Enzyme I of the Phosphoenolpyruvate:Sugar Phosphotransferase System. In Vitro Intragenic Complementation: The Roles of Arg126 in Phosphoryl Transfer and the C-Terminal Domain in Dimerization†

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ABSTRACT: Enzyme I mutants of the *Salmonella typhimurium* phosphoenolpyruvate:sugar phosphotransferase system (PTS), which show in vitro intragenic complementation, have been identified as Arg126Cys (strain SB1690 *ptsI34*), Gly356Ser (strain SB1681 *ptsI16*), and Arg375Cys (strain SB1476 *ptsI17*). The mutation Arg126Cys is in the N-terminal HPr-binding domain, and complements Gly356Ser and Arg375Cys enzyme I mutations located in the C-terminal phosphoenolpyruvate(PEP)-binding domain. Complementation results in the formation of unstable heterodimers. None of the mutations alters the K_m for HPr, which is phosphorylated by enzyme I. Arg126 is a conserved residue; the Arg126Cys mutation gives a V_{max} of 0.04% wild-type, establishing a role in phosphoryl transfer. The Gly356Ser and Arg375Cys mutations reduce enzyme I V_{max} to 4 and 2%, respectively, and for both, the PEP K_m is increased from 0.1 to 3 mM. It is concluded that this activity was from the monomer, rather than the dimer normally found in assays of wild-type. In the presence of Arg126Cys enzyme, V_{max} for Gly356Ser and Arg375Cys enzymes I increased 6- and 2-fold, respectively; the K_m for PEP decreased to <10 μM, but the K_m became dependent upon the stability of the heterodimer in the assay. Gly356 is conserved in enzyme I and pyruvate phosphate dikinase, which is a homologue of enzyme I, and this residue is part of a conserved sequence in the subunit interaction site. Gly356Ser mutation impairs enzyme I dimerization. The mutation Arg375Cys also impairs dimerization, but the equivalent residue in pyruvate phosphate dikinase is not associated with the subunit interaction site. A 37 000 Da, C-terminal domain of enzyme I has been expressed and purified; it dimerizes and complements Gly356Ser and Arg375Cys enzymes I proving that the association/dissociation properties of enzyme I are a function of the C-terminal domain.

Enzyme I of the phosphoenolpyruvate(PEP):sugar phosphotransferase system (PTS)[1] is a PEP-dependent protein kinase that phosphorylates histidine-containing protein, HPr, in the first step of this sugar transport and phosphorylation system (*1*). The PTS is responsible for the transport of a number of hexoses, hexitols, and disaccharides in a variety of bacterial species including *Escherichia coli* and *Salmonella typhimurium*, in which the PTS plays a significant role in catabolite repression and chemotaxis (for reviews, see refs *2*−*5*). Enzyme I has been purified from both *E. coli* (*6*, *7*) and *S. typhimurium* (*8*), and was shown to be a dimer of 63 kDa subunits, which dissociated at low temperature. The reassociation of the dimers is slow at higher temperatures (>15 °C), and understanding the hysteretic monomer−dimer conversion in enzyme I allowed for quantitative assays (*9*) and the conclusion that the dimer was the active form (*7*, *8*, *10*). The kinetic properties of the enzymes from *E. coli* and *S. typhimurium* are very similar when comparable assay conditions are used (*7*, *8*), and the two enzymes can be used interchangeably (*9*). The enzymes have very high sequence homology (*11*−*13*).

The tertiary structure of the complete enzyme I has not been obtained, but the structure and properties of the N-terminal fragment, containing the HPr-binding domain and the active site $N^{\epsilon 2}$-P-histidine domain (residue 189), have been described (*13*−*17*). Structural information about the C-terminal domain is available from a homologue, pyruvate phosphate dikinase (*18*, *19*).

The PTS has an extensive regulatory influence on carbohydrate metabolism (*2*−*5*), which has led to the enzyme I reaction being viewed as a logical site for regulation. The monomer−dimer equilibrium has been proposed as a mechanism of regulation (*20*). Mg^{2+} and phosphorylation by PEP stabilize enzyme activity (*7*); however, no other significant physiological effectors of enzyme I activity or dimerization

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[1] Abbtrviations: DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPr, histidine containing phosphocarrier protein of the PTS; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; PTS, PEP:sugar phsophotransferase system; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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have been found (*7*, *8*, *21*, *22*). The dimerization has been investigated for both the *E. coli* and *S. typhimurium* enzymes by a variety of biophysical techniques (*23*−*29*) and less extensively with enzyme I from *Staphylococcus carnosus* (*30*). These studies show a 240-fold range of K'_{eq} values for the monomer−dimer interaction, which is influenced by PEP concentrations and phosphorylation state of the subunits. The apparent rate constants for association and dissociation are consistent with hysteretic behavior and are lower than similar constants for other proteins (*29*). Efficient phosphorylation of enzyme I by PEP requires dimerization (*6*−*8*). Further, Kukuruzinska et al. (*23*, *24*) proposed that enzyme I subunits may dissociate to phosphorylate HPr and associate to reinitiate the reaction cycle (*29*).

The relationship between PEP concentrations, dimerization, activity, and/or regulation of enzyme I has yet to be convincingly established as a physiological phenomenon. To gain further insight into these relationships, two mutants of enzyme I from *S. typhimurium*, which were known to affect dimerization and activity, were selected for further characterization and investigation. In addition, the activity of these mutants could be complemented by other mutants in vitro. In this paper, the sites of mutation for three of the enzyme I mutants that allow for intragenic complementation are identified and reveal an important residue for phosphoryl transfer in the HPr-binding domain and two residues that affect dimerization. In addition, the 37 kDa C-terminal domain of enzyme I from *E. coli* has been cloned and purified, shown to dimerize, and provided complementing activity for the mutant enzymes I with impaired dimerization.

EXPERIMENTAL PROCEDURES

Materials. E. coli HPr was produced from the *ptsH* gene in pUC19 under the control of its own promoter and purified as previously described (*31*). Vent and Deep Vent polymerases obtained from New England Biolabs were used for DNA amplifications by the polymerase chain reaction (PCR). Restriction endonucleases and other molecular biological enzymes were obtained from either Pharmacia or New England Biolabs. [^{32}P]PEP was prepared as previously described (*32*). Other radiochemicals were obtained from New England Nuclear. Western blot reagents: horseradish peroxidase conjugated goat anti-mouse antibody was from Bio-Rad, and the chemiluminescence reagents were from Boehringer-Mannheim.

Strains. *E. coli* strain ESK238 was a spontaneous *ptsI* mutant of strain BL21 (*33*) selected by streptozotocin resistance (*34*) on LB medium containing 50 μg/mL streptozotocin. *S. typhimurium* strains SB1476, SB1681, SB1690, and SB2227 were kindly provided by Drs. Norman Meadow and Saul Roseman, Johns Hopkins University. Strains SB1476 and SB1681 are *ptsI* mutants in wild-type *S. typhimurium* LT2 (*35*) and strains SB1690 and SB2227 are *ptsI* derivatives of strain SB3507, which contains *trp223* (*36*).

Isolation of the ptsI Genes from S. typhimurium. ptsI genes were obtained from chromosomal DNA by suspension of a colony in 0.05 mL of H_2O, and adding 1 μL of this suspension to 15 μL of H_2O in a PCR reaction tube. Denatured DNA was obtained by incubating at 95 °C for 5 min, cooling on ice, followed by the addition of reagents to give 20 mM Tris-HCl buffer, pH 8.8, 10 mM KCl, 10 mM $(NH)_4SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each dNTP, 10 pmol of the primers, and 0.5 units of Deep Vent DNA polymerase to a total volume of 25 μL. The PCR program was 30 cycles: 30 s each at 95 and 52 °C and 90 s at 72 °C. The primers used for PCR provided *Nde*I and *Bam*HI restriction endonuclease sites for the 5′- and 3′-end, respectively. The PCR reaction product, 1750 bp, was separated on 1% agarose gels and purified by the Agarose Gel DNA Extraction Kit (Boehringer Mannheim). The isolated DNA was first cloned by blunt end ligation into pT7Blue using the Perfectly Blunt Cloning Kit (Novagen), and then transferred into pT7-7 using the *Nde*I and *Bam*HI restriction endonuclease sites. The plasmids produced were pT7-7(EIst), pT7-7(EIstI16), pT7-7(EIstI17), pT7-7(EIstI34), corresponding to *ptsI* from *S. typhimurium* strains, wild-type, SB1681, SB1476, and SB1690, respectively. I16, I17, and I34 refer to the original allele numbers (*36*). *ptsI* genes containing both the I34 and I16 or I17 mutations were constructed by isolation of the *Eco*RI restriction endonuclease DNA fragment from both pT7-7(EIstI16) and pT7-7(EIstI17) followed by replacement in pT7-7(EIstI34) to produce pT7-7(EIstI16,I34) and pT7-7(EIstI17,I34).

Isolation of E. coli ptsI Gene Fragments. The *E. coli ptsI* gene was amplified by PCR from ∼10 ng of pTSHIC9, which contained *ptsHIcrr* (*37*). The conditions were similar to those described above. The amplified DNA product was ligated into pT7-7 using the *Nde*I and *Bam*HI restriction endonuclease sites to give pT7-7(EIec). The *E. coli ptsI* gene sequence (*11*) was confirmed. The 5′-end gene fragments for the production of the N-terminal portion of enzyme I were amplified by PCR from pTSHIC9 in a similar manner using two 3′-end primers that provided for a *Bam*HI restriction site and termination at residues 220 and 250, respectively. The PCR products were ligated into pT7-7 using the *Nde*I and *Bam*HI restriction endonuclease sites. The two plasmids pT7-7(EIecN219) and pT7-7(EIecN249) produced enzyme I N-terminal fragments from residue 1−219 and residue 1−249, respectively.

The C-terminal fragments, residues 239−575 and 334−575, were cloned as follows. Residue 334 is a methionine, and the codon is part of a *Nco*I site. The fragment *Nco*RI−*Bam*HI was ligated into pET11d vector (Novagen) to give pET11(EIecC334). Residue 239 is also a methionine, and site-directed mutagenesis was used to change the DNA sequence to give a *Nde*I site; the *Nde*I−*Bam*HI fragment was ligated into pT7-7 to give pT7-7(EIec239).

Site-Directed Mutagenesis. Mutants in *ptsI* were created using the Quik Change Site-Directed Mutagenesis Kit obtained from Strategene. The method was as specified by the manufacturer, and the gene or fragment of the gene was fully sequenced to confirm the alteration.

DNA Sequencing. The sequencing was carried out either by use of the Pharmacia Sequenase methodology or using an Applied Biosystems 373 (Stretch) DNA sequencer at the Plant Biotechnology Institute, NRC Canada, Saskatoon. A series of primers was used to sequence *ptsI* in ∼400 base stretches, and both strands were sequenced.

Overexpression of Wild-Type, Mutant Enzymes I and Enzyme I Fragments. The *ptsI* gene or derivatives were induced by the addition of 0.5−1 mM isopropylthiogalactoside to 1.5 L mid-log phase cultures of *E. coli* strain ES238 carrying the appropriate pT7-7 and pET11d derivatives. The

LB media (*38*) was in 6 L Erlenmeyer flasks in a rotary shaking incubator (300 rpm), and induction continued for 3 h at 37 °C.

Purification of Wild-Type and Mutant Enzyme I. Cells were harvested by centrifugation, washed, resuspended, broken by passage through a French pressure cell, and membranes removed by centrifugation as previously described (*7*). The membrane-free crude extract (approximately 150 mL from 30 g wet wt cells) was applied to a 200 mL Q-Sepharose (Pharmacia) column equilibrated with 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, and 0.2 mM dithiothreitol (DTT), and eluted with a 0 to 0.5 M KCl gradient. Enzyme I eluted at ~0.4 M KCl, and SDS−PAGE was used to detect fractions with essentially homogeneous enzyme I protein. Fractions with pure enzyme I were pooled, incubated for 10 min at 37 °C with 5 mM $MgCl_2$ and 2 mM PEP, cooled to 4 °C, and concentrated using an Amicon concentrator with a PM-10 membrane. The preparation was stored at −20 °C following either dialysis against with 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, and 0.2 mM DTT or lyophilization.

Purification of N-Terminal Fragments. The purification was as described for enzyme I with the addition of molecular sieve chromatography on an AcA44 Ultrogel column (5 × 100 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.2 mM DTT, 0.1 M KCl, 5 mM $MgCl_2$, and 20 mM ϵ-aminocaproic acid (a protease inhibitor).

Purification of the C-Terminal Fragments. Unless stated otherwise, conditions for purification were the same as employed for enzyme I. The smaller of the two fragments, C27, (residues 334−575) was found in an aggregate, which was solubilized by 3 M urea, and from this solubilized aggregate, cell membranes could be removed. Further purification by passage through Q-Sepharose, to which the fragment did not bind, yielded a preparation that was about 50% pure. The larger fragment, C37, (residues 239−575) was soluble and was purified by Q-Sepharose chromatography, elution ~0.2 M KCl, and after concentration, by Ultrogel AcA44 gel filtration chromatography. Yields were ~100 mg/30 g (wet wt) cells.

Enzyme I Assays. The sugar phosphorylation assays with α-[^{14}C]methylglucoside for crude extracts or [^{14}C]glucose were used with partial purified or pure enzyme I (*9*). With pure enzyme I, the spectrophotometric assay using coupling to lactate dehydrogenase assay was carried out in 1 mL volumes as described previously (*9*), except that 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesufonic acid (Hepes) buffer, pH 7.0, was used in place of phosphate buffer and 16 mM $(NH_4)_2SO_4$ was included. Sulfate is a competitive inhibitor of HPr leading to an increase in the HPr K_m. Conditions for the preincubation and dilution of enzyme I to maximize dimer formation, stability, and activity were as described previously (*8*, *39*). Briefly, enzyme I at concentrations of >1 mg/mL was preincubated at room temperature or 37 °C in the presence of 5 mM $MgCl_2$ and 2 mM PEP. Dilution of enzyme I, for the purpose of assay, was into 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.2 mM DTT, 5 mM $MgCl_2$, and 2 mM PEP at room temperature. If dilutions were carried out with buffers and protein preparations made sterile by filtration, the dilute wild-type enzyme I preparations were stable for several days at room temperature. If placed on ice, the dilute enzyme I dissociated, and reassociation at higher temperatures did not produce the original activity. All solutions used in the spectrophotometric assay were filtered to remove particles that interfered with the spectrometer readings (at least 0.45 μm filters).

Determination of Enzyme I Kinetic Parameters. The determination of K_m and V_{max} was as described previously (*31*). Analysis was by double reciprocal plots ($1/v$ vs $1/S$) with at least eight substrate concentrations and linear regression was applied. Reproducibility of K_m determinations was within ±10%. It was important to determine V_{max} values on enzyme I freshly prepared from either frozen or lyophilized preparations (*7*, *9*, *39*).

Molecular Sieve Chromatography. Chromatography was carried out using a Pharmacia GradiFrac at either room temperature or in a cold room (4 °C). For the N-terminal fragments, a Sephadex-75 column equilibrated with 50 mM potassium phosphate buffer, pH 7.5, with 0.1 M KCl, 1 mM EDTA, and 1 mM DTT was used. For the enzyme I mutants, the Sephadex-75 column was used at 4 °C and a Sepharose-12 column was used at room temperature for which 0.02% sodium azide was added to the buffer. Samples (0.1 mL) of enzyme I preparations (2 mg/mL) at the appropriate temperature were applied and eluted at 0.2 mL/min, and 0.5 mL fractions were collected when necessary. The column was calibrated for each set of experiments using the following standards: Blue Dextran, 2×10^6 Da; enzyme I, 127 000 Da; ovalbumin, 45 000 Da; soya bean trypsin inhibitor; 21 000 Da. Protein elution was detected at 280 nm.

Determination of the Pyruvate Phosphate Dikinase Subunit Interaction Site. The coordinates for the *Clostridium symbosium* pyruvate phosphate dikinase structure (*19*) are referenced as 1DIK. The methods used for the determination of the site of interaction have been described previously (*40*, *41*).

Western Blotting. The monoclonal antibody specific for *E. coli* enzyme I, Jel45 was selected using enzyme I purified from *E. coli* strain P650 (*7*). The isolation of the hybridoma cell line, production, and purification of the monoclonal antibody was identical to that described for HPr-specific monoclonal antibodies (*42*), except that enzyme I replaced HPr. Western blot experiments were performed using a Bio-Rad Minigel apparatus for protein separation by SDS−PAGE and subsequent transfer onto nitrocellulose filters. Detection was by standard methods using powdered milk to block the filter and horseradish peroxidase conjugated goat anti-mouse antibodies were used to detect bound Jel45.

Other Methods. General molecular biological methods were as described by Sambrook et al. (*38*). SDS−PAGE and autoradiography of [^{32}P]phosphoproteins containing phosphohistidines have been described (*43*). Pure HPr, enzyme I, C-terminal, and N-terminal fragment protein concentrations were determined using the method of Waddell (*44*); $A_{215nm} - A_{225nm} = 1A = 0.14$ mg/mL.

RESULTS

Properties of the S. typhimurium ptsI Strains. Mutants of *ptsI*, from *S. typhimurium* have been ordered by deletion mapping (*36*). Two of the mutants, strains SB1476 and SB1681, produced enzyme I with "leaky" activity as

Table 1: Characterization of *S. typhimurium ptsI* Mutations

enzyme I strain, allele	DNA sequence change	protein sequence change
SB1681, *ptsI16*	residue 1066 G → A	Gly356 → Ser
SB1476, *ptsI17*	residue 1123 C → T	Arg375 → Cys
SB1690, *ptsI34*	residue 376 C → T	Arg126 → Cys

determined by assays of crude extracts, and their significant physiological effects in respect to PTS sugar transport and regulation have been described (*35*). Subsequently, it was determined that the enzyme I in crude extracts from strains SB1476 and SB1681 did not form dimers during molecular sieve chromatography at room temperature, and the low activity of these mutants could be complemented in vitro by the addition of enzyme I in the crude extracts of either strain SB1690 or SB2227. These latter strains had no detectable enzyme I activity, but did dimerize during molecular sieve chromatography (unpublished results; E. B. Waygood, J. C. Cordaro, and S. Roseman referred to in ref *8*).

Identification of the S. typhimurium Mutations. The *ptsI* genes were isolated and cloned from *S. typhimurium* strains SB1476, SB1681, SB1690, and the wild-type strain SB3507 by amplification of the chromosomal *ptsI* DNA using the polymerase chain reaction as described in the Experimental Procedures. Both strands of the cloned genes were sequenced, and single point mutations were identified (Table 1). The wild-type gene sequencing confirmed the published sequence (*13*). The *ptsI39* gene from strain SB2227 has resisted cloning attempts. The mutant enzymes I will be identified as Gly356Ser, Arg375Cys, and Arg126Cys (Table 1) in this paper.

Selection and Characterization of Strain ESK238. To obtain pure *S. typhimurium* enzyme I from the *ptsI* gene in the pT7-7 vector, chromosomal production of wild-type *E. coli* enzyme I was eliminated. *E. coli* strain ESK238 is a *ptsI* derivative of strain BL21, which is a common host for pT7-7 and was selected by streptozotocin resistance (*34*) as described in Experimental Procedures. In this strain, no enzyme I activity was detected by sugar phosphorylation assays. On autoradiographs following SDS−PAGE, no [^{32}P]-PEP-dependent [^{32}P]phosphoproteins could be detected at 63 kDa, i.e., enzyme I, nor any of the other PTS proteins, the phosphorylation of which is dependent upon enzyme I. No enzyme I protein was detected by western blotting using the monoclonal antibody Jel45, which is specific for enzyme I. Other PTS proteins, HPr and IIA^{glucose}, were normal as detected by activity or by [^{32}P]phosphoproteins following the addition of active enzyme I to a crude extract of strain ESK238.

Temperature-Dependent Dimerization. Wild-type and mutant enzymes I overexpressed efficiently and were purified as described in the Experimental Procedures yielding 50−100 mg of homogeneous protein/L of culture. SDS−PAGE showed that all preparations had single bands, $M_r \approx 63$ kDa, and displayed a monomer at $M_r \approx 70$ kDa as determined by nondenaturing molecular sieve chromatography at 4 °C. (The *S. typhimurium* and *E. coli* monomer M_r deduced from the gene sequences are 63.2 and 63.5 kDa, respectively). Molecular sieve chromatography at room temperature showed that Arg126Cys enzyme I formed dimers in a manner similar

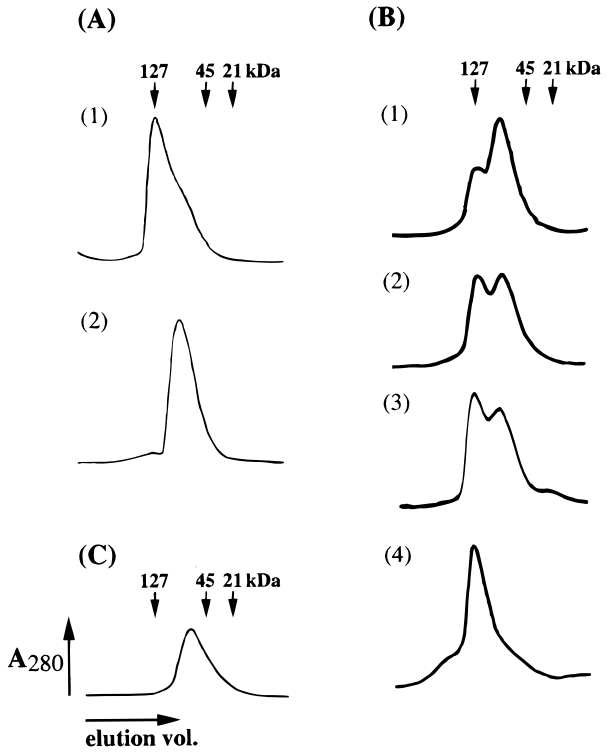

FIGURE 1: Molecular sieve chromatography at room temperature. Proteins were eluted from a Sepharose 12 column calibrated with standards of 127, 45, and 21 kDa as indicated. (A) (1) Arg126Cys enzyme I eluted at ~127 kDa; (2) Gly356Ser enzyme I eluted at ~85−90 kDa. (B) Different ratios of Arg126Cys and Gly356Ser enzymes I were mixed in 0.1 mL on ice, and then equilibrated to room temperature, loaded and eluted. Ratios: (1) 1:1; (2) 4:1; (3) 7:1; all with the column equilibrated with buffer; (4) 4:1, column equilibrated with buffer with 10 mM PEP and 5 mM $MgCl_2$. (C) C37 C-terminal fragment eluted at 75 kDa, the position expected for a dimer. Detection was at 280 nm; peak height maximum ~0.1 A except for (B4) which was ~0.15 A and (C) which was ~0.05 A.

to wild-type enzyme I, 127 kDa, while Gly356Ser enzyme I had impaired dimerization leading to an elution peak of about 85−90 kDa. (Figure 1A). Arg375Cys enzyme I was susceptible to proteolytic degradation at room temperature, giving some protein elution corresponding to lower molecular weights. However, detectable activity corresponding to a broad peak eluting at about 70−85 kDa was found (results not shown).

Kinetic Properties. The enzyme I reaction, HPr + PEP ↔ P−HPr + pyruvate, was measured by a spectrophotometric assay in which pyruvate production is coupled to lactate dehydrogenase. Enzyme I preparations were kept phosphorylated and at room temperature, which are conditions that lead to maximal activity (*8*, *9*, *39*). All three mutants of enzyme I had HPr K_m values that were identical to wild-type (Table 2). Gly356Ser and Arg375Cys enzymes I had PEP K_m values that were about 30-fold higher than wild-type, while Arg126Cys enzyme I had a PEP K_m that was 10-fold lower than wild-type. Gly356Ser and Arg375Cys enzymes I V_{max} were 4 and 2% of wild-type as expected for "leaky" mutants. Arg126Cys V_{max} was 0.04% of wild-type, which is consistent with the absence of detectable activity in strain SB1690 crude extract. The measurement of these K_ms was not affected by instability in the assays as described below for the activity of heterodimers. As all the enzyme I

Table 2: Kinetic Properties of Enzyme I Mutants

enzyme I	HPr K_m (μM)	PEP K_m (mM)	V_{max} (μmol/min/mg)	V_{max} (%)
wild-type	12[a]	0.10	159	100
Gly356Ser	11	3.0	7.0	4.4
Arg375Cys	12	3.1	3.0	1.9
Arg126Cys	12	0.01	0.08	0.04
Gly356Ser + Arg126Cys[c]	12	<0.01[b]	40	25
Arg375Cys + Arg126Cys[c]	12	<0.01[b]	5	3
Gly356Ser + C37[c,d]	12	<0.01[b]	34	21

[a] HPr K_m values have been previously reported as 4-6 μM (*7*, *8*, *31*, *37*). In these assays, 16 mM $(NH_4)_2SO_4$ was included. Sulfate is a competitive inhibitor of HPr (E. B. Waygood, unreported results) and the increased K_m to 12 μM meant that higher HPr concentrations could be used for K_m determinations, removing some of the practical difficulties associated with measurements at very low micromolar concentrations. [b] These are estimates. Activity at lower PEP concentrations was dependent upon a state of association (see text). [c] The protein ratios were 2:1; Gly356Ser or Arg375Cys were at 1. [d] C37 is the C-terminal fragment.

mutants have some activity, incubation with [^{32}P]PEP gave [^{32}P]P-enzyme I which was detected by SDS−PAGE and autoradiography (results not shown).

In Vitro Intragenic Complementation. The assay conditions used for complementation included 30 μM HPr and 20 mM PEP, which gave near maximal activity for all the enzymes I. To demonstrate effective in vitro intragenic complementation between the pure enzyme I mutant proteins, Arg126Cys must be mixed with either Gly356Ser or Arg375Cys while on ice. This temperature leads to monomer formation. The mixtures were then incubated at room temperature (i.e., the condition that should lead to both homodimer and heterodimer formation) before introduction into the assay. The in vitro intragenic complementation between Arg126Cys enzyme I and either Gly356Ser or Arg375Cys enzymes I is shown in Figure 2. The amounts of Arg126Cys enzyme I, which were used to stimulate Gly356Ser or Arg375Cys enzymes I, had no detectable enzyme I activity during the typical time course of the measurement. The extent of stimulation of the leaky activities was greater for Gly356Ser enzyme I. In both cases, not only was there a change in V_{max}, but the K_m for PEP was altered (Table 2). If the complementation (Figure 2) was carried out at 1 mM PEP, which resulted in very little activity for Gly356Ser or Arg375Cys enzymes I due to the differences in PEP K_ms (Table 2), the extent of stimulation was about 30- and 10-fold, respectively. In contrast, when Arg126Cys enzyme I was mixed with Gly356Ser or Arg375Cys enzyme I at room temperature, no complementation was observed in assays containing 20 mM PEP, because the dimers (or at least Arg126Cys enzyme I) did not dissociate. Further, if following the room temperature mixing, the assay was allowed to proceed for as long as 30 min and no complementation was observed. This suggests that, at saturating PEP concentrations, no dissociation of the subunits occurs during enzyme turnover which could lead to heterodimer formation.

The activity of the heterodimers was influenced by the association/dissociation properties of the subunits during the course of the assay. At saturating 20 mM PEP concentrations, enzyme I activity for the complementing mixtures of

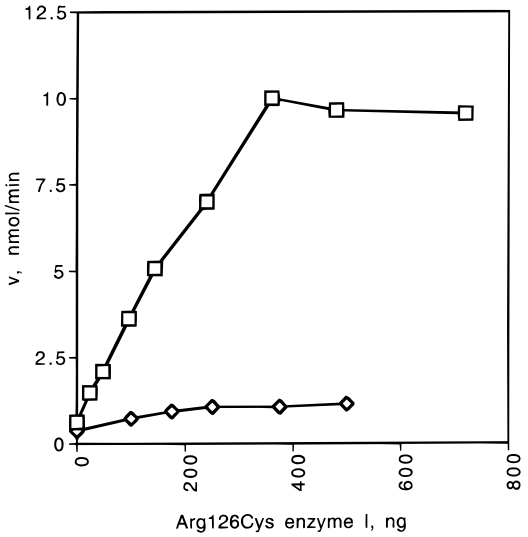

FIGURE 2: In vitro complementation. Gly356Ser and Arg375Cys enzymes I (50 μg/mL) were preincubated on ice with increasing amounts of Arg126Cys enzyme I as described in Experimental Procedures. These mixtures were equilibrated at room temperature, and then 250 ng Gly356Ser (□) or Arg375Cys (◇) enzymes I with Arg126Cys enzyme I were assayed with 30 μM HPr and 20 mM PEP. The amounts of Arg126Cys enzyme I added to the assay did not yield any measurable activity.

Arg126Cys enzyme I with either Gly356Ser or Arg375Cys enzymes I was stable, and was similar in this respect to wild-type enzyme I. At lower PEP concentrations, the presentation of the activity for the heterodimers was remarkably different (Figure 3A). The heterodimer was unstable as activity declined much more rapidly than would be accounted for by substrate utilization. In the presence of Arg126Cys enzyme I, the PEP K_m for both Gly356Ser and Arg375Cys enzymes I improved from 3 mM to <10 μM (Table 2). However, the rapid decline in activity of the heterodimer in the assay, demonstrated in Figure 3A, was so pronounced at lower PEP concentrations, the initial velocity measurements could not be reliably obtained. Thus, the PEP K_m (Table 2) determined for the heterodimer is at best an estimate, and may reflect the state of subunit association more than the direct effect of PEP binding with the active site.

A simple experiment was performed to ascertain whether the decline in activity of the heterodiners was due to subunit dissociation following the dilution into the assay mix or whether catalytic turnover was needed. If the heterodimers were diluted into the assay mixture with 20 mM phsopho-enolpyruvate and no HPr and incubated for 10 min, no significant loss of the initial enzyme activity was observed when HPr was added (Figure 3B). This suggests that, at high PEP concentrations, dilution of the heterodimer does not cause dissociation. However, if the same procedure was done with 1 mM PEP, the heterodimer initially had no activity after HPr addition. The activity improved over a few minutes to give a stable velocity at a much reduced rate. When the heterodimer was added to an assay with HPr present, maximal initial velocity was observed immediately, but this activity was unstable and declined rapidly. This indicates that the association is influenced by PEP concentration and that at lower PEP concentrations, dilution causes dissociation. Turnover did not appear to be a major influence.

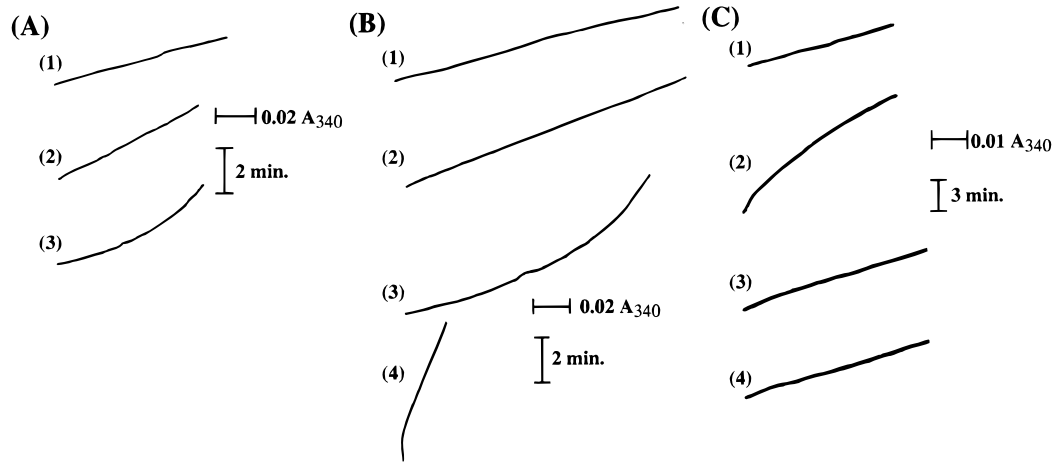

FIGURE 3: Enzyme I activity in an assay. The recording chart traces, for the ΔA at 340 nm of enzyme I activity, demonstrate. (A) Heterodimer instability: 50 ng of wild-type enzyme I (1), 2 μg of Gly356Ser enzyme I (2), and the Gly356Ser/Arg126Cys heterodimer, 250 ng of each subunit (3). Assays contained 30 μM HPr and 1 mM PEP. (B) Effect of dilution: the heterodimer (250 ng of each subunit) activity was measured with 20 mM PEP immediately (1) and after 10 min in the assay without HPr, which was then added to start the reaction 2; or with 1 mM PEP immediately (3) and after 10 min without HPr (4). (C) Effect of the temperature of preincubation. The recording chart traces of the initial velocity of 50 ng of wild-type enzyme I preincubated at room temperature (1) or on ice (2); 2 μg of Gly356Ser enzyme I at room temperature (3) or ice (4). Arg375Cys enzyme I had similar behavior to Gly356Ser enzyme I.

To attempt to show an active heterodimer eluting from a molecular sieve column, Gly356Ser and Arg126Cys enzymes I were mixed on ice and then equilibrated to room temperature using the same preincubation conditions as employed for the assays before application to the column. When activity measurements were made, the activity was found at a position about midway between the elution positions of Arg126Cys enzyme I (127 kDa) and Gly356Ser enzyme I (85−90 kDa). This result would occur when the trailing and leading edges of the two enzymes mix in fractions. However, the distribution of protein was different from expectations. In Figure 1B, the protein profiles for the Arg126Cys enzyme I and Gly356Ser enzyme I mixtures at 1:1, 4:1, and 7:1 ratios are shown. The effect of mixing Arg126Cys enzyme I with Gly356Ser enzyme I was to cause more of the protein to elute at the 85 kDa position than at the 127 kDa position at which Arg126Cys enzyme I should be found as a dimer (Figure 1A). At 1:1, little of the protein was found at 127 kDa, and at 7:1, the amount of dimer was only slightly greater than a peak at the lower molecular weight. We interpret this to mean that the association of Gly356Ser protein with Arg126Cys protein leads to a dynamic process of association and reassociation during the 3 h that it took the proteins to elute, the net effect of which is the propensity toward a lower molecular weight. This indicates that heterodimers do form. Because the assay results described above indicated that high PEP concentrations stabilize the heterodimers, the molecular sieve column was equilibrated with buffer containing 10 mM PEP and 5 mM $MgCl_2$. Under this condition, more protein eluted at 127 kDa (Figure 1D), but the enzyme I activity was still in an intermediary position (result not shown).

Are Gly356Ser and Arg375Cys Enzymes Active as Dimers or Monomers? The consequences of dilution and temperature in respect to enzyme I activity are shown in Figure 3C. When wild-type enzyme I was diluted at room temperature and subsequently measured at room temperature, a stable initial velocity was observed. If the dilution was on ice, without any room-temperature equilibration, the subsequent initial activity was slow and increased as the assay proceeded. Typically, small volumes (5−50 μL) of enzyme I were added to a 1 mL assay at room temperature, and thus temperature equilibration of the enzyme I should have occurred during the mixing of the cuvette. Thus, the increase in activity is consistent with a slow, hystertic association of subunits, and the maximal velocity obtained was substantially less than found when the same amount of warm enzyme I was added to the assay. The protein concentrations of enzyme I subunits associating in a preincubation mixture (50 μg/mL) was 200-fold greater than in the assay (0.25 μg/mL). The higher activity obtained with warm enzyme I suggests that turnover at high PEP concentrations does not cause dissociation of preformed dimers, because such should result in an activity of equal magnitude to that obtained from association of the cold enzyme I subunits in the assay.

This behavior of a slow increase in activity has been accepted as the formation of active enzyme I dimers. For Gly356Ser and Arg375Cys enzymes I, the lower V_{max} values (Table 2) could be due to either of the following: activity associated with the monomer or activity associated with a small population of dimers. When the same preincubation treatments, cold and warm, were applied to Gly356Ser or Arg375Cys enzymes I, the measured activity showed no dependence upon the preincubation condition, i.e., no slow increase in activity due to dimer formation (Figure 3C). This concurs with the proposal that the activity measured in these two mutants is that of the monomeric form of enzyme I.

Double Mutation Is Inactive. Both the Arg126Cys and Gly356Ser mutations were constructed in *S. typhimurium ptsI* by isolation of restriction endonuclease fragments and ligation. The overexpressed protein with two "complementary" mutations in the same subunit was purified to homogeneity. The activity of the double mutant protein was poorer than Arg126Cys enzyme I. Kinetic constants were not obtained. The enzyme I protein with the double mutation could not complement any of the enzymes I containing a single site mutation. These results confirm that the complementation requires the interaction of two different subunits.

Mutations in E. coli ptsI. Enzyme I from either *E. coli* or *S. typhimurium* have essentially the same properties and are

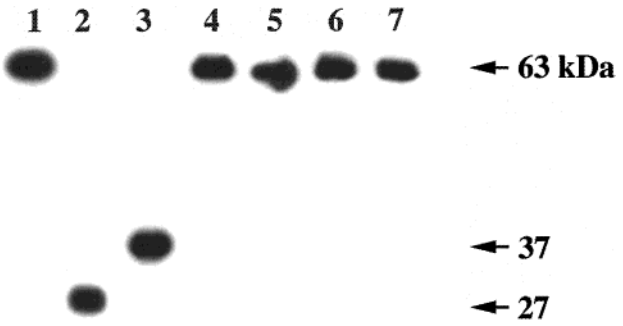

FIGURE 4: Enzyme I immunoreactivity. Enzyme I wild-type, mutant enzymes I, and the C-terminal fragments were separated by SDS−PAGE, electroblotted onto nitrocellulose filters and reacted with the enzyme I-specific monoclonal antibody, Jel45. Lane 1, *E. coli* wild-type; lane 2, C27 C-terminal domain from *E. coli*; lane 3, C37 domain from *E. coli*; lane 4, *S. typhimurium* Gly356Ser; lane 5, *S. typhimurium* Arg375Cys; lane 6, *S. typhimurium* Arg126Cys; lane 7, *S. typhimurium* wild-type. The monoclonal antibody was selected against *E. coli* enzyme I. The amount of protein in each lane was 50 ng.

used interchangeably. Their amino acid sequences differ in 16 conservative substitutions out of 575 residues (*13*). Mutations identical to the *S. typhimurium* Arg126Cys(*ptsI34*) and Gly356Ser(*ptsI16*) were constructed in *ptsI* from *E. coli* by site-directed mutagenesis. Although not purified to homogeneity, the in vitro complementation was observed in sugar phosphorylation PTS assays (*9*) and it was similar to that observed for the *S. typhimurium* enzyme I mutants under the same conditions of assay and purity.

N-Terminal Fragments Do Not Stimulate. Two N-terminal fragments, N24, residues 1−219 (M_r 23.8 kDa) and N27, residues 1−249 (M_r 27.2 kDa) of *E. coli* enzyme I were isolated as described in the Experimental Procedures. These fragments are smaller than N-terminal fragments, residues 1−258 and 1−269 described by others (*13*, *16*, *17*), and these smaller fragments can be phosphorylated by P−HPr, but not PEP, as shown for other N-terminal fragments. The molecular masses determined by molecular sieve chromatography were N24, 43 kDa (4 °C) and 44 kDa (23 °C); N27, 36 kDa (4 °C) and 40 kDa (23 °C), which is consistent with an elongated ellipsoid monomer revealed by the structures of the 1−258 fragment (*16*, *17*). A similar aberrant molecular mass, 35 kDa, and the lack of dimerization for the 1−269 fragment have been reported (*13*). When either of the two *E. coli* N-terminal fragments were added to Arg126Cys, Gly356Ser, or Arg375Cys enzymes I of *S. typhimurium*, they did not cause any stimulation of activity.

C-Terminal Fragments. Two C-terminal fragments of enzyme I from *E. coli*, residues 334−575, C27 (M_r 27.6 kDa) and 239−575, C37 (M_r 37.6 kDa), were cloned and expressed as described in the Experimental Procedures. Both fragments began with methionines found in the enzyme I sequence. C27, although it was expressed well, was found to aggregate. The aggregation made purification difficult and C27 was purified to about 50% homogeneity. The C37 was purified to homogeneity. Both C27 and C37 react with the enzyme I specific monoclonal antibody Jel45 (Figure 4), while the N-terminal does not (results not shown). C27 does not contain all of the conserved essential residues of the PEP-binding site as determined by the pyruvate phosphate dikinase structure (*19*) and sequence comparisons (*18*); residues 296 and 332 and associated sequences are missing (Figure 5). C27 did not complement any mutant enzyme I, nor did C27 appear to dimerize.

The C37 C-terminal domain can replace Arg126Cys enzyme I in the complementation of Gly356Ser and Arg375Cys enzymes I in a manner similar to that shown in Figure 2, and kinetic values for C37 C-terminal domain and Gly356Ser were obtained (Table 2). The instability of the heterodimer in the assay was also found. C37 C-terminal domain, when mixed with either N-terminal fragment, did not have measurable activity in the spectrophotometric assay. The C37 C-terminal domain elutes from room-temperature molecular sieve column corresponding to the size of the dimer (Figure 1C). These results show that the C-terminal domain, which binds PEP, has all the necessary properties to cause dimerization as would be predicted from the pyruvate phosphate dikinase structure (*19*).

DISCUSSION

The results presented in this paper involve experiments with enzyme I from both *S. typhimurium* and *E. coli*. The mutations described in *S. typhimurium* have been reproduced in *E. coli* and show similar properties, and the highly conserved sequence homologies (*18*) suggest that information about the enzyme from one species is the same for the other species.

Structure of Enzyme I. The structure of pyruvate phosphate dikinase (ref *19* and Figure 6A) and the sequence homology with enzyme I (ref *18* and Figure 5) indicate that the two subunits of enzyme I act independently in the sense that the active-site components do not come from two different subunits. To accomplish sugar phosphorylation and translocation, PEP binding at the C-terminal domain of enzyme I leads to the phosphorylation of His189 (Figure 6B) and phosphoryl transfer following HPr binding at the N-terminal domain of the same subunit. While the N-terminal domain of pyruvate phosphate dikinase has about 300 extra residues and little sequence homology with enzyme I, the phosphohistidine domain and the C-terminal PEP-binding domains are very similar (ref *18* and Figure 5). The C-terminal PEP-binding domain in pyruvate phosphate dikinase consists of an α/β-barrel with the binding site in the center of the barrel. In the dimer, the α/β-barrels bind to each other side-by-side, at right angles to each other. The subunit interaction sites in pyruvate phosphate dikinase are highly conserved in enzyme I (*18*, Figures 5 and 7). A structural homologue of the α/β-barrel is found for the PEP-binding site in pyruvate kinase (*19*). Many pyruvate kinases are stable tetramers, however, the ribose 5-phosphate-activated pyruvate kinase from *E. coli* has a reversible association/dissociation between dimers and tetramers (*45*). Together, the pyruvate phosphate dikinase structure and the N-terminal fragment structures of enzyme I (*14*, *15*) provide a putative structure of enzyme I.

Role of Arg126 in Phosphoryl Transfer. The residue, Arg126, is located in the HPr-binding domain of enzyme I on the other side of a cleft from the active-site His189 (Figure 6B). Arg126 is conserved in enzyme I (*18*), but had not been identified as a residue involved in HPr binding nor had any particular role been suggested for it from other structural evidence (*14*, *15*, *46*). However, more recently, Garrett et al. (*47*) have determined the structure of the complex of enzyme I N-terminal domain with HPr by multidimensional

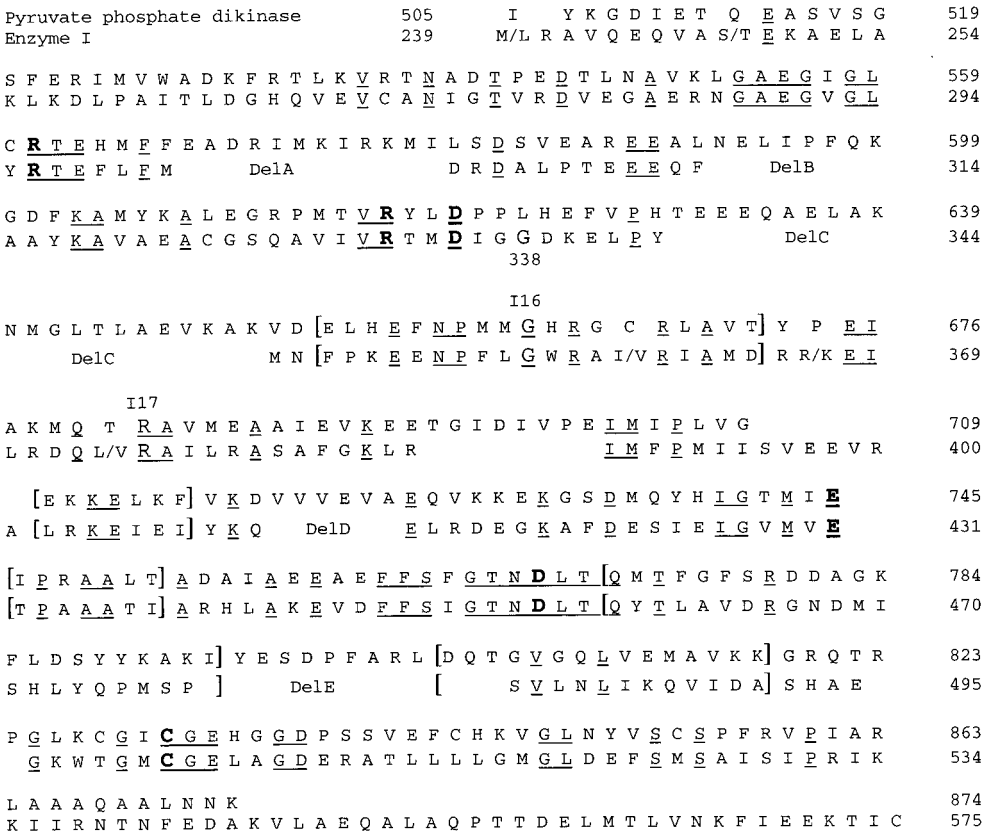

FIGURE 5: PEP-binding domain sequence alignment. The sequence alignment between *C. symbiosum* pyruvate phosphate dikinase and *E. coli* enzyme I PEP-binding domains was generated by alignment of all currently known enzymes I, pyruvate phosphate dikinase, and PEP synthase sequences. The sequence deletions, Del A−E, in the alignment are identified in red in the structure of the pyruvate phosphate dikinase C-terminal domain (Figure 7). Between residues 685/387 and 710/402 two offsetting deletions are found; these sequences correspond to a helix that terminates in DelD (Figure 7). The enzyme I sequences from *E. coli* and *S. typhimurium* differ at five residues and are indicated for example as *M*/*L*, respectively. Residues conserved between enzyme I and pyruvate phosphate dikinase are underlined; bolded residues are those identified to be involved in phophoenolpyruvate binding (*19*); residues in the square brackets are those involved in the subunit interaction site in pyruvate phosphate dikinase; I16 and I17 identify the residues that are mutated: Gly356Ser and Arg375Cys, respectively. 338 identifies the glycine residue which when mutated impairs dimerization (*52*).

NMR. In this structure, enzyme I Arg126 side chain was found to be hydrogen bonded to the backbone carbonyl of Leu14 and the side chain of Gln51 of HPr. The mutant Arg126Cys enzyme I has a wild-type K_m for HPr, but the V_{max} is greatly affected (Table 2), which suggests that Arg126 has a significant role in the phosphotransfer between His189 of enzyme I and His15 of HPr. Prior to the report of the HPr−enzyme I complex (*47*), in which neither enzyme I His189 nor HPr His15 is phosphorylated, we concluded that Arg126 was a good candidate for stabilizing phosphorylated His189 rather than the nonconserved Lys69 that has been proposed (*48*). It would be surprising that Arg126Cys mutation does not affect HPr K_m if Arg126 has a significant role in HPr binding rather than catalysis. Thus, the possibility that Arg126 has a different interaction in the presence of a P-histidine(s) at the active site must be considered. The mutation to Arg126 does not affect the dimerization, which is expected from both the properties of isolated N-terminal fragments reported here and elsewhere (*13*, *49*) in that they have they have no dimerization potential, and from the structure of the homologue, pyruvate phosphate dikinase (*19*).

Roles of Gly356 and Arg375. The structure of the C-terminal region of enzyme I is not known; however, the structure of the homologous pyruvate phosphate dikinase is described (*19*). This structure was examined to gain insights into the locations of the residues homologous to Gly356 and Arg375. The interaction site between the subunits of pyruvate phosphate dikinase was determined from the tertiary structure using a standard method (*40*, *41*), and the information about these residues and the contacts is summarized in Table 3. The residues involved in subunit interaction form a set of five sequences (Figure 5), which are conserved between enzyme I and pyruvate phosphate dikinase (*18*). The locations of these sequences on the C-terminal domain of pyruvate phosphate dikinase are shown in Figure 7 (yellow and amber; amber residues conserved), and while the interaction sequences are not part of the PEP-binding site (purple residues), they are clearly very close in the structure. Sequence 1 (Table 3), containing Gly663 (Gly356 in enzyme I), is at the axis of 2-fold symmetry between the subunits in the crystal (*19*). Residues in this sequence interact with residues from both sequence 1 and sequence 4 of the other subunit. Several residues interact with the same residue in the other subunit (called "self-interacting" in Table 3). In the sequence alignment of enzyme I and pyruvate phosphate dikinase C-terminal domain, five deletions in enzyme I are found (Figures 5 and 7). These are identified by red in the structure of the C-terminal domain (Figure 7), and removal of these sequences, followed by energy minimization (*50*), suggested only one significant structural change. Deletion E leads to both a movement and local disruption of the structure on either side of the deletion, thus affecting the conserved

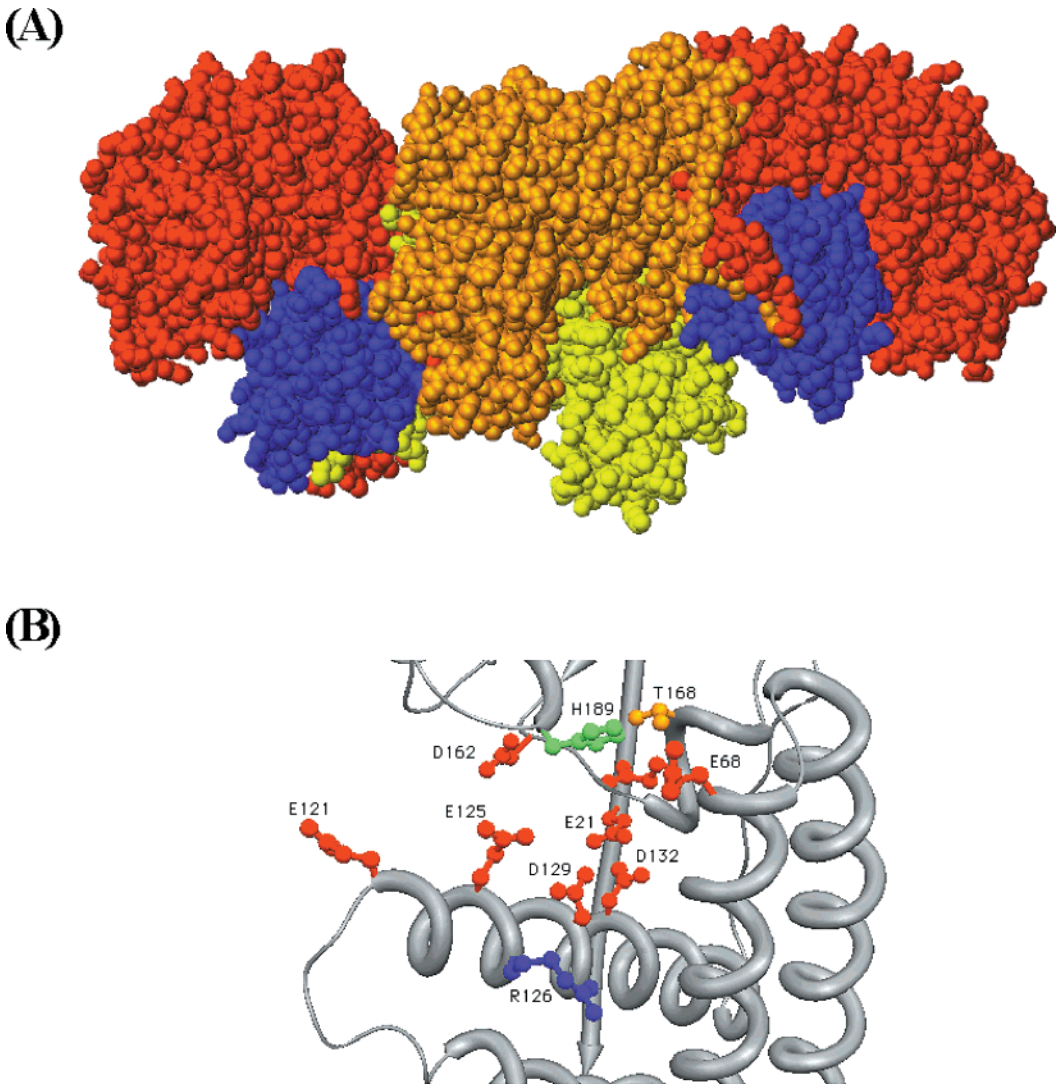

FIGURE 6: Pyruvate phosphate dikinase and enzyme I N-terminal domain structures. (A) The space-filling structure of the dimer of pyruvate phosphate dikinase: ATP-binding domain (residues 1−400), red; P-histidine domain (residues 401−504), blue; the interacting PEP-binding domains (residues 505−874), amber and yellow. Linker regions between each domain are not delineated in this presentation. (B) Enzyme I N-terminal domain active site. The His189 (green) active-site region from the structure of the N-terminal domain of *E. coli* enzyme I. The view presented is the same as shown previously (*14*) except that Arg126 is identified (blue). Glu167, a residue near His189 in the figure, is not labeled due to crowding. Acidic residues are red; Thr168 is amber.

subunit-binding site. In this analysis, none of these deletions has a significant affect on the conserved residues of the PEP-binding site in pyruvate phosphate dikinase (*19*). The equivalent mutations in pyruvate phosphate dikinase, Gly663Ser and Arg682Cys, to the enzyme I mutations, Gly356Ser and Arg375Cys, were examined by inspection and energy minimization, and neither revealed explanations about why these changes would disrupt the interaction of subunits in pyruvate phosphate dikinase. The location of the Gly356Ser mutation in one of the conserved subunit interaction sequences provides a potential explanation for interference in the dimerization process, but clearly, the details of the enzyme I structure will be needed.

The Arg375Cys mutation, which is also impaired in dimerization and activity, is more difficult to understand. The alignment in Figure 5 identifies the enzyme I Arg375 as a conserved residue equivalent to Arg682 in pyruvate phosphate dikinase; the structural location is indicated in Figure 7. Arg682 in pyruvate phosphate dikinase is in neither the site of subunit interaction site nor the PEP-binding site.

The low activity of Gly356Ser and Arg375Cys enzymes I could arise from two events: the activity could be due to the residual monomer activity or a small population of active dimers. No indication of instability in a kinetic assay of Gly356Ser and Arg375Cys enzymes I was found, (Figure 3A), nor was there any temperature dependence for preincubation (Figure 3C) in contrast to the behavior of the heterodimers and wild-type enzyme I. These results indicate that the activities of Gly356Ser and Arg375Cys enzymes I are from the monomers, which have nearly identical PEP K_ms of 3.0 and 3.1 mM. These mutant enzymes I fail to dimerize properly (Figure 1B); the mutations are in distinctly different locations in the C-terminal, yet they produce the same kinetic effect. These results are compatible with the conclusion that the activity of Gly356Ser and Arg375Cys enzymes I comes from active monomers.

Mechanism of in Vitro Complementation. The in vitro complementation described in Figure 2 and Table 2 requires the mixing of Arg126Cys enzyme I with either of the other two mutant enzymes I under the conditions that lead to

Table 3: Summary of the Subunit Site of Interaction in Pyruvate Phosphate Dikinase

description	sequence grouping				
	1	2	3	4	5
residues in PPDK[a]	654−672	710−716	746−752	772−794	804−818
residues in enzyme I	347−365	403−409	432−438	458−479[b]	480−491
no. of residues making contacts	13	4	5	15	7
conserved residues[c]	7	2	3	2	2
self-interacting residues[d]	Phe658 Asn659 Met662			Met773	Met814
interacting sequence number[e]	seq 1 and 4	seq 4	seq 4 and 5	seq 1, 2, and 3	seq 3 and 5
no. of vdw[f]	98 (10)	15	43	117 (6)	18 (1)
no. of H-bonds[f]	12 (1)	1	2	9	5
no. of ion pairs[f]			3		3

[a] PPDK, pyruvate phosphate dikinase. [b] The alignment of enzyme I with *C. symbiosum* pyruvate phosphate dikinase is uncertain after residues ~470 and 784, respectively. The sequences of all the enzymes I show a deletion in this part of the sequence. [c] Residues conserved between *C. symbiosum* pyruvate phosphate dikinase and enzyme I in the interaction sequences. [d] Residues in each subunit which interact with the identical residue. [e] Does not include "self-interacting" residues. [f] Except for the bonds involving the "self-interacting" residues, each bond reported is given twice, once in each sequence. The numbers in parentheses are those involving the "self-interacting" residues. The total number of interactions: van der Waals, 154; H-bonds, 15; ion pairs, 3. Full details are available as Supporting Information.

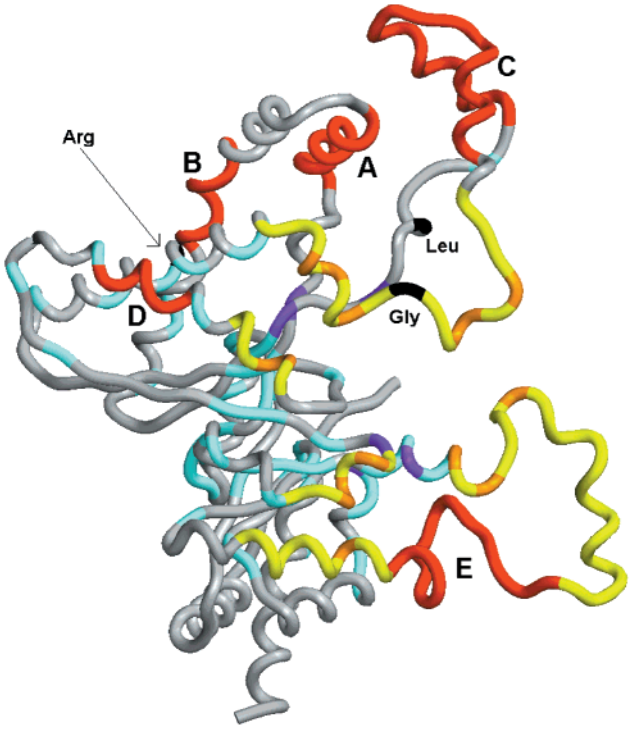

FIGURE 7: Pyruvate phosphate dikinase C-terminal domain. The structure of the C-terminal domain of pyruvate phosphate dikinase beginning at residue 505. The residues color coded in this structure are identified in Figure 5. Purple, conserved residues that are part of the PEP binding site (*19*); yellow, residues that are in the subunit interaction sequences and amber are those conserved in enzyme I and pyruvate phosphate dikinase; pale blue, other residues that are conserved in the two enzymes; red, sequences A−E are residues that are deleted in enzyme I; black, are the residues of characterized mutants, Gly is Gly356 (I16), Arg is Arg375 (I17), and Leu is Gly 338 in enzyme I.

Arg126Cys enzyme I forming monomers and then reassociating to form a mixture of heterodimers with the other mutant enzyme I subunits, homodimers of Arg126Cys subunits, and presumably monomers of the other mutant enzyme I. The improvement in activity (Figure 2) and kinetic parameters (Table 2) must come from the heterodimers. The heterodimers are not as stable as the homodimers of either wild-type or Arg126Cys enzyme I as demonstrated by activity assays (Figure 3) and the molecular sieve chromatography (Figure 4B). It is clear that high PEP concentrations help to stabilize the heterodimer.

A priori, the easiest way by which in vitro complementation between subunits could be explained would be an active site shared between two subunits. The structure of the homologue pyruvate phosphate dikinase (Figure 6A) suggested that this is not the case for enzyme I. Complementation relies upon dimerization, suggesting that the wild-type C-terminal domain of Arg126Cys enzyme I causes dimer formation with the two mutant enzymes I resulting in a conformational change that improves PEP binding (lower K_m). This event leads to activity through the subunits of either Gly356Ser or Arg375Cys enzyme I. This proposal is confirmed by the cloning of the C-terminal domain of enzyme I, which dimerizes readily (Figure 1C) and complements either Gly356Ser or Arg375Cys enzyme I (Table 2) in a manner identical to Arg126Cys enzyme I. A general conclusion is that any mutation in the first 250 residues of enzyme I, that does not interfere with protein production or folding, should be able to complement either Gly356Ser or Arg375Cys enzymes I.

Others have shown that Gly338Asp mutation in *E. coli* enzyme I yields an inactive enzyme (*51*) which can be complemented by the His189Ala mutation in another enzyme I subunit or by wild-type (*52*). The analysis of the complementation assumed that the K_A for the homodimer subunits is the same in the heterodimer, but subsequently, Gly338Asp enzyme I was reported to have impaired dimerization (*49*). The impairment to dimerization of Gly338Asp enzyme I subunits is probably similar to the properties described here. When the residues identified as deletion C in pyruvate phosphate dikinase (Figure 5) are missing in enzyme I, Gly338 would lead almost directly to the conserved subunit interaction sequence that contains the conserved Gly356 residue described in this paper. Gly338 is identified in black as Leu, the residue found in pyruvate phosphate dikinase (Figure 7).

With the assay procedures described in this paper, we were not able to observe complementation between the separately cloned N-terminal and the C-terminal in contrast to the recently reported complementation by similar domain con-

structs. The methodology was different: [^{32}P]phosphoprotein detection and in vivo overproduction were used to show complementation, not enzymatic assays (*53*).

Enzyme I Mechanism and Regulation. A possible link between the reaction mechnaism of enzyme I and subunit dissociation has been proposed as a means by which enzyme I and thus the PTS could be regulated. The evidence for this has been reviewed (*29*). However, no effector molecule has been identified. The K_A for the subunit association is 3×10^6 M^{-1} (*29*), and the internal concentration of enzyme I is in a range of 2−5 μM (*10*, *54*) which suggests that, in the absence of PEP, about 15−25% of enzyme I would be in the monomer form. Because phosphorylation leads to a decrease in K_A, the proportion of monomers could increase to 60−80%, which could make dissociation a viable option for both mechanism and regulation. In contrast, the "stabilizing" effect of phosphorylation has been used to improve purification, and to derive reproducible kinetic measurements for this work and previously (*7*−*9*, *39*).

The results presented here with the mutant enzymes I and the formation of heterodimers demonstrate that enzyme I may have a homotropic cooperative interaction with PEP, which is mediated through either dimerization or a conformational change in the PEP-binding site or both in concert. Preincubation of enzyme I with saturating PEP leads to a dimeric enzyme with activity characterized by a PEP K_m of 0.1 mM; the two enzymes I that have impaired dimerization have a PEP K_m of 3 mM (Table 2). These PEP concentrations (0.1−3 mM) are approximately in the physiological range of PEP concentrations in *E. coli* (*55*). This potential regulatory mechanism may not be influenced by HPr concentrations as no subunit interactions involve the N-terminal domain. While the state of enzyme I dimerization is affected by PEP concentrations, the results presented here do not support the mechanistic proposal (*23*, *24*) that enzyme I must disassociate to phosphorylate HPr.

If the enzyme I monomer/dimer ratio is a function of the PEP concentration, then activity would be dependent on the ability of PEP both to affect dimerization as well as to act in the catalytic mechanism. This implies that a reexamination of both previous measurements and conclusions, with respect to enzyme I activities, will be necessary. For example, previous arguments suggested (*40*) that in vivo conditions seem compatible with wild-type enzyme I existing only as a dimer. However, this view was biased by the use of fully phosphorylated enzyme I for activity measurement. Enzyme I kinetics have been investigated under conditions where both monomers and dimers form, but the monomer was considered to be inactive. Moreover, in these measurements, the monomer/dimer distribution was presumably a function of the temperature and concentration of enzyme added to an assay (*10*, *22*, *56*, *57*). In experiments estimating the levels of enzyme I activity in either *E. coli* or *S. typhimurium* under different physiological conditions, fully phosphorylated enzyme I was measured. Thus measurements have determined the total amount of enzyme I present (*54*), and this is not sensitive to the potential in vivo activity derived from the monomer/dimer distribution.

In conclusion, the susceptibility of the heterodimer to dissociation and the range of PEP K_m values from 0.1 mM for wild-type dimeric enzyme I to 3 mM for Gly356Ser and Arg375Cys monomeric enzymes I, may offer a means by which the regulation and mechanism of enzyme I can be understood.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

The interacting residues and the type of interaction involved between the subunits of pyruvate phosphate dikinase are described. The information is summarized in Table 3. This material is available free of charge via the Internet at http://pubs.acs.org.

NOTE ADDED IN PROOF

The C-terminal and N-terminal domain fragments show complementation when [^{32}P]PEP is used.

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