Location of the Catalytic Site for Phosphoenolpyruvate Formation within the Primary Structure of *Clostridium symbiosum* Pyruvate Phosphate Dikinase. 1. Identification of an Essential Cysteine by Chemical Modification with [1-^{14}C]Bromopyruvate and Site-Directed Mutagenesis†

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ABSTRACT: Pyruvate phosphate dikinase (PPDK) 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 reaction takes place according to the following steps: (1) E + ATP + P_i ⇌ E-PP•AMP•P_i, (2) E-PP•AMP•P_i ⇌ E-P + AMP + PP_i, and (3) E-P + pyruvate ⇌ E + PEP, where E represents free enzyme; E-PP, pyrophosphorylenzyme; and E-P, phosphorylenzyme. Steps 1 and 2 comprise the nucleotide partial reaction, and step 3 comprises the pyruvate partial reaction. The present studies were carried out to locate amino acid residues within the primary structure of *Clostridium symbiosum* PPDK participating in the catalysis of the pyruvate partial reaction. The enzyme was treated with the affinity label [1-^{14}C]bromopyruvate, reduced with $NaBH_4$, proteolyzed with trypsin, and chromatographed on an HPLC column. The radiolabeled tryptic peptide isolate was sequenced to reveal Cys 831 as the site of alkylation. Using PCR techniques Cys 831 was replaced by Ala, and the C831A PPDK mutant formed was then subjected to kinetic analysis. Rapid quench studies of single turnover reactions on the enzyme showed that the mutant is as efficient as wild-type PPDK in catalyzing the nucleotide partial reaction while it is unable to catalyze the pyruvate partial reaction. These results were interpreted as evidence for a role of Cys 831 in pyruvate/PEP binding and/or catalysis.

Pyruvate phosphate dikinase (PPDK)[1] catalyzes the interconversion of ATP, pyruvate, and P_i with AMP, PEP, and PP_i in some microorganisms (including parasitic protozoa) (Reeves et al., 1974; Hrdý et al., 1993) and in C_4 plants (Wood et al., 1977). The phosphoryl transfers which occur in this reaction are mediated by an active site histidine which first abstracts the P_β−P_γ pyrophosphate moiety from ATP and then delivers two phosphoryl groups to P_i and pyruvate, respectively (Scheme 1). The intermediacy of both a pyrophosphorylenzyme and phosphorylenzyme in this three-step reaction is well established (Wood et al., 1977; Carroll et al., 1989, 1990; Thrall et al., 1993). For convenience the first two sites are combined to form what is referred to as the nucleotide partial reaction (E + ATP + P_i ⇌ E-P + AMP + PP_i). This partial reaction can be studied separately from the pyruvate partial reaction (E-P + pyruvate ⇌ E + PEP) using isotope exchange or transient kinetic techniques (Wang et al., 1988; Mehl et al., 1994).

A study of the domain structure of *Clostridium symbiosum* PPDK using proteolysis techniques has suggested a four-domain structure beginning with a 25 kDa domain at the N-terminus, followed by a 13 kDa and then an 18 kDa domain in the center of the amino acid sequence, terminating with a 35 kDa domain (see Chart 1) (Carroll et al., 1994). The catalytic histidine at amino acid position 455 is located within the 18 kDa domain (Chart 1). This domain and the 35 kDa domain show sequence homology with the bacterial PEP-utilizing enzymes, enzyme I of the sugar:PEP phosphotransferase system and PEP synthetase (Pocalyko et al., 1990; Wu & Saier, 1990; Niersbach et al., 1992). Chemical modification of PPDK with [^{14}C]oAMP inactivates the enzyme and results in the transfer of radiolabel specifically to the 25 kDa domain whereas treatment with the photoaffinity label [α-^{32}P]azidoATP inactivates the enzyme and ^{32}P labels both the 25 kDa and 13 kDa domains (Carroll, 1991). These results suggest that the 25 kDa domain and, possibly, the 13 kDa domain contain the ATP binding site (Chart 1).

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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; PITC, phenyl isothiocyanate; CD, circular dichroism.

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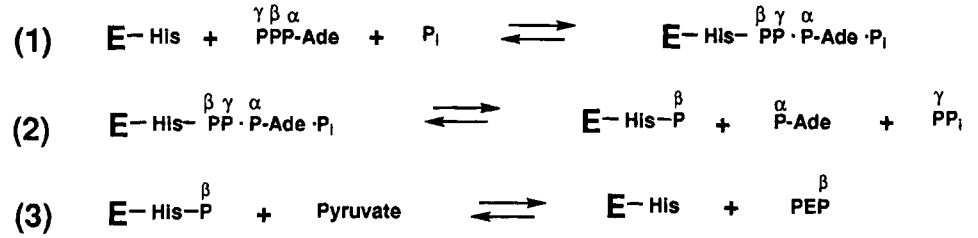
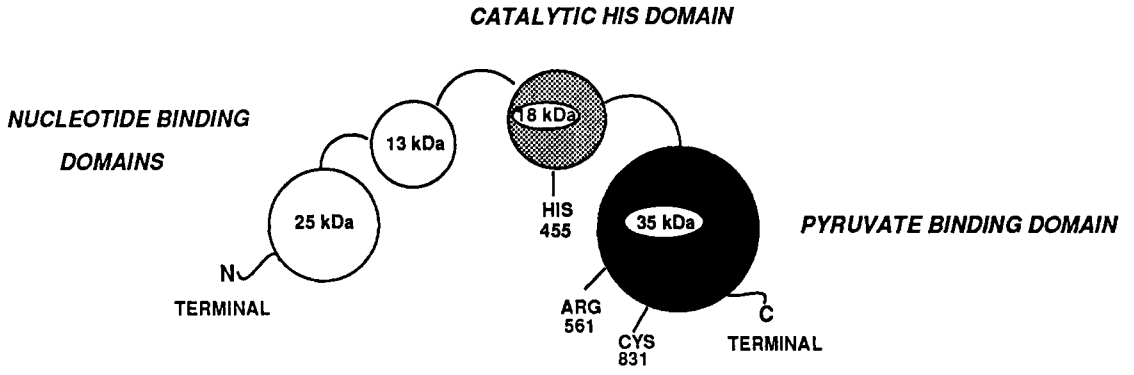
Scheme 1: Chemical Steps of PPDK Catalysis (Wood et al., 1977; Carroll et al., 1989, 1990; Thrall et al., 1993)[a]



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

Chart 1: Structure and Function of *C. symbiosum* PPDK Domains Suggested by Proteolysis and Chemical Modification Studies (Carroll et al., 1994)[a]



[a] For ease of illustration, the proteolytic fragments are represented as spatially separate structural domains connected by loops. The ATP binding site is shown to be located on the 25 kDa/13 kDa domains, the pyruvate binding site on the 35 kDa domain, and the catalytic histidine on the 18 kDa domain.

The PEP/pyruvate binding site on *C. symbiosum* PPDK is (as illustrated in Chart 1) believed to be located on the 35 kDa C-terminal domain. The first hint of this location arose from the recognition of the sequence identity existing between enzyme I of the sugar:PEP phosphotransferase system and *C. symbiosum* PPDK residues 238−874[2] comprising the 18 kDa (catalytic His containing) domain and the 35 kDa C-terminal domain of PPDK (Pocalyko et al., 1990). Further indication of the location of the PEP/pyruvate binding site on the 35 kDa domain derives from the affinity labeling (and inactivation) of *C. symbiosum* PPDK with [1-^{14}C]bromopyruvate specifically at the 35 kDa domain (Carroll et al., 1994).

The present studies were carried out for the purpose of identifying amino acids located in the PEP/pyruvate binding/catalytic site of *C. symbiosum* PPDK. This information, in conjunction with the X-ray crystal structure of this protein, will assist us in determining the precise site of catalysis of the E + PEP ⇌ E-P + pyruvate partial reaction. In this paper we present evidence that the chemical modification of PPDK with [1-^{14}C]bromopyruvate occurs at Cys 831. In addition, the results from site-directed mutagenesis studies are presented which indicate that Cys 831 is essential for catalysis of the E + PEP ⇌ E-P + pyruvate partial reaction but not the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction. In the paper which follows (Yankie et al., 1995) we report the essential role of an Arg residue, contained within a highly conserved Gly-rich motif (resembling a P-loop) located in the 35 kDa C-terminal domain (residues 553−563), in catalysis of the E + PEP ⇌ E-P + pyruvate partial reaction.

[2] The original sequence for *C. symbiosum* PPDK reported by Pocalyko et al. (1990) has been extended in the this paper to 874 amino acids.

MATERIALS AND METHODS

General. PPDK was prepared from *Escherichia coli* JM 101 carrying the plasmid pACYC184-D12 as described in Pocalyko et al. (1990). The enzyme used in these experiments had a specific activity between 20 and 25 units/mg. [1-^{14}C]Bromopyruvate was prepared as described in Carroll et al. (1994). [^{32}P]PEP was prepared according to the method of Carroll et al. (1989). [γ-^{32}P]ATP was purchased from NEN.

Production of Tryptic Peptides from [1-^{14}C]Bromopyruvate-Treated PPDK. PPDK (2.6 mg) was incubated in a 1-mL reaction containing 7.5 mM $MgCl_2$, 30 mM NH_4Cl, 35 μM [1-^{14}C]bromopyruvate (2.5 × 10^6 cpm), and 50 mM K^+Hepes (pH 7.0). After incubation at 30 °C for 30 min, 100 μL of 500 mM $NaBH_4$ in H_2O was added. The reduction was allowed to proceed for 8 min at room temperature. Ammonium sulfate (0.52 g) was added to the reaction to bring the solution to 70% saturation (47.2 g/L solution). After gentle mixing for 10 min, the solution was centrifuged at 14 000 rpm for 15 min in a bench top microcentrifuge. The protein pellet was suspended in 600 μL of 0.4 M Tris·HCl (pH 8.6) and 8 M urea. To the resulting solution was added 5 μL of 0.4 M DTT. The reduction was carried out at 55 °C for 2 h in a water bath. Following reduction, the solution was allowed to cool to room temperature (approximately 20 min). Six microliters of 1 M iodoacetic acid in H_2O was added, and the carboxymethylation was allowed to proceed at 25 °C for 30 min. The solution was diluted to a final volume of 1 mL with 0.4 M Tris·HCl (pH 8.0), and 0.48 g of $(NH_4)_2SO_4$ was added to bring the solution to 70%

saturation. The resulting solution was mixed and centrifuged as before. The pellet was resuspended in 0.5 mL of 0.4 M Tris·HCl (pH 8.0) and 8 M urea and incubated at 37 °C for 5 h. The solution was then diluted to a final volume of 2 mL with 0.4 M Tris·HCl (pH 8.0). Four additions of trypsin (2% by weight with respect to the denatured PPDK) were made over a 36-h period (37 °C). The digested enzyme was stored at −80 °C until it was chromatographed.

Isolation and Sequencing of the [1-^{14}C]Bromopyruvate-Labeled Peptide. The tryptic digest (see above) was separated on a C_{18} reversed-phase column (Vydac, 300 A, 5 μm, 25 × 0.46 cm), using a Beckman System Gold HPLC apparatus equipped with a model 126 pump and a model 167 variable wavelength detector. Two solvents were used: solvent A, 0.12% trifluoroacetic acid in H_2O; solvent B, 0.1% trifluoroacetic acid in acetonitrile. All elution profiles were monitored at 225 and 280 nm. Aliquots of the digested enzyme were applied to the column and separated by a gradient as follows: 5% B for 5 min, then increasing linearly to 75% B in 70 min; flow rate, 0.75 mL/min. Fractions were manually collected, and the ^{14}C radioactivity distribution was determined. The pooled radiolabeled fractions were twice purified on the same C_{18} column using the following gradient: 10% B for 5 min, then increasing linearly to 50% B in 60 min; flow rate, 0.75 mL/min. The purified ^{14}C-labeled peptide was sequenced by using an Applied Biosystems 470A gas phase protein sequencer equipped with an Applied Biosystems 120A online PTH analyzer. Forty percent of PTH-amino acid derivative generated from each cycle, which was not delivered to the PTH analyzer, was collected manually and used for determination of the radioactivity.

Synthesis of S-(3-Lactic acid)cysteine. The *S*-(3-Lactic acid)cysteine standard was synthesized using the method of Barnett et al. (1971) with some modifications. *N*-Acetylcysteine (1.00 mmol) was dissolved in water (10 mL), and 0.1 M NaOH (10 mL) was added to adjust the pH to 6.0. A solution of bromopyruvate (1.0 mmol) in water (10 mL) was treated in the same way (0.1 M NaOH, 9.5 mL) to adjust the pH to 6.0. The two solutions were mixed, and the pH of the resulting mixture was measured at 3. After the pH was adjusted to 6.0 with 0.1 M NaOH, $NaBH_4$ (5 mmol) was added, and the solution was stirred at 25 °C for 10 h. The solution was treated with Dowex-50W (H^+ form) and subsequently evaporated to dryness *in vacuo*. Methanol was added and evaporated several times to remove boric acid, and then 6 M HCl was added. The solution was heated at reflux for 24 h under nitrogen at 110 °C and evaporated to dryness *in vacuo*. Residual HCl was removed from the concentrate by adding and evaporating water several times to give *S*-(3-lactic acid)cysteine (in over 90% yield): ^{1}H NMR (500 MHz in D_2O, 1:1 mixture of two diastereomers A and B) δ 2.27 (dd, J = 14.3, 6.7 Hz, 1H, H-3), 2.36 (dd, J = 14.3, 5.7 Hz, 1H, H-3), 2.39 (dd, J = 14.3, 4.2 Hz, 1H, H-3), 2.43 (dd, J = 14.3, 3.8 Hz, 1H, H-3), 2.48 (dd, J = 15.0, 7.8 Hz, 1H, H-4), 2.51 (dd, J = 15.0, 7.4 Hz, 1H, H-4), 2.61 (dd, J = 15.0, 4.4 Hz, 1H, H-4), 2.65 (dd, J = 15.0, 4.4 Hz, 1H, H-4), 3.68 (dd, J = 7.8, 4.4 Hz, 1H, H-5), 3.71 (dd, J = 7.4, 4.4 Hz, 1H, H-5), 3.86 (dd, J = 6.7, 3.8 Hz, 1H, H-2), 3.9 (dd, J = 5.7, 4.2 Hz, 1H, H-2); ^{13}C NMR (100 MHz in D_2O) δ 34.5 and 34.6 (diastereomers A and B, C-5), 38.2 and 28.6 (diastereomers A and B, C-2), 54.0 and 54.9 (diastereomers A and B, C-3 or C-4), 72.0 and 72.3 (diastereomers A and B, C-3 or C-4), 172.4 and 177.9 (diastereomers A and B, C-1 or C-6); CIMS *m/z* (relative intensity) 210 (MH^+, 6), 192 (22), 174 (27), 162 (15), 148 (22), 123 (42), 104 (100).

Preparation and Identification of the PTH Derivative of S-(3-Lactic acid)cysteine. The PTH derivative of *S*-(3-lactic acid)cysteine was prepared as follows: 10 μL of 100 μM *S*-(3-lactic acid)cysteine in water was mixed with 40 μL of Redry solution (H_2O/methanol/TEA = 1:7:1, v/v), and the resulting solution was dried *in vacuo*. To the dried mixture were added 20 μL of PITC/Redry solution (PITC/H_2O/methanol/TEA = 1:1:7:1, v/v), and the reaction was allowed to proceed at 37 °C for 50 min. The solution was dried *in vacuo* and subsequently resuspended in 100 μL of 20% TFA in water (v/v). The reaction was heated at 55 °C for 30 min before it was evaporated to dryness *in vacuo*. Traces of TFA were removed by resuspending the mixture in 10% acetonitrile in water (v/v) and subsequently drying *in vacuo*. The resulting PTH derivative of *S*-(3-lactic acid)cysteine was dissolved in 250 μL of 20% acetonitrile in water (v/v), of which 50 μL was manually injected into the Applied Biosystems 120A PTH analyzer.

Construction and Expression of the C831A PPDK Mutant. The C831A PPDK mutant was generated using recombinant PCR techniques (Erlich, 1992). The sequences of the two outside primers were 5′-GAGATCATGATTCCGTTAG-3′ (primer 1) and 5′-CAACTCTGGGGCTTCGC-3′ (primer 2), which corresponded respectively to nucleotides 2173−2191 and 2710−2727 of the cloned PPDK gene sequence (Pocalyko et al., 1990). The sequence of the mutagenic primer corresponded to nucleotide positions 2549−2572 (positive strand): 5′-GTGTGGCATCGCTGGCGAGCACGG-3′ (primer 3). The PCR step was performed according to the manufacturer's instruction (Thermolyne Temp. Tronic). Following each reaction the sample was chromatographed on a 1% LMP agarose gel with TBE buffer (10.8 g/L Tris, 5.5 g/L boric acid, 0.74 g/L EDTA). DNA was extracted using the Geneclean II kit or the MerMaid kit from Bio101. The resulting 580 base fragment was digested with *Kpn*I and *Stu*I. The mutated fragment was used to replace the *Kpn*I/*Stu*I fragment of plasmid pACYC184-D12 (which contains the entire PPDK coding sequence in between the unique *Eco*RI site of plasmid pACYC-184; Pocalyko, 1990). The resulting plasmid, called pACYC184-D12-C831A, was used to transform competent *E. coli* JM101 cells. The sequence of the mutated *Kpn*I−*Stu*I fragment was determined by the chain termination method (Sanger et al., 1977; Tabor & Richardson, 1987) using the Sequenase kit from USB. The cloned cells were grown, harvested, and lysed according to the method of Pocalyko et al. (1990), and the PPDK C831A mutant was purified according to the procedure reported for wild-type PPDK (Wang et al., 1988). Fractions from the DEAE-cellulose column and from the Sephacryl S-200 column which comprised the largest protein peak were pooled and analyzed by SDS−PAGE. R_f values were compared to those of wild type for the purpose of identification. Concentration of the protein samples was achieved using a Centricon device.

Rapid Quench Analysis of Wild-Type PPDK and C831A Mutant PPDK Single-Turnover Reactions. Single turnover time courses were determined using the rapid quench instrument from Kin Tek. Forty microliters of solution containing 10 μM [^{14}C]ATP, 2.5 mM P_i, or 1 μM [^{32}P]PEP,

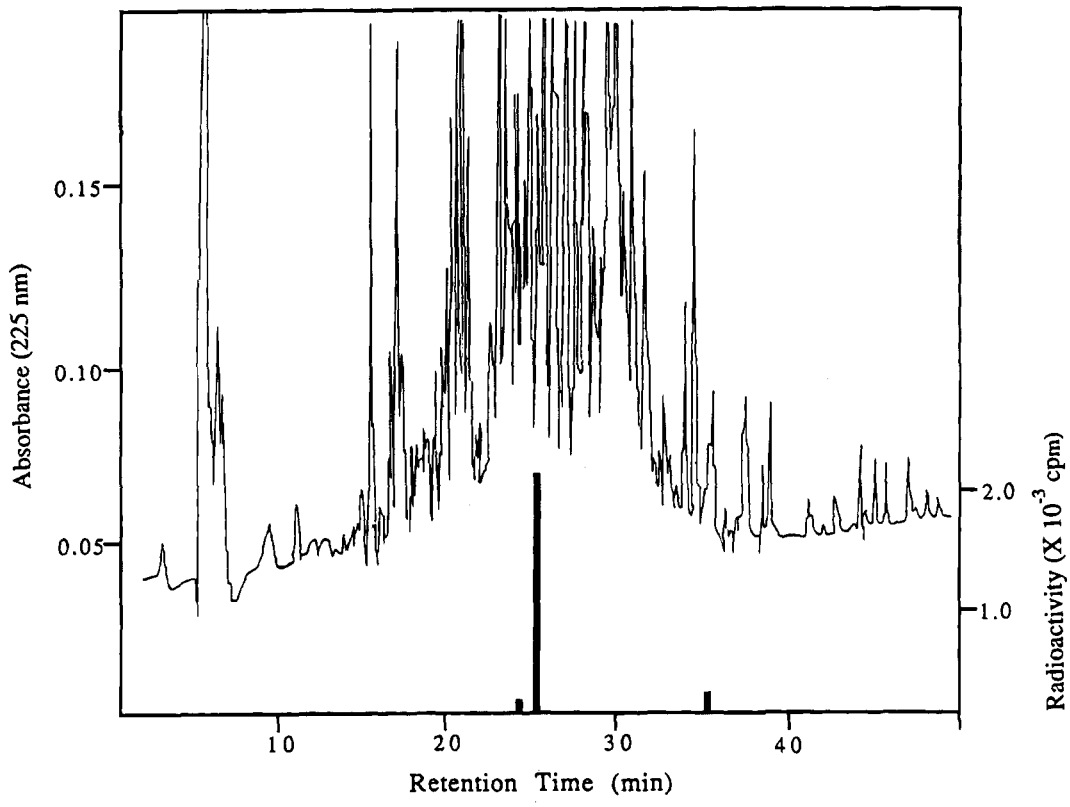


FIGURE 1: Reversed-phase HPLC elution profile of the tryptic peptides of the [1-^{14}C]bromopyruvate/$NaBH_4$/DTT/iodoacetate-treated PPDK. Two hundred microliters of the digested enzyme (see Materials and Methods) were injected. The peptides were eluted with a step gradient of 0.12% TFA in water and 0.1% TFA in acetonitrile as described in Materials and Methods. Plots: (—) OD 225; (■) radioactivity.

in 50 mM K^+Hepes (pH 7.0), was mixed with 40 μL of 80 μM PPDK active sites, 5 mM $MgCl_2$, and 20 mM NH_4Cl in 50 mM K^+Hepes (pH 7.0). The reactions were quenched with 164 μL of 0.6 M HCl. The enzyme was precipitated from the quenched solution by vortexing with 100 μL of CCl_4 and pelleted by centrification. The enzyme pellet was removed from the solution using a micropipet and blotted dry on a Kimwipe. The enzyme pellet was (in the case of the [^{32}P]PEP reaction) dissolved by boiling in 500 μL of 10 N H_2SO_4, and the amount of ^{32}P label present in the sample was determined by liquid scintillation counting. The aqueous supernatant of the CCl_4 mixture was transferred to a fresh test tube and neutralized with 5 M NaOH. The radiolabeled PEP was separated on a Beckman HPLC ultrasil anion-exchange analytical column with 0.4 M KCl and 0.1 M KH_2PO_4 (pH 2.8) serving as eluant. In the case of the [^{14}C]ATP reaction the separation was carried out on a Beckman ultrasphere reversed-phase C_{18} analytical column using 25 mM KH_2PO_4, 2.5% triethylamine, and 5% methanol (pH 4.1) as eluant. Fractions were collected and analyzed by scintillation counting.

RESULTS AND DISCUSSION

The goal of the present work was to locate the pyruvate/PEP binding site within the primary structure of *C. symbiosum* PPDK. This was accomplished by reacting the enzyme with [1-^{14}C]bromopyruvate, cleaving it with trypsin, and then sequencing the radiolabeled peptide isolated from the tryptic digest. The role of the modified residue, Cys 831, in pyruvate binding and/or catalysis was tested by carrying out site-directed mutagenesis and examining the catalytic properties of the Cys 831 → Ala 831 mutant.

Chemical Modification of PPDK with [1-^{14}C]Bromopyruvate. PPDK was alkylated with [1-^{14}C]bromopyruvate, and then, to stabilize the attachment of the pyruvate moiety to the protein (and destroy unreacted reagent), the reaction mixture was reduced with $NaBH_4$, forming the corresponding lactate derivative. The Cys residues of the (modified) protein, which are readily oxidized during handling, were treated with DTT and then with iodoacetic acid (in order to avoid technical difficulties experienced during the isolation of the ^{14}C-labeled tryptic peptide). The resulting protein sample was treated with trypsin, and the digest was then subjected to reversed-phase HPLC. The radioactivity of the column fractions collected was monitored by scintillation counting and the peptide content by UV absorption. The elution profile obtained is shown in Figure 1. The total recovery of radioactivity from the column was 90% of that applied. The most radioactive fraction, which contained 85% of the eluted radioactivity, was rechromatographed twice by reversed-phase HPLC with approximately 90% recovery of the applied radioactivity. Sequence analysis of this fraction showed it to contain two different peptides. The quantities of the two peptides present were sufficiently different as to allow the amino acid residues corresponding to each peptide to be assigned. The sequences of the two peptides are given in Figure 2.

Two separate experiments including labeling, digestion, and HPLC separation were performed, and sequence analysis of the major radioactive fraction obtained from each trial gave the same result shown in Figure 2. Peptide B corresponded exactly to positions 23−32 in the published *C. symbiosum* PPDK primary sequence (Pocalyko et al., 1990). Peptide A, on the other hand, corresponded to

(CM)
Typtic Peptide B: Gly Cys Asn Leu Ala Glu Met Thr Ile.....

PPDK Sequence: ^{21}Gly Lys Gly Cys Asn Leu Ala Glu Met Thr Ile Leu Gly Met Pro

Ile Pro Gln Gly Phe Thr Val Thr Thr Glu Ala Cys Thr Glu Tyr

Tyr Asn Ser Gly Lys Gln^{57}. .....

(CM)
Tryptic Peptide A: Cys Gly Ile (?) Gly Glu His Gly Gly.....

PPDK Sequence: ^{825}Leu Lys Cys Gly Ile Cys Gly Glu His Gly Gly Asp Pro Ser Ser

(Corrected) Val Glu Phe Cys His Lys Val Gly Leu Asn Tyr Val Ser Cys Ser Pro Phe Arg Val Pro Ile

Ala Arg Leu Ala Ala Ala Gln Ala Ala Leu Asn Asn Lys^{874}End

FIGURE 2: Comparison of the sequences of tryptic peptides A and B [observed in the radioactive peptide fraction isolated by HPLC (Figure 1) from the digest of [1-^{14}C]bromopyruvate/$NaBH_4$/DTT/iodoacetate] treated PPDK with the corresponding regions of the (corrected; see supplementary material for details) sequence of the *C. symbiosum* PPDK. The Lys residues flanking the tryptic peptides are underlined. The Cys residues of the tryptic peptide identified as the PTH derivative of *S*-(carboxymethyl)cysteine are denoted as (CM)Cys.

positions 828−835 in the published sequence (Pocalyko et al., 1990) with one exception. The exception is the C-terminal residue of peptide A, identified as Gly. This residue corresponds to position 836 of the PPDK sequence reported to be Glu (Pocalyko et al., 1990). Because of this apparent discrepancy we repeated the sequence determination of the cloned gene in this C-terminal region. The sequence of the positive strand corresponded to that reported by Pocalyko et al. (1990); however, the sequence of the negative strand revealed a compressed GG doublet at nucleotide positions 2575−2576. The G base that was missed in the original sequence resulted in a frame shift and premature termination of the sequence. *The corrected C-terminal sequence (Figure 2) leads to a protein containing 874 amino acids and having MW = 96 028.*

The presence of two Cys-containing peptides (peptides A and B, Figure 1) observed in the radioactive fraction of the trypsin digest of [1-^{14}C]bromopyruvate-treated PPDK required further analysis to be made to demonstrate which peptide contains the radiolabeled Cys residue. Only the first nine amino acid residues could be identified for tryptic peptides A and B, because it was very difficult to isolate a sufficient amount of pure peptide for additional cycles to be carried out. However, PTH amino acid analysis for cycle 1 recorded a clear signal for carboxymethylated cysteine for peptide A, and the analysis for cycle 2 gave the same signal for peptide B. This indicated that the first Cys of peptide A and the only Cys of peptide B were not labeled by the [1-^{14}C]bromopyruvate but instead had been carboxymethylated during the workup with iodoacetic acid. Cycle 4 showed a clear signal of Leu for peptide B but no detectable signal for peptide A. The PPDK amino acid sequence predicts a Cys at position 4 in peptide A. During the sequence analysis, 60% of the PTH-amino acid derivative released from each cycle was used for identification, and the remainder was collected manually for measurement of radioactivity. Approximately 85% of the applied radioactivity was recovered, of which 90% occurred at the fourth cycle and 10% at the fifth cycle. The radioactivity associated with the fifth cycle could be explained as the usual "carryover" of the sample delivery system. These results, therefore, strongly suggested that the fourth amino acid in peptide A corresponds to the Cys residue modified by the [1-^{14}C]bromopyruvate. Peptide B is not ^{14}C labeled and is merely a contaminant of the radiolabeled peptide, peptide A.

S-(3-Lactic acid)cysteine is the expected product when the bromopyruvate-labeled cysteine residue is reduced with $NaBH_4$ and subsequently cleaved from the peptide backbone. This synthetic standard (prepared as described in Materials and Methods) was applied to an amino acid analyzer and a sharp peak, which eluted just before the aspartic acid standard, was detected. This chromatographic behavior is consistent with the fact that *S*-(3-lactic acid)cysteine is more acidic than aspartic acid and, therefore, should migrate faster on the anion-exchange column of the amino acid analyzer. The PTH derivative was prepared from the synthetic *S*-(3-lactic acid)cysteine and subsequently applied (200 pmol) manually to the PTH analyzer [since it is the PTH derivative of the *S*-(3-lactic acid)cysteine which we would be looking for in cycle 4 of the automated analysis of peptide A]. Examination of the elution profile revealed that the PTH derivative of *S*-(3-lactic acid)cysteine could not be assigned as a single component. Three sample peaks were detected, indicating that this PTH derivative is unstable under the conditions of analysis. Fortuitously, peak 1 coeluted with the PTH derivative of Ser and peak 2 with the PTH derivative of Thr. Peak 3 eluted just after the PTH derivative of Asp. The intensities of these peaks showed that they would not be detected unless more than 10 pmol of the PTH derivative is applied. The apparent instability of the PTH derivative of *S*-(3-lactic acid)cysteine would possibly account for why no signal was detected for the fourth amino acid in peptide A, as less than 10 pmol of the peptides was applied to the sequencer. *Taken together, these results allowed the assignment of the PPDK residue specifically alkylated with [1-^{14}C]bromopyruvate as Cys 831.*

Examination of the Role of Cys 831 by Site-Directed Mutagenesis. The studies described in the previous section demonstrate that Cys 831 is the PPDK residue alkylated by treatment with [1-^{14}C]bromopyruvate. The location of this residue in or nearby the pyruvate/PEP binding site of PPDK was first suggested by the observation that bromopyruvate is a competitive inhibitor vs pyruvate and that, once alkylated, the enzyme is catalytically inactive (Yoshida & Wood, 1978). Later, Carroll et al. (1994) reported the selective alkylation at the 35 kDa domain of PPDK by [1-^{14}C]bromopyruvate and the protection against alkylation afforded by PEP.

Cys 831 is conserved among the four known PPDK sequences [*Zea mays* (Hudspeth et al., 1986; Matsuoka et al., 1988), *Flaveria trinevia* (Rosche & Westhoff, 1990), *Clostridum symbiosum* (Pocalyko et al., 1990), and *Entamoeba histolytica* (Bruchhaus & Tannich, 1993)], the *E. coli* PEP synthetase sequence (Niersbach et al., 1992), and the numerous known sequences of enzyme I of the bacterial PEP:sugar phosphotransferase system [see Reizer et al. (1993) for a recent sequence alignment)], suggesting that it may play a direct role in pyruvate binding and/or catalysis. This possibility was examined by substituting the Cys at position 831 with Ala. The C831A PPDK mutant was expressed in *E. coli* JM101 transformed with mutated plasmid pACYC184-D12-C831A and purified by the method of Wang et al. (1988). The yield of mutant protein averaged 15 mg/g of cell compared to 20−25 mg/g of cell obtained with the wild-type clone (*E. coli* JM101 transformed with plasmid pACYC184-D12). The mutant protein's chromatographic properties, UV and CD spectral properties, and stability toward proteolysis (during isolation and storage) were indistinguishable from that of wild-type PPDK.

The catalytic properties of the C831A PPDK mutant were first examined using a spectrophotometric assay which measures multiple turnovers for the full enzymatic reaction in the PEP + AMP + PP_i → pyruvate + ATP + P_i direction (Wang et al., 1988). The formation of pyruvate is detected by monitoring its *in situ* reduction by NADH (0.4 mm) and LDH (10 units/mL) at 340 nm. Wild-type PPDK catalyzes the reaction of 0.5 mM PEP, 0.5 mM AMP, and 1 mM PP_i in solution with 5 mM $MgCl_2$, 40 mM NH_4Cl, and 50 mM imidazole hydrochloride (pH 6.8, 25 °C) with a specific activity ranging from 20 to 30 units/mg. Using these conditions and a high concentration of mutant enzyme (10 μM), we observed a reduction in absorbance at 340 nm proceeding at a rate 14-fold greater than the background rate (where the background rate was measured for the reaction solution containing all components except PP_i). On the basis of this measurement a specific activity = 0.01 unit/mg (roughly 0.03−0.04% the activity of wild-type PPDK) was calculated. The substitution of Cys 831 with Ala was thereby shown to have a substantial inhibitory effect on PPDK catalysis.

Single-turnover experiments using rapid quench techniques in conjunction with radiolabeled substrates were carried out next to dissect which partial reaction(s) (see Scheme 1) was (were) inhibited by the point mutation. The nucleotide partial reaction, comprised of chemical steps 1 and 2 of Scheme 1, was examined by reaction of limiting [^{14}C]ATP (5 μM) with excess enzyme (40 μM active sites) in the presence of saturating P_i (2 mM), $MgCl_2$ (2.5 mM), and NH_4Cl (10 mM) at pH 7.0 (50 mM K^+Hepes buffer, 25 °C). The reaction solution was quenched at varying conversions with acid, releasing [^{14}C]AMP and unconsumed [^{14}C]ATP from the enzyme. The radiolabeled nucleotides were separated by HPLC and quantitated by liquid scintillation counting.

The time courses for a single turnover on the wild-type PPDK and the C831A mutant generated from these experiments, and shown in Figure 3, are essentially identical. A single-exponential fit to the data of Figure 3 provided an observed turnover rate of 6.6 ± 0.7 s^{-1} for wild-type PPDK and 3.2 ± 0.4 s^{-1} for the C831A mutant PPDK. Hence, the efficiency of PPDK catalysis of the nucleotide partial reaction appears to be virtually unaffected by the mutation.

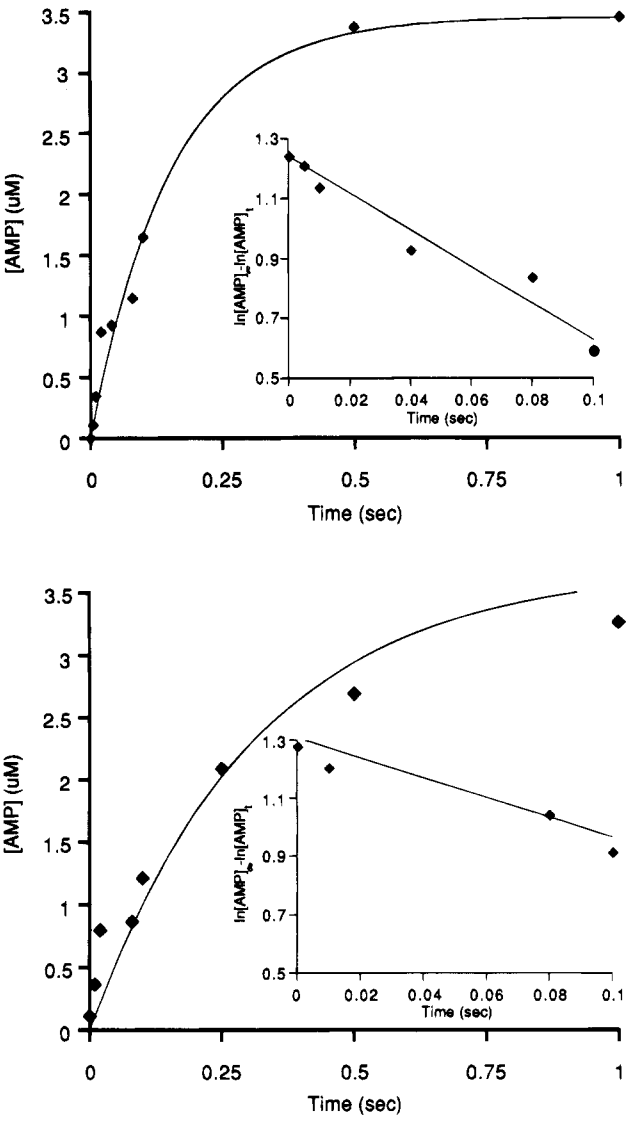


FIGURE 3: Time course for a single turnover of [^{14}C]ATP (5 μM) with 40 μM wild-type PPDK (A, top) or C831A PPDK mutant (B, bottom) active sites in the presence of 2.5 mM P_i, 2.5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) at 25 °C.

Next, the time course for a single turnover of limiting [^{32}P]PEP (1 μM) by excess enzyme (40 μM active sites) in the presence of 2.5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0, 25 °C) was measured. The reaction was quenched at varying conversions with acid and the protein removed for ^{32}P analysis by precipitation/centrifugation. The supernatant was analyzed for [^{32}P]PEP content by HPLC separation in conjunction with scintillation counting. Shown in Figure 4 is the time course for the appearance of ^{32}P-labeled enzyme formed during the reaction. While phosphorylation of the wild-type enzyme had reached equilibrium within 200 ms, essentially no phosphorylated enzyme was formed with the C831A mutant at 1 s (Figure 4) and at 10 s (data not shown). Analysis of the reaction supernatant revealed that 48% of the [^{32}P]PEP had been consumed by the wild-type PPDK (consistent with the known internal equilibrium constant for E·PEP⇌E-P·pyruvate = 1.1; Mehl et al., 1994) while (within limits of detection) no [^{32}P]PEP had been consumed by the C831A mutant enzyme. *Thus, substitution of Cys 831 with Ala has a significant inhibitory effect on catalysis of the pyruvate partial reaction (step 3 of Scheme 1), while having little or no apparent effect on*

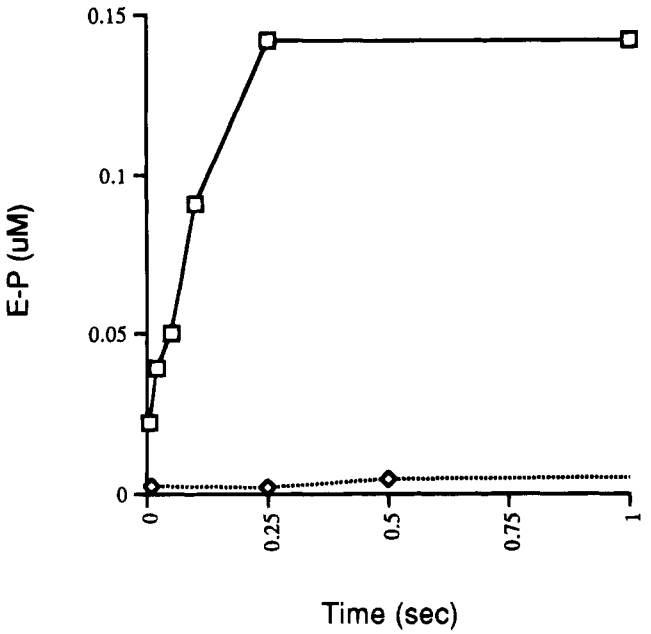


FIGURE 4: Time course for a single turnover of [^{32}P]PEP (1 μM) with 40 μM wild-type PPDK (□) or C831A PPDK mutant (◇) active sites in the presence of 2.5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) at 25 °C.

catalysis of the nucleotide partial reaction (steps 1 and 2 Scheme 1; Figure 3).

CONCLUSIONS

Chemical modification of PPDK with [1-^{14}C]bromopyruvate followed by sequence analysis of a tryptic peptide which retains the radiolabel has, in the present study, allowed us to assign the modified Cys residue as Cys 831. From previous studies it was known that the alkylation of PPDK with bromopyruvate destroys catalytic activity (Yoshida & Wood, 1978; Carroll et al., 1994). It, however, was unclear at the outset of our studies whether the loss of catalytic activity results from the masking of an amino acid residue which directly participates in substrate binding and/or catalysis or from the presence of the appended pyruvate moiety which could potentially inhibit substrate binding and/or catalysis in numerous indirect ways. To distinguish between these two possibilities, the catalytic properties of the Cys 831 → Ala 831 mutant were examined. *On the basis of the data represented in Figures 3 and 4, we propose that Cys 831 plays a role in substrate binding and or catalysis of the pyruvate partial reaction (step 3 of Scheme 1).*

SUPPLEMENTARY MATERIAL AVAILABLE

A figure and a discussion of the corrected nucleotide and amino acid sequence for *C. symbiosum* PPDK (3 pages). Ordering information is given on any current masthead page.

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