Separate Site Catalysis by Pyruvate Phosphate Dikinase As Revealed by Deletion Mutants†

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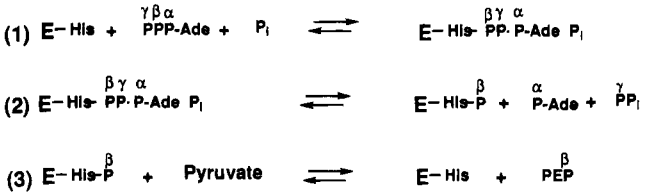
ABSTRACT: Previous studies had indicated that pyruvate phosphate dikinase (PPDK), an enzyme which catalyzes the interconversion of adenosine 5′-triphosphate (ATP), orthophosphate (P_i), and pyruvate with adenosine 5′-monophosphate (AMP), pyrophosphate (PP_i), and phosphoenolpyruvate (PEP), is made up of 25, 13, 18, and 35 kDa domains [Carroll, L. J., Xu, Y., Thrall, S. H., Martin, B. M., & Dunaway-Mariano, D. (1994) *Biochemistry 33*, 1134]. The catalytic histidine (which mediates the phosphoryl group transfers from ATP to P_i and pyruvate) is located on the 18 kDa domain while the 25 and 13 kDa domains appear to contain the ATP binding site and the 35 kDa domain appears to contain the pyruvate binding site, respectively. The goal of this investigation was to examine functional interdependency of the putative ATP and pyruvate binding domains. Two truncated forms of PPDK were created by using recombinant DNA techniques. The 35 kDa (C-terminal) deletion mutant was found to catalyze the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction but not the E-P + pyruvate ⇌ E + PEP partial reaction. The 25 kDa (N-terminal) deletion mutant was found to catalyze the E-P + pyruvate ⇌ E + PEP partial reaction but not the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction. Neither mutant catalyzes the full ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP reaction. These results are interpreted to mean that the ATP and pyruvate binding domains in PPDK are functionally independent, thus providing evidence for separate active sites for catalysis of the two partial reactions.

Pyruvate phosphate dikinase (PPDK)[1] catalyzes the interconversion of ATP, pyruvate, and P_i with AMP, PEP, and PP_i in certain microorganisms and in C_4 plants (Wood et al., 1977). A novel feature of this enzyme is the use of a histidine residue to abstract the P_β−P_γ pyrophosphate unit from ATP and sequentially deliver the γ-P phosphoryl and β-P phosphoryl group to P_i and pyruvate, respectively (Scheme 1). The reaction takes place in three chemical steps involving the formation and reaction of a pyrophosphorylenzyme intermediate and a phosphorylenzyme intermediate (Wood et al., 1977; Carroll et al., 1989; Thrall et al., 1993). For convenience the first two steps 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).

Scheme 1: Chemical Steps of PPDK Catalysis (Wood et al., 1977; Carroll et al., 1989; 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.

Chemical modification (Yoshida & Wood, 1978; Evans et al., 1980), transient kinetic (Thrall & Dunaway-Mariano, 1994), and stereochemical (Cook & Knowles, 1985) studies of the enzyme have indicated that the nucleotide and pyruvate binding sites on the enzyme are nonoverlapping, kinetically independent sites and that the catalytic histidine must, in effect, move between the two sites during turnover. Several models illustrating possible modes of histidine movement between reaction sites have been described (Wood et al., 1977; Carroll et al., 1994). These include simple rotation of the histidine about its C_α−C_β or C_β−C_γ bond, translocation of the histidine via a "swinging peptide arm", and movement of the histidine relative to substrate binding sites by global (domain) conformational changes. Possibly relevant to the mechanism of separate site catalysis is the apparent organization of the PPDK subunit into structural

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[1] Abbreviations: PPDK, pyruvate phosphate dikinase; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; P_i, orthophosphate; PP_i, pyrophosphate; E, free enzyme; E-P, phosphorylenzyme; E-PP, pyrophosphorylenzyme; LDH, lactate dehydrogenase; NADH, dihydronicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride, PVDF, polyvinylidene difluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LB, Luria−Bertani; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; oAMP, 2′,3′-dialdehyde adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Met_0, N-terminal methionine residue; Tween, poly(oxyethylene) (20) sorbitan monolaurate.

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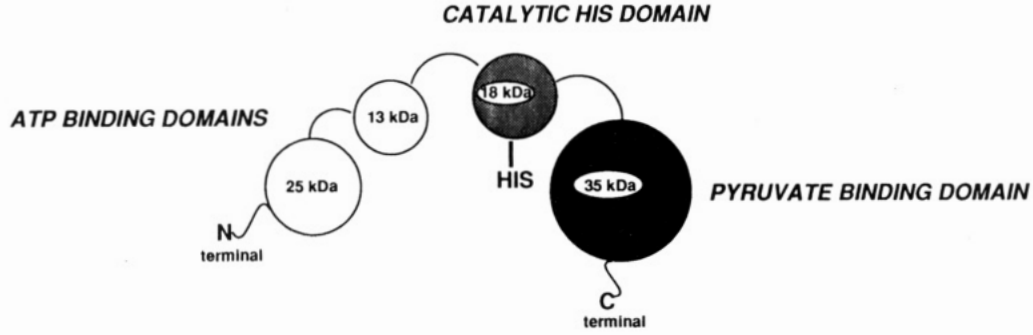
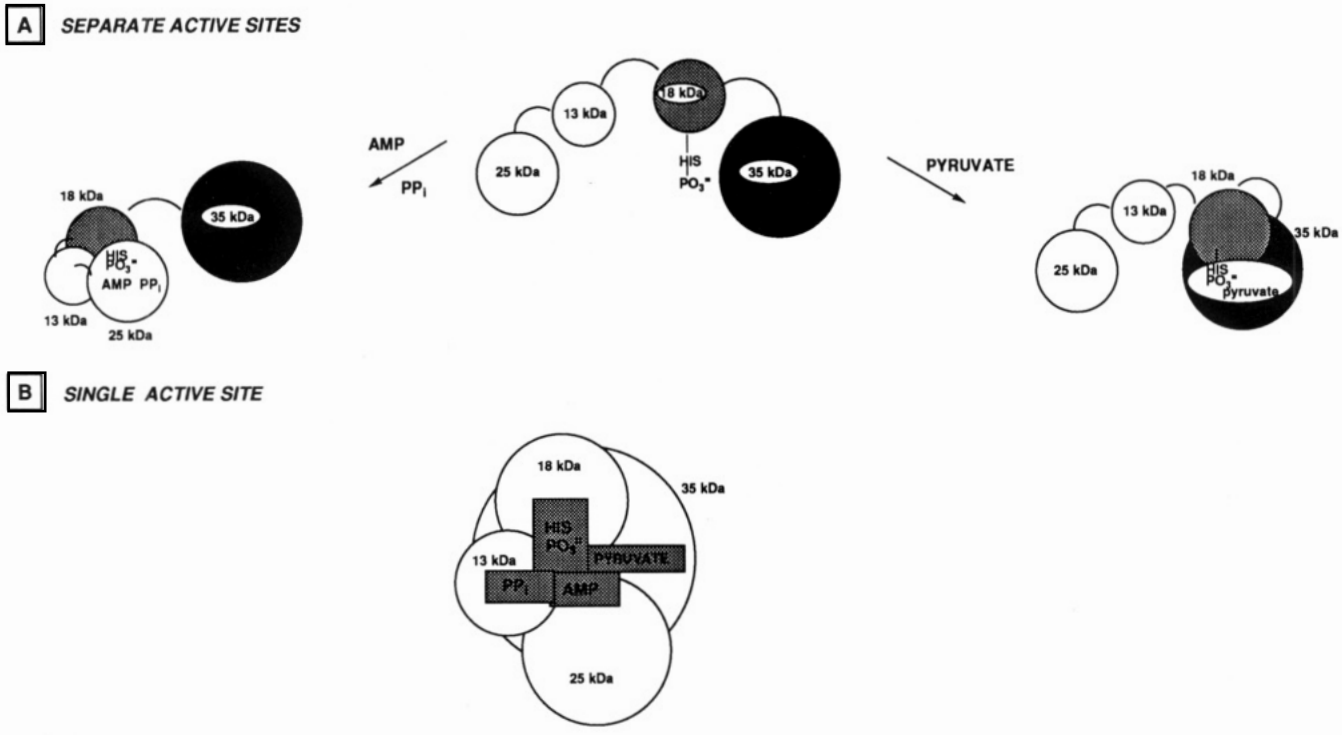
Chart 1: Proposed Model of the *C. symbiosum* PPDK Structural/Substrate Binding Domains Based on Previous Proteolysis, Site-Directed Mutagenesis, and Chemical Modification Studies (Carroll et al., 1994)[a]



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

Scheme 2: Hypothetical Models of the PPDK Active Site Illustrating (A) Separate Sites and (B) a Shared Site for Catalysis of the Nucleotide and Pyruvate Partial Reactions[a]

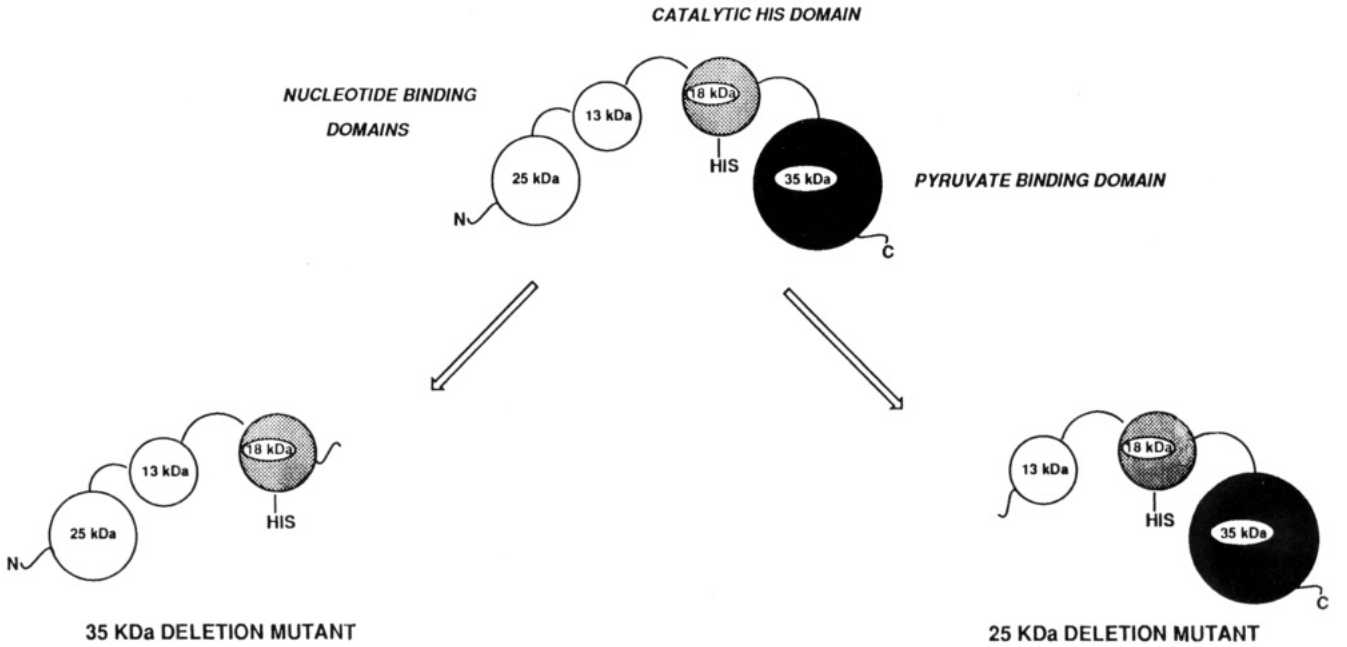


[a] Scheme 1, steps 1 plus 2 and step 3, respectively.

domains. Previous studies of the *Clostridium symbiosum* PPDK (a homodimer of 96 kDa subunits; Pocalyko et al., 1990; Xu et al., 1995) using subtilisin, a nonspecific protease, to cut at accessible "loop or hinge" regions of the protein provided evidence for the four-domain structure represented (in cartoon form) in Chart 1 (Carroll et al., 1994). Reaction of the *C. symbiosum* PPDK with radiolabeled substrates (*viz.*, [^{32}P]PEP or [β-^{32}P]ATP/P_i) to form [^{32}P]phosphoenzyme or with radiolabeled affinity labels (*viz.*, [^{32}P]azido-ATP, [^{14}C]-oAMP, and [^{14}C]bromopyruvate) to modify active site residues covalently allowed us to identify the catalytic histidine, the ATP binding site, and the pyruvate binding site within this four-domain structure (Carroll, 1991; Carroll et al., 1994; Xu et al., 1995; Yankie et al., 1995). These functional sites are, as illustrated in Chart 1, found on the 18 kDa, the 25/13 kDa, and the 35 kDa domains, respectively. Thus, the catalytic histidine and the nucleotide and pyruvate binding sites appear to be located on separate structural domains.

An intriguing model for PPDK catalysis emerging from the discovery of separate structural/functional domains requires the catalytic histidine to commute between the nucleotide site (where it abstracts the P_β–P_γ pyrophosphate unit from ATP) to the pyruvate site [where it delivers the β-P phosphoryl group to pyruvate enol(ate)] via domain movement (see Scheme 2A). This model, which is based on the assumption of geographically separate catalytic sites for the nucleotide and pyruvate partial reactions, predicts that the 35 kDa (pyruvate binding) domain is not required for catalysis of the nucleotide partial reaction and that the 25 kDa (nucleotide binding) domain is not required for catalysis of the pyruvate partial reaction. A more conventional model for PPDK catalysis assumes a single active site which is formed at the interface of the four domains (Scheme 2B). Movement of the catalytic histidine between the nucleotide binding site and the pyruvate binding site within this active site would require comparatively localized conformational

Scheme 3: A Model Illustrating the Hypothetical Domain Structure of the PPDK 25 kDa Deletion Mutant and the PPDK 35 kDa Deletion Mutant



changes possibly involving rotation about the histidine Cα−Cβ or Cβ−Cγ bond or movement of a flexible peptide "arm" attached to the histidine. If it is assumed that removing the 25 or 35 kDa domain from the structure represented in Scheme 2B would disrupt the active site structure in a global sense, then it should be possible to distinguish between the separate active site model of Scheme 2A and the shared active site model of Scheme 2B by measuring the impact of deletion of the 25 or 35 kDa domain of PPDK on catalysis.

In the present study we have constructed mutants of the *C. symbiosum* PPDK which lack the 25 kDa "nucleotide binding" domain (referred to as the 25 kDa deletion mutant) or the 35 kDa "pyruvate binding" domain (referred to as the 35 kDa deletion mutant) (see Scheme 3). These constructions were simplified by the fortunate arrangement of the two target domains at the N-terminus (25 kDa domain) and C-terminus (35 kDa domain) of the protein chain. The catalytic activity of these truncation mutants toward the E + ATP + P_i ⇌ E-P + AMP + PP_i and the E-P + pyruvate ⇌ E + PEP partial reactions was tested as a possible means of distinguishing between the separate active site model of Scheme 2A and the shared active site model of Scheme 2B.

MATERIALS AND METHODS

Construction of the 35 and 25 kDa Deletion Mutants. Deletion of the region encoding the 35 kDa C-terminal domain from the cloned PPDK gene (Pocalyko, 1990) was carried out using recombinant PCR techniques. The sequences of the two outside primers were 5′-GCACACCA-CAGCCTATC-3′ (primer 1) and 5′-GAGAAGAACTCT-GCTTC-3′ (primer 2), which corresponded respectively to nucleotides 926−942 and 2344−2369 of the cloned PPDK gene sequence (Pocalyko et al., 1990). The sequences of the two inside (mutagenic) primers were 5′-GTCAGC-TTA*GAGCTC*TACCATACCATGATACGCTC-3′ (primer 3) and 5′-GTATGGTAG*GAGCTC*TAAGCTGACAAG-TTCAGAACA-3′ (primer 4), which corresponded respectively to nucleotides 1633−1656 and 1645−1668 of the cloned PPDK gene sequence. The two mutagenic primers were designed to insert the sequence 5′-TAG-*GAGCTC*TAA-3′ in between nucleotide 1650 and 1651, which created a *Sac*I restriction site (*GAGCTC*) and two stop codons (TAG and TAA) for the deletion of the C-terminal 35 kDa domain. The PCR step was performed according to the manufacturer's instruction (Thermolyne Temp. Tronic). The 1.4 kb secondary PCR product was digested with *Bst*XI and *Kpn*I, and the 1.3 kb *Bst*XI/*Kpn*I fragment within the wild-type pACYC184-D12 plasmid was replaced by the mutated fragment. The resulting mutant plasmid, called pACYC184-D12-57, was used to transform competent *Escherichia coli* JM101 cells. The mutants were selected for the presence of the new *Sac*I restriction site and confirmed by DNA sequencing by the dideoxy chain termination method (Sanger, 1977) using the modified form of T7 DNA polymerase (Tabor & Richardson, 1987). The entire sequence of the 1.3 kb *Bst*XI/*Kpn*I fragment within plasmid pACYC184-D12-57 was determined to verify that no unwanted mutations were introduced by the PCR amplification.

The 25 kDa (N-terminal) truncation mutant was constructed using plasmid pACYC184-D12, which contains the entire PPDK coding sequence in between the unique *Eco*RI site of plasmid pACYC184. The plasmid pACYC184-D12 was linearized with *Nco*I and then subjected to complete digestion with *Hpa*I. The resulting 7.1 kb *Hpa*I/*Nco*I fragment was purified. The deleted 0.8 kb *Hpa*I/*Nco*I fragment carried the complete coding sequence for the N-terminal 25 kDa domain plus a 30 bp sequence immediately upstream of the initiating ATG of the wild-type PPDK and a 45 bp sequence encoding the N-terminal amino acids of the desired truncation mutant. These sequences, plus an initiating ATG codon, were added back to the plasmid by ligation of the 7.1 kb *Hpa*I/*Nco*I fragment with an 80 bp synthetic linker. The resulting mutated plasmid was named pACYC184-D12-67. The ligation mixture was used to transform competent *E. coli* JM101 cells. The

mutants were selected on the basis of endonuclease restriction map analysis and confirmed by plasmid DNA sequencing using the dideoxy chain termination method referenced above.

Expression of the 25 and 35 kDa Deletion Mutants. A single *E. coli* colony containing the mutated pACYC184-D12 plasmid was used to inoculate 5 mL of LB medium containing 12.5 μg/mL tetracycline. The culture was incubated at 37 °C with vigorous shaking until the cell density corresponded to 1.5 OD units at 600 nm. The cells (50 μL) were pelleted by centrifugation and then resuspended in 30 μL of sample buffer containing 1% SDS, 1% β-mercaptoethanol, 0.25% bromophenol blue, 10% glycerol, and 50 mM Tris·HCl at pH 6.8. After incubation in a boiling water bath for 2 min, the sample was applied to a 12% SDS−PAGE gel. Following electrophoresis the proteins were transfer blotted from the gel to a PVDF membrane. The protein bands corresponding to the 25 and 35 kDa deletion mutants were excised, and the N-terminal sequences of the respective mutant proteins were determined by automated degradation using an Applied Biosystems Model 470 gas-phase protein sequencer.

Preparation of Affinity Columns. The *E. coli* affinity column was prepared by packing a small column with 3 mL of commercial (5 Prime → 3 Prime, Inc.) resin, consisting of lysate from *E. coli* Y1090 coupled to Sepharose 4B, as a 50% (v/v) slurry. This column was used to remove components from antisera which bind to antigens produced by *E. coli*. The column was equilibrated with 10 volumes of PBS (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 3 mM KCl, pH 7.4). The PPDK affinity column was prepared by incubating 30 mg of PPDK in 5 mL of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3) with 1 g of CNBr-activated Sepharose 4B (solid) suspended and washed in 1 mM HCl (2.5 h, 25 °C). The resulting gel was washed according to the manufacturer's instruction and used to pack a 3-mL column. This column was used to purify the PPDK-specific antibodies from the antisera.

Preparation of PPDK-Specific Antibodies. Polyclonal antisera were obtained from Duncroft, Inc., by immunizing New Zealand white rabbits with highly purified PPDK (Wang et al., 1988). Three milliliters of antiserum was diluted to 15 mL with PBS before application to the *E. coli* affinity column (3 mL). The column was washed with 10 volumes of PBS, and the fractions collected were monitored at 280 nm. The fractions comprising the protein peak were pooled and applied to the PPDK affinity column (3 mL). The column was washed with 5 volumes of PBS containing 1 M NaCl, followed by 10 volumes of 50 mM sodium acetate, pH 5.0. The PPDK-specific antibodies were then eluted with 3 volumes of 100 mM glycerin (pH 3). The elute was adjusted to pH 7.5 with 1 M NaOH and stored in 0.02% sodium azide at 4 °C.

Immunoblot Assay. Protein samples were separated by 12% SDS−PAGE and electrophoretically transferred to S&S NC nucleic acid and protein transfer membranes. The membranes were treated with 3% (w/v) non-fat dried milk in PBS for 1 h at 25 °C and then incubated for 2 h with a 1:1000 dilution of the purified PPDK-specific antibodies (see above) in PBS. Excess antibodies were removed by washing the membranes three times with PBS containing 0.4% Tween (ICI). The membranes were then treated with horseradish peroxidase-labeled goat anti-rabbit IgG and tetramethylbenzidine membrane peroxidase substrate according to the vendor's protocol (Kirkegard & Perry Laboratories, Inc.). After the development of the enzyme reaction, the membranes were washed with water and air-dried. Preimmune serum was used as a control as it did not react with wild-type PPDK.

Purification of the 25 and 35 kDa Deletion Mutants. The mutant proteins were purified from lysed cells containing protease inhibitors (1 mM 1, 10-phenanthroline, 1 mM benzamidine hydrochloride, 1 mM PMSF and 50 mg/L trypsin inhibitor) using the method of Wang et al. (1988). The cell-free lysate was fractionated with ammonium sulfate. The 35 kDa deletion mutant precipitated in the 50−70% fraction (as did wild-type PPDK) whereas the 25 kDa deletion mutant precipitated in the 0−50% fraction. After dialysis the mutant proteins where chromatographed on a DEAE-cellulose column (30 × 2.6 cm) using a 88−500 mM KCl gradient. Both deletion mutants eluted at 300 mM KCl (whereas wild-type PPDK eluted at 200 mM KCl). The fractions containing the respective PPDK deletion mutants proteins were pooled, concentrated using an Amicon device, and then applied to a Sephadex G-200 column (equilibrated with a buffer containing 20 mM imidazole, 100 mM KCl, 0.1 mM Na_2EDTA, and 0.7 mM β-mercaptoethanol at pH 6.4). The same buffer was used to elute the proteins from the column. Column fractions were analyzed by using 12% SDS−PAGE separation and the immunoblot assay.

Determination of the Catalytic Activity of the 25 and 35 kDa Deletion Mutants. The [^{32}P]PEP and [β-^{32}P]ATP were prepared according to Carroll et al. (1989). The partial reactions were carried out with either 5 μL of crude cell lysate [from cells pelleted from 250 mL of culture displaying an absorbance of 1.5 at 600 nm, suspended in 25 mL of 3 mM β-mercaptoethanol and 50 mM K^+Hepes (pH 7.0), and lysed in a French pressure cell at 12 000 psi] or 15 μL of a DEAE-cellulose column fraction in combination with 10 mM [β-^{32}P]ATP (2×10^6 cpm), 10 mM K_2HPO_4, 2.5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) (25-μL total reaction volume) or with 10 mM [^{32}P]PEP (4×10^6 cpm), 2.5 mM $MgCl_2$, 10 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0) (30-μL total volume). The reaction solutions were incubated at 25 °C for 10 min before being analyzed by 12% SDS−PAGE. Following the electrophoresis the proteins were transfer blotted from the gel to a PVDF membrane. The air-dried membrane was placed against a Kodak X-ray film and exposed at room temperature for 3−6 days. Following the development of the film the membrane was stained with Coomassie blue stain. Control reactions were performed in parallel using wild-type PPDK.

RESULTS AND DISCUSSION

Whether or not the nucleotide partial reaction (E + ATP + P_i ⇌ E-P + AMP + P_i) and the pyruvate partial reaction (E-P + pyruvate ⇌ E + PEP) (Scheme 1) take place at the same active site on the enzyme (Scheme 2B) or at geographically separate active sites (Scheme 2A) was tested by measuring the ability of the 35 and 25 kDa PPDK deletion mutants (Scheme 3) to catalyze each of the two partial reactions. The separate active site model (Scheme 2A) predicts that the 25 kDa deletion mutant will catalyze [^{32}P]E-P formation from [^{32}P]PEP but not [^{32}P]E-P formation from [β-^{32}P]ATP + P_i. Likewise, the 35 kDa deletion mutant

should be effective in forming the [^{32}P]E-P from [β-^{32}P]ATP + P_i but ineffective at catalyzing [^{32}P]E-P formation from [^{32}P]PEP. In contrast, if the active site is shared, as is illustrated in Scheme 2B, the two PPDK deletion mutants would be unable to catalyze either partial reaction since the active sites of both mutants would be disrupted through the removal of a section from each. The goal of the present work, therefore, was to prepare truncated forms of PPDK devoid of the 25 kDa N-terminal (ATP binding) domain or 35 kDa C-terminal (pyruvate binding) domain and test their catalytic activities.

Earlier attempts made in our laboratory to purify "native" N-terminal and C-terminal truncation fragments of PPDK generated from subtilisin digests of the holoenzyme were, for various technical reasons, unsuccessful (Noh and Dunaway-Mariano, unpublished data). Recombinant DNA techniques were therefore used in place of the limited proteolysis method to obtain PPDK fragments missing one of the substrate binding domains. The known subtilisin cleavage sites served as a useful guide in the design of a gene which, upon expression, could potentially generate large quantities of truncated PPDK. In previous studies recombinant DNA techniques have been used successfully to probe for, or to prove the domain structure of, many proteins including yeast tRNA ligase (Apostol et al., 1991), bacterial adenylate kinase (Labruyene et al., 1990), yeast phosphoglycerate kinase (Minard et al., 1989), and bacterial Ca^{2+}/calmodulin-dependent protein kinase II (Hagiwara et al., 1991).

Construction and Expression of the Deletion Mutants. *C. symbiosum* PPDK had been originally cloned in *E. coli* using the moderate copy number plasmid pACYC184 (Pocalyko, 1990). The observed level of expression of the PPDK in the clone was unusually high (>50% of the total protein). Experimentation with the PPDK encoding insert, and with the promoter region located within this insert, led us to the conclusion that the high level of expression observed was probably the product of a highly effective ribosome binding site [the nucleotide sequence (Pocalyko et al., 1990) in this region is a close match with the *E. coli* Shine−Delgarno consensus sequence] and/or the strong CAT promoter of the plasmid under which control the PPDK gene was fortuitously placed during cloning (Carroll, Xu, Yankie, and Dunaway-Mariano, unpublished data). In an attempt to preserve this high level of expression in creating the PPDK deletion mutants, we engineered the original PPDK−pACYC184 construct (called pACYC184-D12) so that expression of the gene would remain under the control of the same promoter and ribosome binding site.

Previous proteolysis experiments using subtilisin indicated that the C-terminal 35 kDa domain of PPDK starts at Ala 528 (Carroll et al., 1994). In other words, the subtilisin cleavage site, which leads to the release of the C-terminal 35 kDa domain, is located between Trp 527 and Ala 528. Two stop codons and a *Sac*I restriction site were therefore inserted in between the codon for Trp 527 and the codon for Ala 528 by using recombinant PCR techniques. Two stop codons rather than one (*viz.*, TAG and TAA) were introduced to avoid an eventual translation read through. The *Sac*I restriction site was introduced to simplify our initial screen for the desired mutant (pACYC184-D12 lacks a *Sac*I site). This insertion mutation resulted in a premature termination of PPDK translation at Trp 527, giving rise to a truncated form of PPDK containing the 25/13 kDa ATP binding domains and the 18 kDa catalytic histidine-containing domain.

The 25 kDa deletion mutant, on the other hand, was obtained by deleting the N-terminal 25 kDa ATP binding domain from the holoenzyme. This was achieved by removing a 0.8 kb *Hpa*I/*Nco*I fragment from plasmid pACYC184-D12. This fragment contains the coding region for Met_0-Ala-Thr 241 as well as a 30 bp sequence (containing the ribosome binding site) immediately upstream of the initiation codon ATG coding for Met_0. Since the N-terminal 25 kDa domain ends at Met 226 (subtilisin cut PPDK between Met 226 and Asp 227) and not at Thr 241, an 80 bp linker containing the 30 bp sequence upstream of the initiation codon plus a sequence coding for Met_0 Asp 227...Thr 241 of PPDK was prepared. This 80 bp synthetic linker was added to the linearized pACYC184-D12 plasmid, replacing the 0.8 kb *Hpa*I/*Nco*I fragment.

The expression levels of the two deletion mutants in *E. coli* JM101 were examined and compared with that of the wild-type PPDK. An equal quantity of cells taken from cultures of cells transformed with pACYC184 plasmid carrying the wild-type PPDK gene, the 25 kDa deletion mutant gene, or the 35 kDa deletion mutant gene were lysed, and the crude cell lysate was subjected to SDS−PAGE analysis. The positions of the deletion mutant proteins on the gel were identified by an immunoblot assay employing antibodies raised against native PPDK and then by N-terminal sequencing of the protein transferred from the gel to a PVDF membrane. The N-terminal sequence determined for the 35 kDa deletion mutant is Ala-Lys-Trp-Val-Tyr-Lys..., which corresponds to the N-terminal sequence of the wild-type enzyme (Pocalyko et al., 1990). The N-terminal sequence determined for the 25 kDa deletion mutant is Met-Asn-Asp-Ile-Pro-Gly-Asp-Trp-Gly-Thr-Ala, which corresponds to residues 226−236 of the wild-type PPDK (the N-terminal 25 kDa domain ends at residue 226). As estimated from the band intensities of the Coomassie blue stained SDS−PAGE gel, the expression level dropped from greater than 50% of the total protein as observed for the expression of wild-type PPDK to roughly 10% for the 25 kDa deletion mutant and 5% for the 35 kDa deletion mutant.

These results indicate that the 25 and 35 kDa deletion mutants are expressed correctly in the cloned *E. coli* cell but at a lower level than had been expected. Whether specific properties of the truncated forms of the encoding mRNA or of the translated mutant protein are responsible for the observed lower expression levels is not currently known. We do know however, as discussed below, that the deletion mutant proteins are more susceptible to proteolysis and to aggregation, either of which processes could contribute to lower yields of isolated protein.

Partial Purification of the 25 and 35 kDa Deletion Mutants. Initial attempts that were made to purify the two deletion mutant proteins using the method described for wild-type PPDK (Wang et al., 1988) resulted in rapid loss of the mutant proteins. Modification of the purification procedure to include protease inhibitors greatly improved the yields. The relative sensitivity of the deletion mutants (compared to wild-type PPDK) to proteolytic digestion suggests a comparatively less compact or stable structure.

Following the precipitation of the PPDK deletion mutants from the cell lysate with ammonium sulfate, DEAE-cellulose

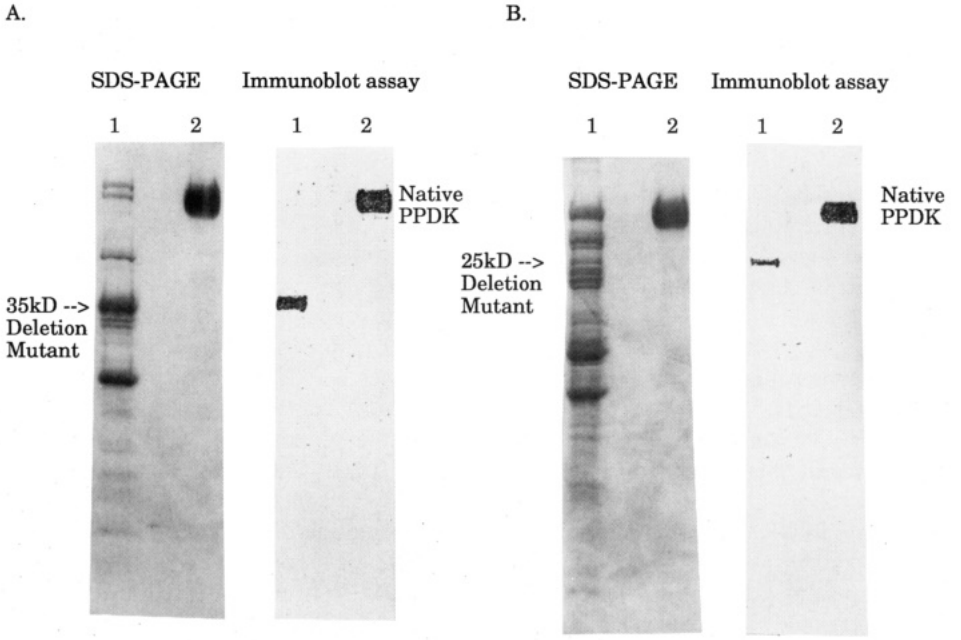


FIGURE 1: SDS−PAGE analysis of the (A) PPDK 35 kDa deletion mutant and (B) PPDK 25 kDa deletion mutant fractionated on DEAE-cellulose columns. The Coomassie blue stained PVDF membrane is shown along with the PPDK antibody immunoblot of the membrane where wild-type PPDK (lane 2) is compared with the PPDK deletion mutant (lane 1).

chromatography was carried out. The column fractions were analyzed by SDS−PAGE in conjunction with the immunoblot assay. As indicated in Figure 1, the mutant proteins were only partially purified by this step. In contrast, the wild-type PPDK eluted from the column with greater than 95% purity. The difference observed in the level of purification achieved with the wild-type PPDK vs the deletion mutants is no doubt due at least in part to the higher level of the wild-type PPDK present in the crude protein fraction loaded onto the DEAE-cellulose column.

Next, Sephadex G-200 column chromatography of the two partially purified PPDK deletion mutants (obtained by concentration of the DEAE-cellulose column fractions) was carried out in an attempt to further purify these proteins as well as to measure their native molecular weights. Both mutant proteins eluted from the gel filtration column as a very broad peak which began at the void volume of the column. This behavior suggested that the two deletion mutants aggregate when concentrated and/or stick to the gel filtration matrix and/or to other cellular proteins during chromatography. Wild-type PPDK (a homodimer of 96 kDa subunits) does not exhibit such behavior, suggesting that deletion of the 25 and 35 kDa domains may have left adhesive surfaces on the truncated proteins. The catalytic activity of the PPDK deletion mutants (described below) was therefore examined using the partially purified protein obtained from the DEAE-cellulose column fractions (without concentration) since further purification without the loss of functional protein did not seem possible.

Determination of the Catalytic Activity of the PPDK Deletion Mutants. Using the partially purified deletion mutants and the spectrophotometric assay (in which pyruvate formation from reaction of AMP, PEP, and PP_i is coupled to pyruvate reduction by LDH/NADH; Wang et al., 1988) to measure PPDK activity, we were unable to detect catalysis of the full (i.e., PEP + PP_i + AMP → pyruvate + P_i + ATP) reaction.

Next, we examined catalysis of the two partial reactions. In the nucleotide (ATP + P_i) partial reaction the β-phosphoryl group of ATP is transferred to the catalytic histidine of the enzyme, ultimately giving rise to the E-P intermediate (steps 1 and 2 of Scheme 1). The E-P formed then reacts, in the second partial reaction, with pyruvate to form PEP and the free enzyme (step 3 of Scheme 1). Previous studies have shown that catalysis of the nucleotide or pyruvate partial reactions can be monitored using [β-^{32}P]ATP or [^{32}P]PEP as reactants and SDS-PAGE of the radiolabeled enzyme coupled with autoradiographic techniques as the assay method (Carroll et al., 1994).

Accordingly, the partially purified deletion mutants (from the DEAE-cellulose column chromatographies) were reacted with [β-^{32}P]ATP + P_i or with [^{32}P]PEP. After a specified period of time, the reaction mixtures were separated on 12% SDS−PAGE gels and subsequently transfer blotted to PVDF membranes. The membranes were first used to make autoradiograms and then stained with Coomassie blue dye. The stained PVDF membranes and the corresponding autoradiograms are shown in Figure 2. In the case of the 35 kDa deletion mutant, ^{32}P-labeled protein is formed with [β-^{32}P]ATP + P_i but not with [^{32}P]PEP. In the case of the 25 kDa deletion mutant, ^{32}P-labeled protein was formed with [β-^{32}P]ATP + P_i but not with [^{32}]PEP (because of the closely spaced protein bands on the gel, the identity of the radiolabeled protein was verified using immunoblot techniques as described in the Materials and Methods section). As expected, the wild-type PPDK (control) was radiolabeled with either of the two ^{32}P-labeled reactants (Mehl et al., 1994; Carroll et al., 1994; Thrall et al., 1993).

These data show that the 25 kDa deletion mutant protein is unable to catalyze the E + ATP + P_i ⇌ E-P + AMP + PP_i partial reaction but is still an active catalyst in the E + PEP ⇌ E-P + pyruvate reaction. This result suggests that the 25 kDa (ATP binding) domain is required for the nucleotide partial reaction but not for the pyruvate partial reaction. Likewise, the 35 kDa deletion mutant was able to

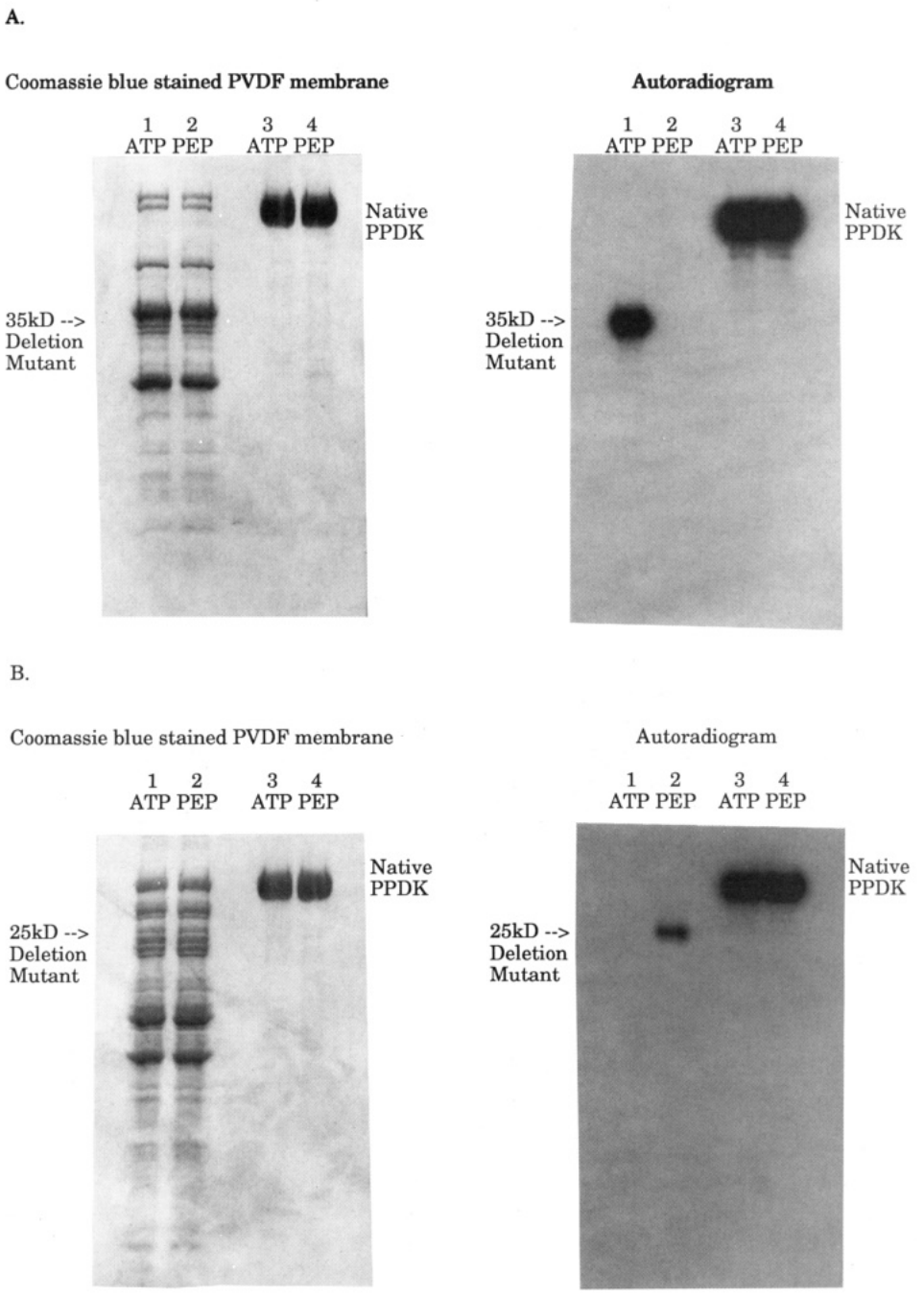


FIGURE 2: Coomassie blue stained PVDF membrane and autoradiogram of the PVDF membrane obtained from the SDS−PAGE gel generated from the reaction of wild-type PPDK with [^{32}P]PEP (lane 4) or with [β-^{32}P]ATP + P_i (lane 3) and from the reaction of the deletion mutant (A for the 35 kDa deletion mutant; B for the 25 kDa deletion mutant) with [^{32}P]PEP (lane 2) or [β-^{32}P]ATP + P_i (lane 1). In each case, the wild-type PPDK and the PPDK deletion mutants had been subjected to DEAE-cellulose column chromatography just prior to reaction.

catalyze the nucleotide partial reaction, but it was unable to catalyze the pyruvate partial reaction, suggesting that the 35 kDa (pyruvate binding) domain is required for the pyruvate partial reaction but not the nucleotide partial reaction. These same results were observed for the two PPDK deletion mutants assayed using crude protein from freshly lysed cells (data not shown), suggesting that the purification steps did not themselves destroy catalytic activity in the two truncated enzymes.

Originally, we had planned to compare the catalytic efficiency of the truncated mutants toward the partial reactions with the catalytic efficiency of wild-type PPDK using rapid quench techniques (Mehl et al., 1994). However, the tendency of the deletion mutants to unfold and to aggregate made the preparation of pure, active mutant protein samples seemingly impossible. Thus, from the results presented in this paper we provide qualitative (not quantitative) data which, nevertheless, identifies the retention and loss of the catalytic modes of the wild-type enzyme in the deletion mutants.[2]

SUMMARY

Thus, in summary, the N-terminal and C-terminal domains of PPDK form, in conjunction with the 18 kDa histidine-containing domain (and the 13 kDa domain), active sites which are involved in the catalysis of the E + ATP + P_i $\rightleftharpoons$ E-P + AMP + PP_i partial reaction and the E-P + pyruvate $\rightleftharpoons$ E + PEP partial reaction, respectively. While catalysis

of a given partial reaction can take place in the absence of either the N-terminal segment or the C-terminal segment, both segments must be present for catalysis of the overall (ATP + P_i + pyruvate ⇌ AMP + PP_i + PEP) reaction. These findings are more consistent with the model of separate active sites illustrated in Scheme 2A than with the shared active site model of Scheme 2B.

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[2] On the basis of the present studies we conclude that the 35 kDa deletion mutant is active toward autophosphorylation with ATP/P_i but totally inactive toward autophosphorylation with PEP. Hence, the 35 kDa domain is necessary for the pyruvate partial reaction but not the nucleotide partial reaction. What we do not know from these studies is whether or not removal of the 35 kDa domain lowers the rate of catalysis of the nucleotide partial reaction. While knowledge of the relative catalytic efficiencies of the wild-type PPDK and the PPDK deletion mutants is desirable, the conclusions drawn in this study are not dependent on this. Until a method for assaying the number of fully functioning active sites in the heterogeneous protein sample becomes available, a quantitative determination cannot be made.

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