA, 0.5 mM DTT, and 2 mM $MgCl_2$. After incubation for 30 min, the samples were mixed with 10 μL of 2× SDS loading buffer containing 100 mM DTT. SDS−PAGE was carried out on 4−20% gradient gels (Novex). The gels were stained with Coommassie blue and dried. They were then exposed to X-ray film for 3 h.

Phosphoryl Transfer from Enzyme IIA^{glc} to Enzyme I Proteins. [^{32}P]PEP (20 μM), prepared as described above, diluted with unlabeled PEP (80 μM) was incubated with EnzI (5 μg), HPr (5 μg), and enzyme IIA^{glc} (90 μg) in a total volume of 500 μL at room temperature in 0.1 M Tris·HCl, pH 7.5, 1.0 mM EDTA, 0.5 mM DTT, and 2 mM $MgCl_2$. After incubation for 4 h, the reaction mixture was fractionated by FPLC (MonoQ 5/5). A gradient (20 mL, from 10 mM Tris·HCl, pH 7.5, containing 0.1 M NaCl to the buffer containing 0.3 M NaCl) was used to separate the different proteins. The ^{32}P-labeled enzyme IIA^{glc} was collected and concentrated using a Microcon 3 filtration device (Amicon). Samples of EnzI proteins (1 μg) were incubated in a total volume of 10 μL with ^{32}P-labeled enzyme IIA^{glc} (1 μg) and HPr (0.1 μg) at room temperature in 0.1 M Tris·HCl, pH 7.5, 1.0 mM EDTA, 0.5 mM DTT, and 2 mM $MgCl_2$. After incubation for 30 min, the samples were mixed with 10 μL of 2× SDS loading buffer containing 100 mM DTT. SDS−PAGE was carried out on 4−20% gradient gels (Novex). The gels were stained with Coommassie blue and dried. They were then exposed to X-ray film for 8 h.

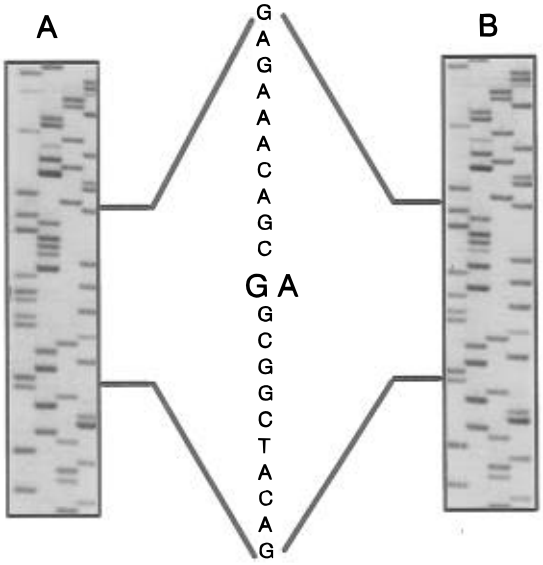

FIGURE 2: Demonstration of the *ptsI* mutation in strain 1103. Sequencing of clones of the *ptsI* gene from strains 1100 (panel A) and 1103 (panel B) was performed as described under Experimental Procedures. The region of the sequence in the gels from nucleotides 1518−1538 of the Genbank sequence for *ptsI* are highlighted. The four lanes in each gel correspond, from left to right, to termination with G, A, T, or C.

Other Methods. Western blotting and immunostaining was carried out as previously described (Zhu et al., 1994). Oligonucleotides were prepared on an Applied Biosystems Model 394 DNA synthesizer. Recombinant DNA methods were by standard procedures (Sambrook et al., 1989). Protein concentration was determined by the method of Bradford (1976).

RESULTS

Characterization of E. coli Strain 1103. The original description of *E. coli* strain 1103 (Fox & Wilson, 1968) presented evidence that it contained a leaky, revertible mutation in the gene encoding EnzI of the PTS. Previous studies in this laboratory (Peterkofsky & Gazdar, 1978) showed that the strain exhibited approximately 5% of the level of PTS activity compared to the wild-type strain (*E. coli* 1100) and that the affinity of the EnzI for PEP was drastically reduced. Since there was little information available concerning the nature of the PEP binding site in EnzI, it appeared that elucidation of the site of mutation in strain 1103 would provide some information about that site. Therefore, the sequence of the region of the chromosomal DNA from strain 1103 containing the *ptsI* gene was determined and compared to the wild-type *ptsI* sequence (see Experimental Procedures). The sequence encoding EnzI was identical to the wild-type sequence with one exception. There was a transition replacement of base G1528 to A (see Figure 2). The result of this mutation is that residue 338 of EnzI is changed from Gly to Asp.

Mutagenesis of the ptsI Sequence. The finding that the explanation for decreased activity of EnzI in strain 1103 was

FIGURE 3: Fermentation properties of *E. coli* strains transformed with pAP100 mutants. Strain TP2819 (Δ*ptsI*, *crr*) (see Table 2) was transformed with pAP100 (see Figure 1) derivatives harboring various mutations at Gly338. Cultures of the transformants were grown in LB medium at 37 °C overnight. A drop of each culture was deposited on a plate containing MacConkey agar supplemented with 1% mannitol. The plates were incubated overnight at 37 °C. The spot identifications are as follows: 1, TP2819; 2, strain 1103; 3, strain 1100; G, pAP100 encoding wild-type enzyme I; A, pAP100(G338A); V, pAP100(G338V); E, pAP100(G338E); D, pAP100(G338D); DI, pAP100(G338D, M561I); R, pAP100-(G338R); H, pAP100(G338H); N, pAP100(G338N).

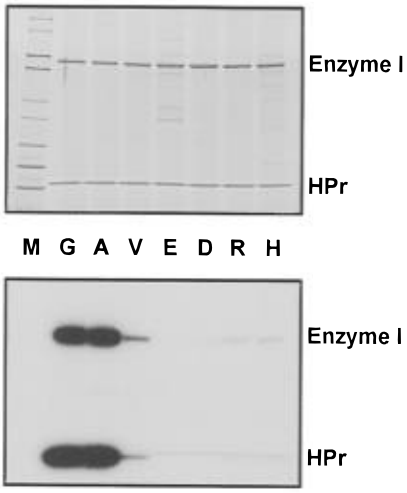

FIGURE 4: Autophosphorylation by PEP and phosphoryl transfer to HPr of enzyme I proteins mutated at G338. Purified EnzI proteins mutated at Gly338 were incubated with [^{32}P]PEP and HPr as described under Experimental Procedures. The samples were then deposited on SDS−PAGE gels and electrophoresed. The gel was then stained with Coommassie Blue (upper panel) and the stained gel was exposed to X-ray film (lower panel). The lane designations are as follows: M, molecular weight markers; G, wild-type EnzI; A, EnzI(Gly338Ala); V, EnzI(Gly338Val); E, EnzI(Gly338Glu); D, EnzI(Gly338Asp); R, EnzI(Gly338Arg); H, EnzI(Gly338His). The positions of EnzI and HPr on the gel and autoradiogram are indicated.

associated with an amino acid replacement at Gly338 prompted experiments designed to characterize the importance for activity of the Gly338 region of EnzI. For mutagenesis of that region, a new plasmid vector, pAP100 (see Figure 1) which contains the *pts* promoter fused to the sequence encoding EnzI, was constructed. Using this plasmid as a template, mutagenesis was carried out to produce a collection of modified versions of pAP100. As described in Experimental Procedures and Table 1, Gly337 was mutated to Asp, and Gly338 was mutated to Ala, Val, Glu, Asp, Arg, His, and Asn.

PTS Activity of ptsI Mutants. The modified forms of pAP100 containing replacements at Gly338 were used to transform a strain of *E. coli* (TP2819) which harbors a deletion in *ptsI*−*crr* (Levy et al., 1990) (see Table 2). These strains were grown in LB medium supplemented with the appropriate antibiotics and then spotted onto MacConkey indicator plates. Figure 3 shows the results of the fermentation test on a mannitol indicator plate (identical results were obtained if the indicator sugar was fructose, data not shown). It is clear from this experiment that mutation of Gly338 to Ala leads to retention of PTS activity. However, mutagenesis of Gly338 to Val, Glu, Asp, Arg, His, or Asn results in loss of EnzI activity.

Expression and Purification of Mutated Proteins. The *Nde*I−*Bam*HI fragments from the various mutated forms of pAP100 (Figure 1) were used to reconstruct pPR6, an expression vector in which the gene encoding EnzI is under the control of the λP_L promoter. As outlined in Table 2, the mutated forms of pPR6 were used to transform strain TP2819 previously transformed with pRK248 (expressing a temperature sensitive form of the λ repressor). Heat induction of such strains, as previously described (Reddy et al., 1991; Zhu et al., 1995) at 42 °C for 2 h (see Experimental Procedures) resulted in good expression of all the mutated forms of EnzI (except for Gly338Asn) as judged by SDS−PAGE of cell pellets. All the expressed proteins were purified to near homogeneity by a previously published method involving chromatography on DEAE-cellulose and AcA44 columns (Reddy et al., 1991). Western blotting using rabbit antiserum directed against wild-type EnzI showed positive reactions with all the mutated proteins (data not shown).

Biological Activity of Mutated Enzyme I Proteins. The data of Figure 4 deal with the capability of the mutated EnzIs to be phosphorylated by PEP and to carry out phosphoryl transfer to HPr. There is a normal level of autophosphorylation and phosphoryl transfer by the protein in which Gly338 is mutated to Ala, a substantially decreased activity in the mutant containing Val, a trace of activity in mutants containing Arg or His, and essentially no activity in the Glu or Asp mutants.

The studies shown in Figure 4 demonstrate a major effect of mutation at Gly338 on the autophosphorylation reaction but not on the phosphoryl transfer reaction from P-EnzI to HPr, since the equilibrium between the phospho- forms of EnzI and HPr is similar at both high and low levels of autophosphorylation of EnzI (Figure 4). The data in Figure 5 provide strong support for that conclusion. Phosphoryl transfer from the phosphorylated form of enzyme IIA^{glc} via HPr to the collection of EnzIs was examined. Not only the wild-type protein (labeled G) but all the proteins mutated at Gly338 were active as phosphoryl acceptors from P-HPr.

Importance of Other Residues in the Vicinity of G338. The sequence of EnzI in the vicinity of Gly338 is D(335)IGGDK-(340). In order to determine whether Gly338 is uniquely important for EnzI function or whether other residues in that region are also critical, further mutagenesis experiments were carried out (see Experimental Procedures). Gly337 in pAP100 was changed to Asp. Fermentation tests similar to those performed in Figure 3, carried out with this mutant, showed that it did not allow fermentation (data not shown).

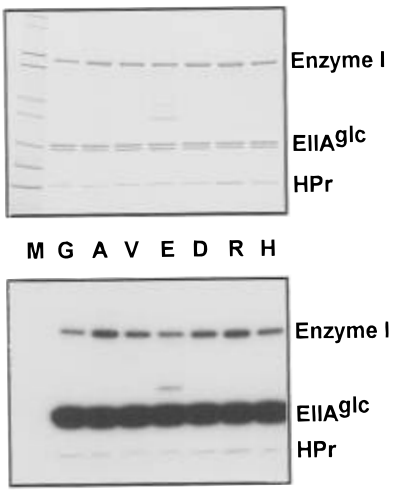

FIGURE 5: Phosphoryl transfer from enzyme IIA^{glc} to enzyme I proteins. Purified EnzI proteins mutated at Gly338 were incubated with ^{32}P-labeled enzyme IIA^{glc} and HPr as described under Experimental Procedures. The samples were then deposited on SDS−PAGE gels and electrophoresed. The gel was then stained with Coommassie Blue (upper panel) and the stained gel was exposed to X-ray film (lower panel). The lane designations are as follows: M, molecular weight markers; G, wild-type EnzI; A, EnzI-(Gly338Ala); V, EnzI(Gly338Val); E, EnzI(Gly338Glu); D, EnzI-(Gly338Asp); R, EnzI(Gly338Arg); H, EnzI(Gly338His). The positions of EnzI, enzyme IIA^{glc} ($EIIA^{glc}$), and HPr on the gel and autoradiogram are indicated.

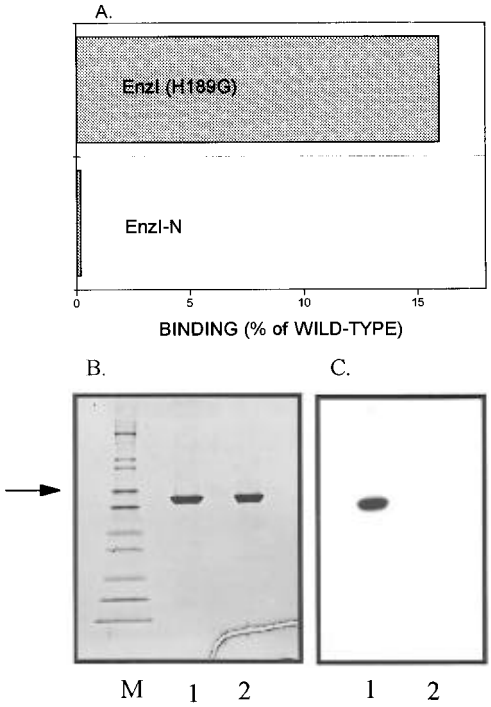

FIGURE 6: Phosphoenolpyruvate binding to enzyme I and EnzI-N. (Panel A) Binding studies. In a total volume of 70 μL, 100 μg of wild-type EnzI or EnzI(His189Gly) or 50 μg of EnzI-N were incubated with [^{32}P]PEP (1 mM, 3×10^4 dpm/nmol) at room temperature in 0.1 M potassium phosphate, pH 7.5, 1.0 mM EDTA, 0.5 mM DTT, and 2 mM $MgCl_2$. After 30 min, the reaction mixtures were deposited on Bio-Spin 30 (Bio-Rad) (EnzIs) or Bio-Spin 6 columns (EnzI-N) and centrifuged at 1000*g* for 2 min. Radioactivity in aliquots (1 μL) of the eluents was determined by scintillation counting. The radioactivity in the wild-type EnzI corresponded to essentially complete binding. (Panel B) SDS−PAGE of enzymes I after incubation with PEP. Aliquots (7.5 μL) of the remaining portions of the samples containing wild-type or EnzI(His189Gly) were run on denaturing SDS−polyacrylamide gels (4−20%). The gel was stained with Coommassie Blue. M, size markers (Novex); lane 1, wild-type EnzI; lane 2, EnzI(His189Gly). Horizontal arrow corresponds to 66.3 kDa. (Panel C) Autoradiography of the stained gel. The gel from panel B was exposed to X-ray film for autoradiography.

These data indicate that both Gly337 and Gly338 are important for the PTS activity of EnzI.

Importance of the Carboxy-Terminal Domain of Enzyme I for Interaction with Phosphoenolpyruvate. A mutant form of EnzI in which the active site His (His189) was changed to Gly was expressed and purified to homogeneity (see Experimental Procedures). Wild-type EnzI, the His189Gly mutant form of EnzI and the amino-terminal domain of EnzI (EnzI-N) were tested for binding by a gel filtration assay (panel A) and phosphorylation by SDS−PAGE analysis (panel B) by PEP. The data in Figure 6A show that EnzI (His189Gly) can bind PEP, while EnzI-N cannot. The capability of the EnzI-N preparation used in these studies to be phosphorylated by HPr (as in Figure 5) was confirmed. This is further evidence that the carboxy-terminal domain of EnzI is important for the interaction of the protein with PEP.

Panels B and C of Figure 6 show that the interaction of PEP with wild-type EnzI (lane 1) leads to a covalent adduct, while the interaction with EnzI (His189Gly) (lane 2) is noncovalent. This is consistent with the model that the initial binding of PEP to EnzI requires the carboxy-terminal domain; the autophosphorylation which follows is at His189.

DISCUSSION

The data presented here demonstrate unequivocally that residues Gly337 and Gly338 are important for the biological activity of EnzI. In the case of Gly338, it was shown that a replacement of the Gly by an Ala residue can be tolerated, but other replacements tested result in substantial or total loss of activity. Using purified EnzI proteins with a variety of amino acid replacements at residue 338, it was possible to determine the function of Gly338 in the catalytic mechanism of EnzI. When the various EnzI species were tested for autophosphorylation by PEP and phosphoryl transfer to HPr, only those species containing Gly or Ala at position 338 were active (Figure 4). These data were essentially in agreement with the fermentation patterns of intact cells containing the various mutant forms of EnzI (Figure 3). Figure 4 shows that while different species of EnzI became autophosphorylated to different degrees, the relative distribution of label between EnzI and HPr (an index of phosphoryl transfer) was similar. These studies strongly suggest that Gly338 mutations influence the autophosphorylation activity of EnzI but not its phosphoryl transfer activity. The data of Figure 5, in which phosphoryl transfer to EnzI from enzyme IIA^{glc} via HPr was studied, validate that conclusion.

EnzI has been characterized as consisting of two domains (LiCalsi et al., 1991; Lee et al., 1994). The amino-terminal half of the protein (residues 1−260) contains the active site (His189). While the isolated amino-terminal domain retains the capability to be reversibly phosphorylated by HPr, it is incapable of autophosphorylation by PEP. It has therefore been suggested that a region important for PEP binding is within the carboxy-terminal domain. Gly338 is found in that domain. Previous data (Peterkofsky & Gazdar, 1978) showed that strain 1103, in which Gly338 of EnzI is mutated to Asp, is characterized by an altered affinity for PEP; this

is consistent with the idea that Gly338 is important for PEP binding to EnzI. The studies described in Figures 4 and 5 provide a strong argument that Gly338 functions in the PEP binding and/or autophosphorylation reaction(s) but not in the phosphoryl transfer from EnzI. It is worth noting that the properties of the EnzI proteins mutated at Gly338 (except for Gly338Ala) described here are essentially identical to those of the isolated amino-terminal domain of EnzI (LiCalsi et al., 1991; Seok et al., 1996).

Besides EnzI, there are two classes of bacterial proteins that are mechanistically related. Pyruvate phosphate dikinase (PPDK) (Xu et al., 1995) catalyzes the reversible interconversion of ATP, pyruvate and P_i with AMP, PEP, and PP_i. There is an additional similarity to EnzI in that one of the partial reactions involves a PEP-dependent autophosphorylation of a His residue in a catalytic domain of the enzyme. PEP synthetase (PPS) (Niersbach et al., 1992) catalyzes the formation of PEP, AMP, and P_i from ATP and pyruvate. In the case of this enzyme as well, the enzyme is autophosphorylated on a His residue by PEP. With the aim of searching for PEP binding domains, it is therefore useful to compare the sequences of EnzI, PPDK and PPS.

There are several regions of the carboxy-terminal domain of EnzI that show sequence similarities with PPDK and PPS (see Figure 7). There is a region (from residues 288−300 of *E. coli* EnzI) that bears a great similarity to a phosphate binding loop, generally characterized as GxxxxGK(S,T). In this case, the motif appears to be GxExxGLxRxE (see Figure 7A).

The region of *E. coli* EnzI which includes Gly338 shows some conservation among the three classes of enzymes that are related (see Figure 7B). The suggested motif derived from the alignment is VRxxD. It is worth noting that Gly residues 337 and 338 of EnzI appear to be important for function, but these residues are not conserved in either PPDK or PPS. It is therefore possible that the effects of the Gly337 and Gly338 mutations are indirect, affecting the activities of the conserved residues V, R, and/or D.

An additional region of absolute conservation occurs in the region of *E. coli* EnzI starting at residue 350 (see Figure 7C). The conserved motif suggested from the alignment is ExNPxxGxR. The characteristic feature of the motifs shown in Figure 7A−C is the presence of an Arg residue and one or more acidic residues. It is possible that the presence of the basic−acidic residue pairs leads to ion-pair associations.

A different type of highly conserved sequence is found in the region of *E. coli* EnzI beginning at residue 429 (see Figure 7D). This sequence, which does not contain a basic residue, can be described by the motif MxExPxxAxxA.

Closer to the carboxy terminus of EnzI, there is a conserved sequence that is closer to the form described in A−C in that there is the potential for ion-pair formation (see Figure 7E). The suggested motif is FSxGxNDxxQ$(x)_6$R.

EnzI contains four Cys residues (Saffen et al., 1987), all of which are in the carboxy-terminal domain. Comparison of the sequences of EnzI (Saffen et al., 1987), PPDK (Pocalyko et al., 1990), and PPS (Niersbach et al., 1992) in these four regions shows that only one Cys residue (at position 502 of *E. coli* EnzI) (see Figure 7F) is conserved. In all of the proteins, it is followed by a Gly residue. It is likely, therefore, that Cys502 is the only Cys residue that is important. It should be noted that Cys831 of PPDK has been established to be essential by studies involving chemical modification and site-directed mutagenesis (Xu et al., 1995).

A. Phosphate binding loop regions

```
Enz. I (B. su.) 268 GGEAVGLYRTEFL 280
Enz. I (E. c.)  288 GAEGVGLYRTEFL 300
PPDK   (C. sy.) 553 GAEGIGLCRTEHM 565
PPS    (E. c.)  503 PNEGVGLARLEFI 515
```

B. VRxxD motif

```
Enz. I (B. su.) 309 VVVRTLDIGGDKEL 322
Enz. I (E. c.)  329 VIVRTMDIGGDKEL 342
PPDK   (C. sy.) 614 MTVRYLDPPLHEFV 627
PPS    (E. c.)  575 VIVRLSDFKSNEYA 588
```

C. ExNPxxGxR motif

```
Enz. I (B. su.) 330 EMNPFLGYRAIR 341
Enz. I (E. c.)  350 EENPFLGWRAIR 361
PPDK   (C. sy.) 657 EFNPMMGHRGCR 668
PPS    (E. c.)  600 EENPMLGFRGAG 611
```

D. MxExPxxAxxA motif

```
Enz. I (B. su.) 409 MVEIPSTAVIA 419
Enz. I (E. c.)  429 MVETPAAATIA 439
PPDK   (C. sy.) 743 MIEIPRAALTA 753
PPS    (E. c.)  679 MCEIPSNALLA 689
```

E. FSxGxNDxxQxxxxxxR motif

```
Enz. I (B. su.) 429 FSIGTNDLIQYTMAADR 445
Enz. I (E. c.)  449 FSIGTNDLTQYTLAVDR 465
PPDK   (C. sy.) 763 FSFGTNDLTQMTFGFSR 789
PPS    (E. c.)  698 FSIGSNDMTQLALGLDR 714
```

F. CG motif

```
Enz. I (B. su.) 481 MCGE 484
Enz. I (E. c.)  501 MCGE 504
PPDK   (C. sy.) 830 ICGE 833
PPS    (E. c.)  750 ICGQ 753
```

FIGURE 7: Alignment of regions of the carboxy-terminal domain of enzyme I with homologous sequences from PPDK and PPS. The four sequences matched are EnzI from *Bacillus subtilis* (B.su.) (Reizer et al., 1993), EnzI from *E. coli* (E.c.) (Saffen et al., 1987), pyruvate phosphate dikinase (PPDK) from *Clostridium symbiosum* (C.sy.) (Pocalyko et al., 1990), and phosphoenolpyruvate synthetase (PPS) from *E. coli* (E.c.) (Niersbach et al., 1992). The numbering shown corresponds to the respective residue numbers in each sequence. Those residues that are identical in the matched sequences are reverse shaded.

The alignment analysis shown in Figure 7 suggests that there are six discrete regions of the PEP binding proteins that may be important in the PEP binding and/or autophosphorylation reaction(s). Aside from the studies carried out on Gly337 and Gly338 of EnzI and Cys831 of PPDK, little experimental work has been done to evaluate the importance of these conserved residues. Mutagenic and crystallographic studies on EnzI, PPDK, and PPS should be instructive in evaluating the importance of these regions for the catalytic activities of EnzI, PPDK, and PPS.

REFERENCES

Bernard, H. U., & Helinski, D. R. (1979) *Methods Enzymol. 68*, 482.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Fox, C., & Wilson, G. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 988.

Lee, B. R., Lecchi, P., Pannell, L., Jaffe, H., & Peterkofsky, A. (1994) *Arch. Biochem. Biophys. 312*, 121.

Levy, S., Zeng, G., & Danchin, A. (1990) *Gene 86*, 27.

LiCalsi, C., Crocenzi, T. S., Freire, E., & Roseman, S. (1991) *J. Biol. Chem. 266*, 19519.
Mattoo, R. L., & Waygood, E. B. (1983) *Anal. Biochem. 128*, 245.
Niersbach, M., Kreuzaler, F., Geerse, R. H., Postma, P. W., & Hirsch, H. J. (1992) *Mol. Gen. Genet. 231*, 332.
Peterkofsky, A., & Gazdar, C. (1978) *J. Supramol. Struct. 9*, 219.
Pocalyko, D. J., Carroll, L. J., Martin, B. M., Babbitt, P. C., & Dunaway-Mariano, D. (1990) *Biochemistry 29*, 10757.
Postma, P. W., Lengeler, J. W., & Jacobson, G. R. (1993) *Microbiol. Rev. 57*, 543.
Reddy, P., Peterkofsky, A., & McKenney, K. (1989) *Nucleic Acids Res. 17*, 10473.
Reddy, P., Fredd-Kuldell, N., Liberman, E., & Peterkofsky, A. (1991) *Protein Express. Purif. 2*, 179.
Reizer, J., Hoischen, C., Reizer, A., Pham, T. N., & Saier, M. H., Jr. (1993) *Protein Sci. 2*, 506.
Saffen, D. W., Presper, K. A., Doering, T. L., & Roseman, S. (1987) *J. Biol. Chem. 262*, 16241.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Seok, Y.-J., Lee, B. R., Zhu, P.-P., & Peterkofsky, A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Shah, S., & Peterkofsky, A. (1991) *J. Bacteriol. 173*, 3238.
Xu, Y., Yankie, L., Shen, L., Jung, Y., Mariano, P., Dunaway-Mariano, D., & Martin, B. (1995) *Biochemistry 34*, 2181.
Zhu, P.-P., Reizer, J., & Peterkofsky, A. (1994) *Protein Sci. 3*, 2115.
Zhu, P.-P., Lecchi, P., Pannell, L., Jaffe, H., & Peterkofsky, A. (1995) *Protein Express. Purif. 6*, 189.
Zuber, M., Patterson, T. A., & Court, D. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4514.

BI952052K