Location of the Catalytic Site for Phosphoenolpyruvate Formation within the Primary Structure of *Clostridium symbiosum* Pyruvate Phosphate Dikinase. 2. Site-Directed Mutagenesis of an Essential Arginine Contained within an Apparent P-Loop†

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ABSTRACT: Pyruvate phosphate dikinase catalyzes the interconversion of adenosine 5′-triphosphate (ATP), orthophosphate (P_i), and pyruvate with adenosine 5′-monophosphate (AMP), pyrophosphate (PP_i), and phosphoenolpyruvate (PEP). The Arg 561 residue of *Clostridium symbiosum* PPDK is contained within a Gly-rich stretch of sequence spanning positions 553−563 (*viz.*, GAEGIGLC*R*TE) located in the 35 kDa C-terminal domain of the enzyme. The possible role of this stretch of sequence as a phosphate binding loop participating in catalysis of the PEP/pyruvate partial reaction (*viz.*, E + PEP ⇌ E-P + pyruvate, where E-P represents enzyme phosphorylated at the catalytic histidine) was deduced from the similarity of this sequence to other known phosphate binding loops and by its location in the 35 kDa PEP/pyruvate binding domain of PPDK. To test the proposed role of Arg 561, and hence, the signature sequence, in catalysis of the E + PEP ⇌ E-P + pyruvate partial reaction, the *C. symbiosum* PPDK site-directed mutants Arg 561 → Leu 561 and Arg 561 → Lys 561 were constructed and expressed in *Escherichia coli* JM101. Neither mutant catalyzed the full PPDK reaction, ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP, but both catalyzed the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction as efficiently as wild-type PPDK. Both mutants were shown to be unable to catalyze the PEP/pyruvate partial reaction. On the basis of these results it was proposed that Arg 561 and, possibly, the Gly-rich stretch of sequence spanning positions 553−563 are essential components of the active site of the PEP/pyruvate partial reaction.

The interconversion of ATP,[1] and pyruvate with AMP, PP_i, and PEP is catalyzed in certain microbes and in C_4 plants by the enzyme pyruvate phosphate dikinase (Wood et al., 1977). The reaction consists of three phosphoryl transfers mediated by a catalytic histidine (Wood et al., 1977, Carroll et al., 1989, 1990; Thrall et al., 1993) (Scheme 1A). Proteolysis experiments have suggested that the structure of *Clostridium symbiosum* PPDK (a homodimer) is organized into four domains (Carroll et al., 1994). The catalytic histidine, the ATP/AMP binding site, and the PEP/pyruvate binding site are separately located on the 18 kDa central domain, the 25 kDa/13 kDa domains, and the 35 kDa C-terminal domain, respectively (Carroll, 1991; Carroll et al., 1994). As work on the three-dimensional structure of this protein progresses, identification of the ATP/AMP and PEP/pyruvate binding sites within the primary structure of these domains has become an important goal.

In the accompanying paper (Xu et al., 1995) we reported on the identity of an amino acid residue, Cys 831, in *C. symbiosum* PPDK which, based on chemical modification with [1-^{14}C]bromopyruvate and protection with PEP, was suspected to be in or nearby the PEP/pyruvate binding site (Yoshida & Wood, 1978). Direct participation of this residue, Cys 831, in PEP/pyruvate binding and/or catalysis was tested by examining the catalytic properties of the Cys 831 → Ala 831 site-directed mutant (C831A). C831A was shown to catalyze the nucleotide partial reaction, E + ATP + P_i ⇌ E-P + AMP + PP_i, with the efficiency of the wild-type enzyme but was unable to catalyze the pyruvate partial reaction, E + PEP ⇌ E-P + pyruvate. It was thus concluded that Cys 831, a residue contained within the 35 kDa C-terminal domain, is a component of the PEP/pyruvate binding/catalytic site.

Possibly significant to our search for the PEP/pyruvate binding site on PPDK is the observation that, in addition to the essential Cys 831, the 35 kDa domain of *C. symbiosum* contains a 11 amino acid long stretch of sequence (positions 553−563) which is highly conserved among the four known PPDK sequences (*viz.*, *Zea mays*, *C. symbiosum*, *Entamoeba histolytica*, and *Flaveria trinervia*), the known sequences of enzyme I of the bacterial sugar:PEP phosphotransferase system, and the sequence of *Escherichia coli* PEP synthetase.

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[1] Abbreviations: PPDK, pyruvate phosphate dikinase; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; PEP, phosphoenolpyruvate; P_i, orthophosphate; PP_i, pyrophosphate; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; E-P, phosphorylenzyme; E-PP, pyrophosphorylenzyme; LDH, lactate dehydrogenase; NADH, dihydronicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride, PVDF, polyvinylidene difluoride; LB, Luria−Bertani; PCR, polymerase chain reaction; oAMP, 2′,3′-dialdehyde adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid; TEA, triethylamine; C831A, Cys 831 → Ala 831 PPDK site-directed mutant; R561L, Arg 561 → Leu 561 PPDK site-directed mutant; R561K, Arg 561 → Lys 561 PPDK site-directed mutant; CD, circular dichroism.

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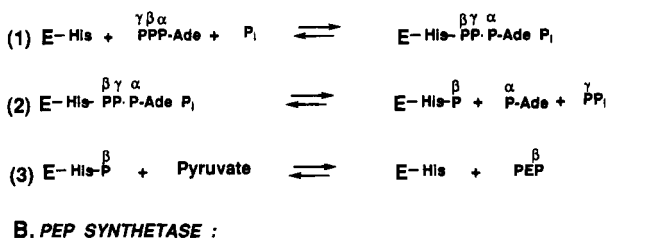
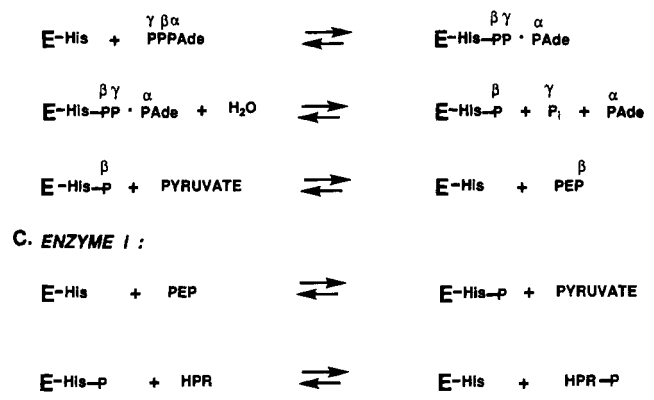
Scheme 1: (A) Chemical Steps of the PPDK Reaction[a] (Wood et al., 1977; Carroll et al., 1989, 1990; Thrall et al., 1993), (B) Phosphoryl Transfer Steps of *E. coli* PEP Synthetase (Cooper & Kornberg, 1974; Narindarasorasak & Bridger, 1974), and (C) Phosphoryl Transfer Steps of Enzyme I of the Sugar:PEP Phosphotransferase System (Postma & Lengler, 1985; Saier, 1989; Postma et al., 1993)

A. *PPDK* :



B. *PEP SYNTHETASE* :



C. *ENZYME I* :

[a] E represents the free enzyme, E-PP represents the pyrophosphorylenzyme, and E-P represents the phosphorylenzyme. Steps 1 and 2 comprise the nucleotide partial reaction, and step 3 comprises the pyruvate partial reaction.

An alignment of these amino acid sequences in this region is shown in Figure 1.[2] The consensus sequence derived from the alignment **GXXGXGLXRXE** resembles phosphate binding loops found in nucleotide/phosphate ester binding proteins (Saraste et al., 1990). We suspected (Pocalyko et al., 1990) that this conserved stretch of sequence represents a functional motif in these enzymes, and therefore we compared their catalytic functions to determine if a common trait exists among them.

Like PPDK, the PEP synthetase catalyzes the pyrophosphorylation of an active site histidine with ATP (Cooper & Kornberg, 1974; Narindrasorasak & Bridger, 1977). The E-PP intermediate formed in the synthetase reaction reacts with H_2O (instead of P_i as in the PPDK reaction), and the E-P intermediate thus formed phosphorylates pyruvate (Scheme 1B). Enzyme I of the sugar:PEP phosphotransferase system, on the other hand, catalyzes phosphoryl transfer from PEP to a catalytic histidine and, hence, to the histidine residue of the next protein in the phosphotransferase system, HPr (Postma & Lengler, 1985; Saier, 1989; Postma et al., 1993) (Scheme 1C). The fact that the sequence motif observed at positions 553−563 of the *C. symbiosum* PPDK is conserved in enzyme I as well as in PEP synthetase

PPDK (C.s.)	553	G A E G I G L C R T E H M	565
PPDK (Z.m.)	627	G A Q G I G L C R T E H M	639
PPDK (E.h.)	553	G A E G I G L C R T E R M	565
PPDK (F.t.)	633	G A Q G I G L C R T E H M	645
PPS (E.c.)	503	P N E G V G L A R L E F I	515
Enz. I (B.s.)	288	G G E A V G L Y R T E F L	300
Enz. I (S.c.)	290	G A E G I G L Y R T E F L	302
Enz. I (S.s.)	290	G A E A V G L Y R T E F L	302
Enz. I (S.t.)	288	G A E G V G L Y R T E F L	300
Enz. I (E.c.)	288	G A E G V G L Y R T E F L	300
Enz. I (A.e.)	300	G A V G V G L Y R S E F L	312
Enz. I (R.c.)	556	G A E G V G L L R T E F L	568

FIGURE 1: Alignment of a conserved region of sequence existing among the amino acid sequences the bacterial, protozoan, and plant pyruvate phosphate dikinases, the *E. coli* of PEP synthetase from *E. coli*, and the enzyme I proteins and protein domains of the bacterial PEP:sugar phosphotransferase system. The abbreviations used in the figure and the references to the published protein sequences are as follows: PPDK, pyruvate phosphate dikinase of *C. symbiosum* (C.s.) (Pocalyko et al., 1990), *F. trinervia* (F.t.) (Rosche & Westhoff, 1990), *E. hystolytica* (E.h.) (Bruchhaus & Tannich, 1993), *Z. mays* (Z.m.) (Matsuoka et al., 1988), PEP synthetase (PPS) of *E. coli* (E.c.) (Niersbach et al., 1992), enzyme I of the PEP:sugar phosphotransferase system of *Alcaligenes entrophus* (A.e.) (Pries et al., 1991), *E. coli* (E.c.) (Saffen et al., 1987; De Reuse & Danchin, 1988), *R. capsulatus* (R.c.) (Wu et al., 1990), *Staphylococcus carnosus* (S.c.) (Kohlbrecher et al., 1991), *Streptococcus salivarius* (S.s.) (Gagnon et al., 1992), *Salmonella tryphimurium* (S.t.) (Byrne et al., 1988, LiCalgi et al., 1991).

suggested that it is involved in binding the phosphoryl group of PEP and/or the phosphoryl group of the E-P intermediate (Scheme 1A). In other words, the common denominator between PPDK, PEP synthetase, and enzyme I appears to be the formation of E-P and/or PEP.

In this paper we describe the catalytic properties of the Arg 561 → Leu 561 (R561L) and Arg 561 → Lys 561 (R561K) *C. symbiosum* PPDK site-directed mutants. Both mutants were found to be nearly as efficient as the wild-type PPDK in catalyzing the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction, yet both were shown to be unable to catalyze the E + PEP ⇌ E-P + pyruvate partial reaction. These results, combined with those reported in the accompanying paper (Xu et al., 1995) describing the essential role of Cys 831, suggest that Arg 561 and Cys 831 are components of the E + PEP ⇌ E-P + pyruvate catalytic site on the enzyme. We propose that the conserved stretch of sequence spanning positions 553−563 constitutes a phosphoryl group binding motif in the 35 kDa domain which is essential to the catalysis of the E + PEP ⇌ E-P + pyruvate partial reaction.

[2] For recent, published full-length sequence alignments of these proteins, see Niersbach et al. (1992), Reizer et al. (1993) and Bruchhaus and Tannich (1993). We note that the alignment of the PEP synthetase sequence used in Figure 1 is different from that reported by Reizer et al. (1993) but the same as that reported by Niersbach et al. (1992).

MATERIALS AND METHODS

Plasmids, Bacterial Strains, and Reagents. Plasmid pACYC184-D12 containing the cloned PPDK gene is described in Pocalyko et al. (1990). *E. coli* strain JM101 was a gift of Dr. John Gerlt. Cells containing pACYC184-D12 were grown to mid-log phase in LB media (containing 12.5 μg of tetracycline/mL) at 37 °C before harvesting. Plasmid DNA for sequencing and mutagenesis was obtained by the method described in Sambrook et al. (1989). Primers for PCR and sequencing reactions were purchased from Midland Certified Reagent Co. Enzymes for DNA manipulations were purchased from Amersham and Promega and used with the supplied buffers. [β-^{32}P]ATP and [^{32}P]PEP were synthesized from [γ-^{32}P]ATP by the method of Carroll et al. (1989). [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from NEN, and all enzymes used in the preparations were from Sigma.

Site-Directed Mutagenesis. The R561L and R561K mutants were generated using recombinant PCR techniques (Erlich, 1992). Reagents used in the PCR reaction were from the GeneAmp kit supplied by Perkin-Elmer Cetus. Each 100-μL reaction contained 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 200 μM each of dATP, dCTP, dGTP, and dTTP, 20 pM each primer, 2.5 units of Taq DNA polymerase, 1.5 mM $MgCl_2$, and 0.5 μg of template DNA. Mutant primers corresponded to bases 1740−1761 in the wild-type PPDK positive strand sequence (Pocalyko et al., 1990). The CGT codon of Arg 561 was changed to CTA in R561L and AAG in R561K. Additional primers were upstream of the unique *Bst*X1 restriction site (nucleotides 926−942) and downstream from the unique *Kpn*I site (nucleotides 2344−2361). Reactions were overlaid with 50 μL of sterile silica oil. Denaturation was achieved at 92 °C, annealing at 40−55 °C depending upon primer composition, and elongation at 72 °C. The cycle number ranged from 25−35. Following each reaction the sample was run on a 1% LMP agarose gel with TBE buffer (10.8 g/L Tris, 5.5 g/L boric acid, 0.84 g/L EDTA). DNA was extracted using the Geneclean II kit from Bio101.

The resulting 1.3 kb fragment containing the mutated R561 and plasmid pACYC184-D12 containing the wild-type PPDK gene (Pocalyko, 1990) were digested with *Kpn*I and *Bst*XI. The mutant fragment was then inserted into the wild-type plasmid DNA using T4 DNA ligase. Following ligation at 37 °C for approximately 4 h, the plasmid was transformed into competent *E. coli* JM101 cells (Sambrook et al., 1989). The sequence of the 1.3 kb region of the resulting mutated pACYC184-D12 plasmid was verified by the chain termination method (Sanger et al., 1977) using the Sequenase kit from USB. The transformed cells were grown, harvested, and lysed according to the method of Pocalyko et al. (1990).

Purification of R561L and R561K. PPDK R561L and R561K were purified by modification of the procedure used by Wang et al. (1988) for purification of wild-type PPDK. The protease inhibitors 1,10-phenanthroline (1 mM), PMSF (1 mM), and benzamidine hydrochloride (1 mM) and trypsin inhibitor (50 mg/L) were added to the extraction buffer and to the DEAE-cellulose column elution buffer at the concentrations indicated. The concentration of protein in the DEAE-cellulose column fractions was determined using the Bio-Rad protein assay system. Those fractions comprising the largest protein peak were pooled and concentrated. An aliquot of this protein was applied to a Sephacryl S-200 column and eluted with a buffer containing 20 mM imidazole hydrochloride, 0.1 mM EDTA, 0.7 mM β-mercaptoethanol, and 100 mM KCl (pH 6.5). Selected fractions (monitored for protein by measuring absorbance at 280 nm) comprising the largest protein peak were pooled and concentrated. The concentrated protein solution was dialyzed against 50 mM K^+Hepes (pH 7.0). The purity of the protein preparations was confirmed by SDS−PAGE analysis.

Rapid Quench Experiments. Reactions were carried out by mixing 40 μL of solution containing enzyme (wild-type PPDK, R561K PPDK, or R561L PPDK) and metal ions (Mg^{2+}, NH_4^+) in buffer (50 mM K^+Hepes, pH 7.0) with 40 μL of solution containing radiolabeled substrate ([^{14}C]ATP, [β-^{32}P]ATP, or [^{32}P]PEP) in buffer (50 mM K^+Hepes, pH 7.0) at 25 °C. The reactions were quenched at varying conversions with 164 μL of 0.6 N HCl. The protein in the quenched samples was precipitated by vortexing with 100 μL of CCl_4 and then collected by centrifugation. The protein pellet was analyzed for radioactivity by dissolving it in boiling 10 N H_2SO_4 and then subjecting the resulting solution to scintillation counting [see Xu et al. (1995)]. The supernatant of the quenched reaction sample was analyzed for product and unconsumed reactant by HPLC separation coupled with scintillation counting as described previously (Mehl et al., 1994). The specific concentrations used in the single turnover reactions are given in the figure headings. The kinetic data were analyzed by linear regression analysis.

Circular Dichroism Spectra. Far-ultraviolet (200−250 nm) circular dichroism spectra of wild-type PPDK, R561L PPDK, and R561K PPDK were recorded on a Jasco 500-C spectropolarimeter. Each enzyme was at a concentration of 0.15 μM active sites in 5 mM potassium phosphate buffer (pH 6.9).

RESULTS

Construction and Expression of the R561K and R561L Mutants. The R561K and R561L mutants of wild-type *C. symbiosum* PPDK were constructed from the pACYC184-D12 clone (Pocalyko et al., 1990) using standard PCR techniques. Expression of wild-type PPDK in transformed *E. coli* JM101 and purification using the procedure described in Wang et al. (1988) yielded 20−25 mg of enzyme/g of cell. The R561L enzyme was purified to a yield of 20−35 mg/g of cell while the R561K mutant was obtained in a yield of 15−20 mg/g of cell.

Wild-type PPDK and the R561K mutant displayed comparable resistance to proteolysis during purification and storage. Each protein was stored in buffer solution at 4 °C for several months without causing noticeable chain cleavage or loss of protein. The R561L mutant, on the other hand, was susceptible to fragmentation during purification and storage despite the inclusion of protease inhibitors in the buffered media. SDS−PAGE analysis (data not shown) of purified R561L stored for 10 days at 4 °C revealed that only about 30% of the intact protein remained. The major protein bands observed on the SDS−PAGE gel corresponded to the 67 kDa (major) and 57 kDa (minor) truncation fragments (missing the N-terminal piece) plus the 25 kDa N-terminal fragment previously observed in subtilisin proteolytic digests of wild-type PPDK (Carroll et al., 1994). The stable 35 kDa (C-terminal) fragment (spanning positions 528−874 of the amino acid sequence) which is generated by the secondary

subtilisin cleavage of the 67 and 57 kDa pieces of wild-type PPDK was, however, not observed in the storage solution of the R561L PPDK mutant. The absence of the 35 kDa fragment from R561L suggests the possibility that the substitution of Arg 561 with Leu destabilizes this domain. Realistically, however, no firm conclusion regarding the impact of the R561 → L561 substitution on the stability of the isolated 35 kDa (pyruvate binding) domain can be drawn from these (limited) results.

The spectrophotometric-based PPDK activity assay (Wang et al., 1988) was used to measure the activity of the R561L and R561K mutants present in crude cellular extract. No activity was detected. Therefore, in the absence of a convenient activity assay, the purification of the mutants by application of the standard column chromatographic procedures used for the purification of cloned wild-type PPDK (Wang et al., 1988) relied on the large quantities of the mutant enzymes present (relative to total cellular protein) and on the similarity of their chromatographic behavior to that of wild-type PPDK. These two factors served as a guide for the selection of the column fractions to be pooled and/or analyzed by SDS−PAGE.

The UV CD spectra (200−250 nm) of freshly prepared mutant proteins were measured along with that of wild-type PPDK. Essentially the same spectra as reported in Pocalyko et al. (1990) were observed for all three enzyme samples. Thus, through this window of observation, no substantial change in the native α/β structure was found to result from the two R561 site-directed mutations.

Catalytic Properties of the R561L and R561K PPDK Mutants. The catalytic properties of the purified R561L and R561K PPDK mutants were scrutinized using steady-state and pre-steady-state kinetic techniques. First, the NADH/LDH coupled spectrophotometric assay, which monitors pyruvate formation from PEP, PP_i, and AMP under "maximum velocity" conditions, was applied (Wang et al., 1988). The specific activity measured for wild-type PPDK in solutions containing 0.5 mM PEP, 10 mM PP_i, 0.5 mM AMP, 10 mM $MgCl_2$, and 40 mM NH_4Cl in 50 mM imidazole hydrochloride (pH 6.8, 25 °C) is equal to 20−30 units/mg. These reaction conditions, in combination with high enzyme concentration (up to 10 μM), failed to support a detectable level of activity in the mutants. Hence, the specific activity of the two mutants for catalysis of the full reaction in the *ATP-forming direction* is less than 0.01 unit/mg (or 0.04% the activity of wild-type PPDK).

Next, we tested the ability of the R561L and R561K PPDK mutants to catalyze *PEP formation* from pyruvate, ATP, and P_i using a limiting amount of [β-^{32}P]ATP (3.7 μM) to react with excess mutant enzyme (16 μM) and cosubstrates (10 mM pyruvate and 11 mM P_i) in 50 mM K^+Hepes (pH 7.0, 25 °C) containing 3 mM $MgCl_2$ and 10 mM NH_4Cl. The time courses for a single turnover on the mutant enzymes (Figure 2) were monitored by measuring the levels of [β-^{32}P]-ATP, radiolabeled enzyme, and [^{32}P]PEP present in the reaction mixtures at varying conversions. The reactions were quenched with acid which releases from the enzyme active site unconsumed [β-^{32}P]ATP and product [^{32}P]PEP. Precipitation of the enzyme followed by scintillation counting of the enzyme pellet to analyze for ^{32}P content allowed us to measure the ratio of free enzyme to pyrophosphorylated enzyme and/or phosphorylated enzyme present in the reaction mixture (see Scheme 1A). *As indicated by the single-turnover profiles shown in Figure 2, both mutants turned over [β-^{32}P]ATP to form ^{32}P-labeled enzyme, but neither mutant was able to catalyze [^{32}P]PEP formation.* This is in contrast to the behavior observed for wild-type PPDK for which catalysis of [^{32}P]PEP formation (from pyruvate) follows the appearance of the ^{32}P-labeled enzyme generated by reaction of [β-^{32}P]ATP and P_i (i.e., steps 1 and 2 of Scheme 1A) (Mehl et al., 1994).

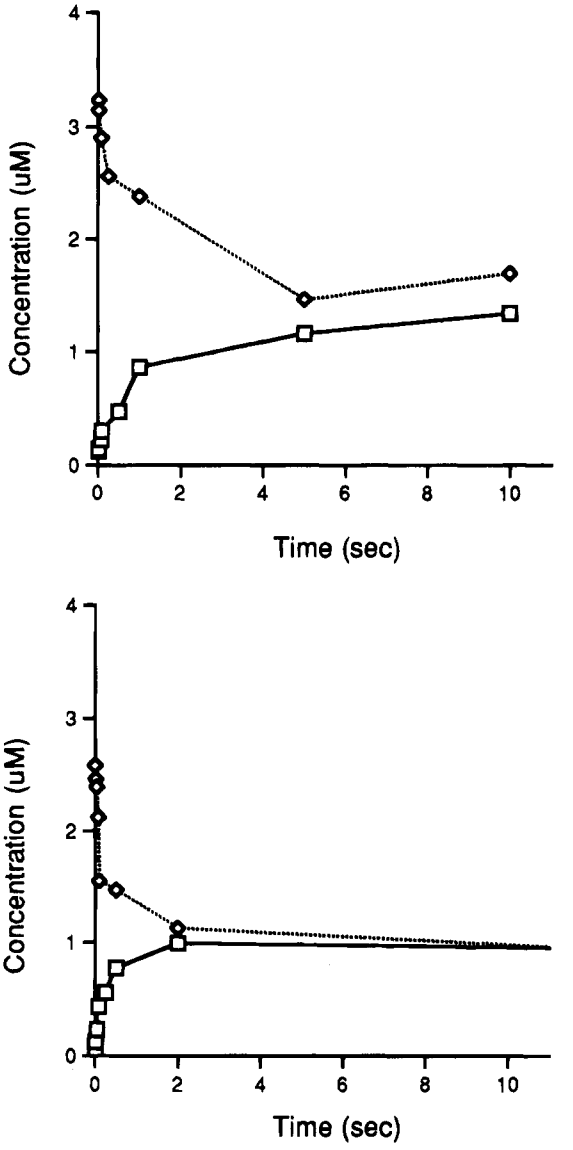


FIGURE 2: Time course for a single turnover of [β-^{32}P]ATP (3.7 μM) with 16 μM R561K (A, top) or R561L (B, bottom) PPDK active sites in the presence of 11 mM P_i, 10 mM pyruvate, 3 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) at 25 °C. Symbols: (◇) ATP; (□) E-P + E-PP.

The results presented above suggest that the R561K and R561L mutant enzymes cannot catalyze the full PPDK reaction, ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP, measured in either direction. The single-turnover experiment with [β-^{32}P]ATP and P_i (Figure 2) indicated that while the mutant enzymes were effective in catalyzing the nucleotide partial reaction (E + ATP + P_i ⇌ E-P + AMP + PP_i), they appeared to be unable to catalyze PEP formation from E-P + pyruvate.[3] Catalysis of the pyruvate partial reaction (step 3, Scheme 1A) by the mutants was examined further by carrying out single-turnover experiments with [^{32}P]PEP. Reaction of 5 μM [^{32}P]PEP with 40 μM enzyme in 50 mM

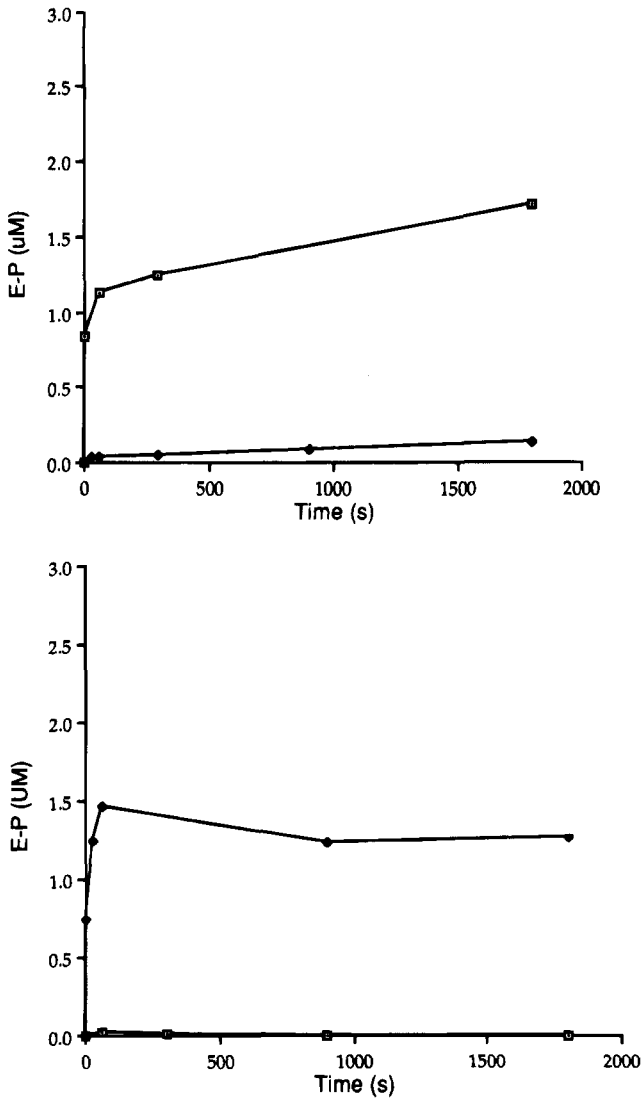


FIGURE 3: Time course for a single turnover of [^{32}P]PEP by wild-type PPDK, R561K PPDK, and R561L PPDK. Reactions contained 40 μM enzyme active sites, 5 μM [^{32}P]PEP, 2.5 mM $MgCl_2$, and 10 mM NH_4Cl in 50 mM K^+Hepes at pH 7.0 and 25 °C. Panels: (A, top) μM E-P formed from wild-type PPDK (□) and R561K PPDK (◆); (B, bottom) μM E-P formed from wild-type PPDK (◆) and R561L PPDK (□).

K^+Hepes (pH 7.0, 25°C) containing 2.5 mM $MgCl_2$ and 10 mM NH_4Cl generated a time course for [^{32}P]E-P formation from wild-type enzyme which plateaued (within the 200-ms time point) at 1.2 μM [^{32}P]E-P (Figure 3). In contrast, no [^{32}P]E-P was observed in the [^{32}P]PEP reaction mixture of the R561L or R561K mutants (Figure 3). The reaction was repeated at higher [^{32}P]PEP concentration (200 μM) (to overcome the possible problem of low E·[^{32}P]PEP concentration arising from reduced substrate binding affinity in the mutants) and in the presence of 50 μM NADH and 22 units/mL LDH [to drive the E + PEP ⇌ E-P + pyruvate reaction to completion by reduction of pyruvate to lactate (Thrall et al., 1993)]. Under these conditions 20 μM [^{32}P]E-P was formed from wild-type PPDK within 200 ms, but none, in excess of that of the control reaction (in which the enzyme was added after the acid quench), was observed for the R561K or R561L PPDK reactions monitored to 4 s. *Thus, both the R561L and R561K PPDK mutants are defective in their ability to catalyze the phosphorylation of the catalytic histidine with [^{32}P]PEP.*

Finally, the efficiency of the R561K and R561L PPDK mutants in catalyzing the nucleotide partial reaction (steps 1 and 2 Scheme 1A) was compared with that of wild-type PPDK. Accordingly, the time course for a single turnover of 5 μM [^{14}C]ATP catalyzed by 20 μM enzyme in the presence 11 mM P_i, 5 mM $MgCl_2$, and 10 mM NH_4Cl in 50 mM K^+Hepes (pH 7.0, 25 °C) was measured for wild-type and mutant PPDK under identical conditions. The time courses obtained (Figure 4) were analyzed by linear regression to yield turnover rates of 9.34 ± 0.08 s^{-1}, 5.4 ± 0.2 s^{-1}, and 5.4 ± 0.6 s^{-1} for wild-type PPDK, R561K PPDK, and R561L PPDK, respectively. *Hence, Arg 561, which borders the Gly-rich signature sequence of the 35 kDa domain plays no apparent role in catalysis of the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction but is essential to the catalysis of the E-P + pyruvate ⇌ E + PEP partial reaction.*

DISCUSSION

Physical and Kinetic Properties of the R561K and R561L PPDK Mutants. In the present study site-directed mutagenesis of Arg 561 of the *C. symbiosum* PPDK was carried out to test the possible role of Arg 561 and, hence, the signature sequence spanning amino acid positions 553−563 (see Figure 1) in catalysis of the ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP reaction. Two mutants were prepared, one in which the Arg residue was replaced with an amino acid carrying the same charge, namely, Lys, and the other in which the Arg residue was replaced with the nonpolar amino acid, Leu. Both mutants were expressed and isolated in high yields, suggesting that *in vivo* they maintain relatively compact, stable structures. The UV CD spectra of the mutants are indistinguishable from that of wild type, giving no indication of altered native structure. On the other hand, the R561L mutant showed a greater susceptibility to proteolytic fragmentation during storage, suggesting that it may have, compared to wild-type PPDK or the R561K mutant, altered structural properties. Thus, while we are reasonably confident that the R561K mutant has retained (in a global sense) the wild-type PPDK native conformation, we are less certain about the R561L mutant.

On the basis of our earlier investigations of the *C. symbiosum* PPDK a structural model was proposed (Carroll, 1991; Carroll et al., 1994) which divides the native structure into four domains with the ATP binding site being located on the 25 and 13 kDa domains, the catalytic histidine located on the 18 kDa domain, and the pyruvate binding site located on the 35 kDa C-terminal domain. Catalysis of the E + ATP + P_i ⇌ E-P + AMP + PP_i and E-P + pyruvate ⇌ E + PEP partial reactions at separate sites on the enzyme is evidenced by the findings from chemical modification (Yoshida & Wood, 1978; Evans et al., 1980), stereochemical (Cook & Knowles, 1985), and transient kinetic (Thrall &

[3] In theory, the failure to observe [^{32}P]PEP formation could be attributed to the failure of the enzyme to catalyze E-PP·AMP·P_i ⇌ E-P·AMP·PP_i (step 2, Scheme 1). However, the high level of accumulation of ^{32}P-labeled enzyme observed suggests that the E·ATP ⇌ E-PP·AMP reaction step, which is thermodynamically unfavorable (Mehl et al., 1994), has been driven by the ensuing step: E-PP·AMP + P_i ⇌ E-P·AMP·PP_i (which is favorable). Hence, the radiolabeled enzyme that is isolated from the single turnover of [β-^{32}P]ATP and P_i is primarily [^{32}P]E-P formed in reaction step 2 of Scheme 1.

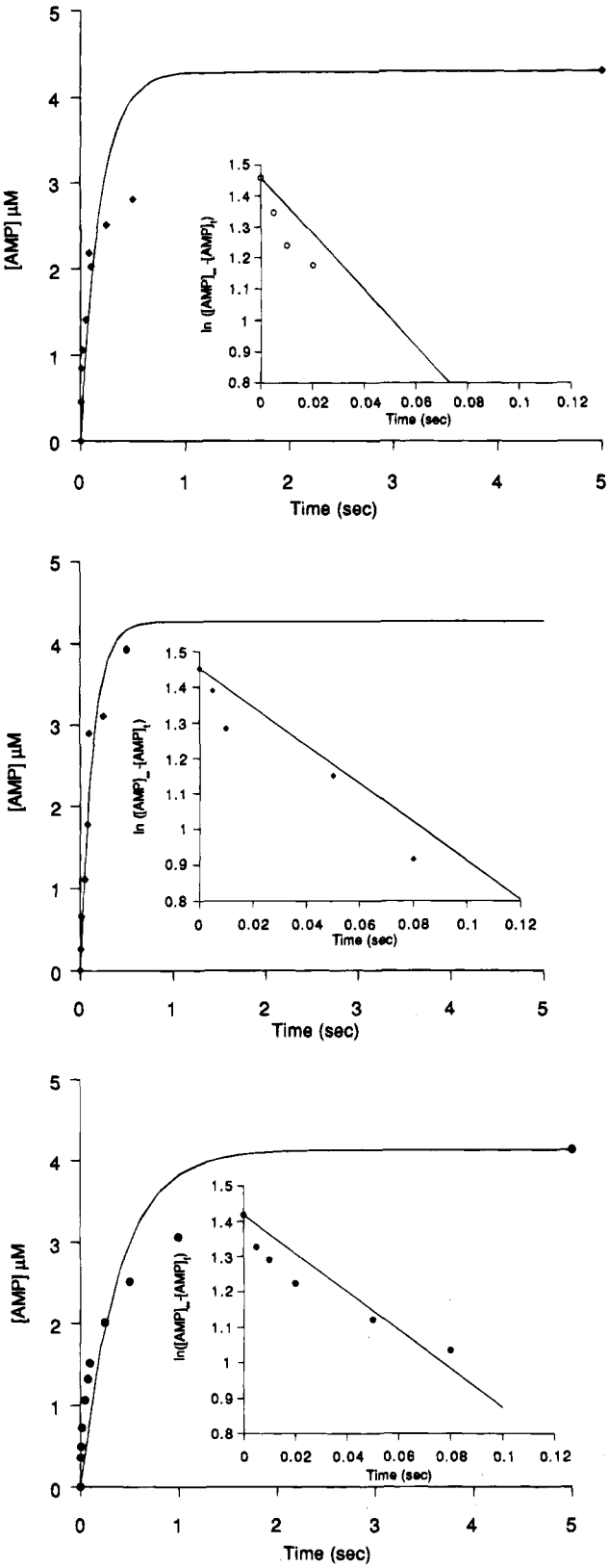


FIGURE 4: Time course for a single turnover of [^{14}C]ATP (5 μM) with 20 μM wild-type PPDK (A, top), R561K PPDK (B, middle), or R561L PPDK (C, bottom) active sites in the presence of 11 mM P_i, 5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) at 25 °C. The lines drawn through the data points were generated by computer fitting the data to a first-order rate equation.

Dunaway-Mariano, 1994) studies. The proposal that these separate active sites are located on separate structural domains originated from the study of the domain structure of this enzyme by limited proteolysis (Carroll et al., 1994). Thus, because the nucleotide and pyruvate partial reactions occur at separate domains (25 and 35 kDa, respectively) which function independent of one another, mutation of the suspected P-loop in the 35 kDa domain should, in theory, only inhibit the pyruvate partial reaction if, indeed, the site of mutation is located in the PEP/pyruvate active site.

Experimentally, we found that amino acid replacement at Arg 561 destroyed catalysis (within the detection limit) of the complete reaction, ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP, without inhibiting catalysis of the E + ATP + P_i ⇌ E-P + AMP + P_i partial reaction. Neither mutant (R561K or R561L) PPDK was able to catalyze the reaction between E-P and pyruvate (to generate PEP) nor the phosphorylation of the catalytic histidine of the free enzyme with PEP (to generate E-P). If, for the moment, it is assumed that replacement of Arg 561 does not trigger long-range conformational changes in PPDK which offset catalysis of the pyruvate partial reaction, these findings suggest that Arg 561 is located in the vicinity of the active site.

Role of Cys 831 and Arg 561 in PPDK Catalysis. The Arg 561 of *C. symbiosum* PPDK is conserved among all known PPDK, PEP synthetase, and enzyme I (of the PEP: sugar phosphotransferase system) sequences,[2] suggesting that it may play a role in the catalytic mechanisms of each of these enzymes. From the studies presented in this paper, and in the accompanying paper (Xu et al., 1995), we have shown that Arg 561 is crucial for the catalysis of the E-P + pyruvate ⇌ E + PEP partial reaction (step 3 Scheme 1A) by the *C. symbiosum* PPDK. An interesting extrapolation of these findings is the proposal that the corresponding Arg residues play similar roles in the catalysis of the phosphoryl transfer between the active site histidine and PEP in enzyme I (Scheme 1B) and between the active site phosphorylhistidine and pyruvate in PEP synthetase (Scheme 1A).

At present, the precise role that Cys 831 may play in PPDK catalysis is unclear and will probably remain so until a crystal structure becomes available. We have looked at the picture of the active site of pyruvate kinase as reported by Larsen et al. (1994) for possible clues. However, pyruvate kinase, which also catalyzes phosphoryl transfer from PEP, does not appear to contain an active site Cys functioning in catalysis.

In contrast, a possible role of Arg 561 in PPDK catalysis is suggested by the Gly-rich stretch of sequence which proceeds it. In Figure 1 we have aligned the short stretch of sequence surrounding Arg 561 in *C. symbiosum* PPDK with corresponding stretches of sequence in PPDK from *Z. mays*, *E. histrolytica* and *F. trinerva*, in *E. coli* PEP synthetase, and in enzyme I of the PEP:sugar phosphotransferase system (from numerous bacterial sources). The consensus sequence which emerges, **GXXGXGLXRXE** is quite similar to the P-loop motif. The P-loop motif, which is found in many phosphate binding proteins (*viz.*, phosphate ester, mononucleotide, dinucleotide binding proteins), consists of a short stretch of sequence which is rich in Gly residues and may or may not be flanked by an amino acid bearing a positively charged (or simply, an H-bond donating) side chain (Saraste et al., 1990; Lacour et al., 1983; Jurnak, 1985; Egner et al., 1987; Dreusicke et al., 1988, Pai et al., 1989, Tong et al., 1990; Hanks et al., 1988; Moller & Amons, 1985; Wierenga et al., 1983; Walker et al., 1982; Pompliano et al., 1990, Kocabivik & Perlin, 1994). In these proteins

binding interactions occur between the phosphate moiety of the bound substrate/cofactor and the amino acids of the P-loop. It is possible that Arg 561 plays a role in PEP or phosphohistidine (of E-P) binding in PPDK analogous to that played by the Lys conserved in the P-loop, (G,A)XXXXGK-(T,S), frequently observed in the nucleotide binding proteins (Saraste et al., 1990). The X-ray crystal structure of PPDK, which is currently under study, should no doubt shed light on this proposal.

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