# Substrate Binding Domains in Pyruvate Phosphate Dikinase†

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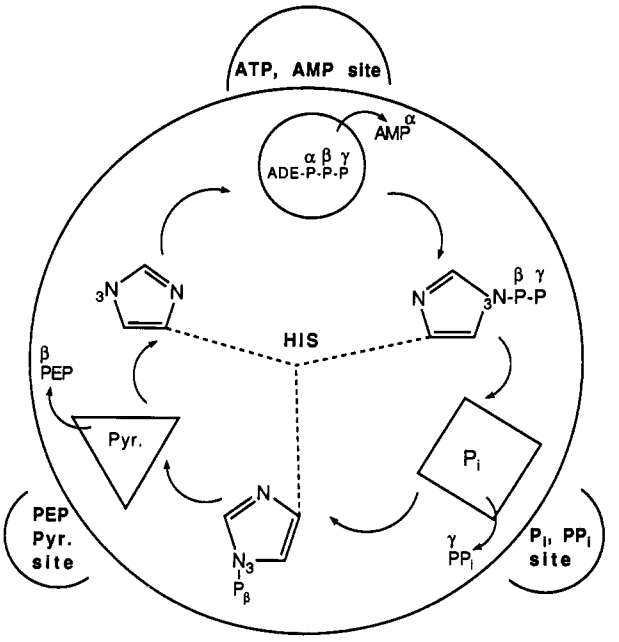
ABSTRACT: Proteolysis of *Clostridium symbiosum* pyruvate phosphate dikinase (PPDK) in its free or phosphorylated state with subtilisin Carlsberg followed two different cleavage pathways. The major pathway involved initial cleavage of the holoenzyme (93 kDa) into a stable 25-kDa N-terminal fragment and transiently stable 67-kDa C-terminal fragment. The 67-kDa fragment was cleaved to generate a stable 35-kDa fragment and an unstable 30-kDa fragment (containing the catalytic histidine). Proteolytic cleavage via the minor pathway divided the holoenzyme into an unstable 37-kDa N-terminal piece (which was further cleaved to the stable 25-kDa fragment produced in the major pathway) and a transiently stable 55-kDa C-terminal fragment. The 55-kDa fragment was then cleaved to produce the stable 35-kDa fragment produced by the major pathway. The cleavage pattern of PPDK complexed with the ATP analog adenyl imidodiphosphate was identical to that of the free enzyme, only the rate of cleavage as slower. In contrast, proteolysis of the phosphorylenzyme–oxalate complex generated the 55-kDa fragment indicating that oxalate binding induces a change in protein conformation. Treatment of PPDK with [1-$^{14}$C]bromopyruvate followed by proteolysis revealed selective radiolabeling of the stable 35-kDa fragment while similar experiments with [$^{14}$C]2′,3′-dialdehyde adenosine 5′-monophosphate resulted in selective radiolabeling of the stable 25-kDa fragment. These results were interpreted to suggest that PPDK contains several structural domains and that the catalytic histidine, the pyruvate binding site, and the ATP binding site may be located on different domains.

Pyruvate phosphate dikinase (PPDK)[1] catalyzes the reversible phosphorylation of pyruvate and orthophosphate using a single molecule of ATP:

$$ATP + P_i + \text{pyruvate} \rightleftharpoons AMP + PP_i + PEP$$

The phosphoryl transfers of the reaction are mediated by a catalytic histidine which first displaces AMP from the β-phosphoryl group of ATP, forming a novel pyrophosphorylenzyme (E-PP). $P_i$ then abstracts the terminal phosphoryl group from the E-PP to generate $PP_i$ and the phosphorylenzyme (E-P). Finally, PEP is formed by phosphoryl transfer from E-P to pyruvate. The ATP, $P_i$, and pyruvate binding sites are separate, noninteracting sites on the enzyme (Milner & Wood, 1976; Yoshida & Wood, 1978; Evans et al., 1980). A model accounting for the movement of the catalytic histidine residue between these sites during turnover was proposed two decades ago by Wood and co-workers as the "swinging arm mechanism" of PPDK catalysis (see Scheme 1) (Wood et al., 1977). This model assumes that the catalytic histidine is contained within a flexible peptide region which can move freely between reaction sites. Although there is no direct evidence for such a swinging arm in PPDK catalysis, analogous swining arm models have been proposed for biotin-dependent carboxylases (Samols & Wood, 1988), the pyruvate dehydrogenase complex (Volpe & Vagelos, 1973), and the fatty acid synthase complex (Reed, 1974).

Scheme 1: "Swinging Arm Mechanism" of PPDK Catalysis Adapted from Wood et al. (1977)




In recent years our laboratory has been engaged in the study of the nature of separate site catalysis in the PPDK reaction. Although considerable evidence for separate ATP, $P_i$, and pyruvate binding sites exists (Thrall & Dunaway-Mariano, 1994), precisely how far apart these sites are on the protein is not known. The stereochemistry of β-P transfer from the ATP to pyruvate (two in-line displacements giving overall retention of configuration) (Cook & Knowles, 1985)

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[1] Abbreviations: PPDK, pyruvate phosphate dikinase; E-PP, pyrophosphorylenzyme; E-P, phosphorylenzyme; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; PEP, phosphoenolpyruvate; $PP_i$, inorganic pyrophosphate; $P_i$, inorganic phosphate; NADH, dihydronicotinamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid; LDH, lactate dehydrogenase; AMP-PNP, adenyl imidodiphosphate; PMSF, phenylmethanesulfonyl fluoride; oAMP; 2′,3′-dialdehyde adenosine 5′-monophosphate; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; PVDF, poly(vinylidene difluoride); SDS–PAGe, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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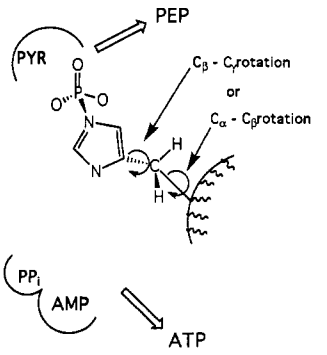
suggests that the phosphohistidine residue essentially undergoes a 180° rotation while moving between the ATP and pyruvate sites.[2]

In addition to the swinging arm model which involves local conformational changes affecting a flexible "arm" region (Scheme 1), we have considered two additional models to depict movement of the histidine between the ATP and pyruvate sites. First, simple bond rotation between the $C_\beta$–$C_\alpha$ or $C_\gamma$–$C_\beta$ bonds of the catalytic histidine residue could link the nucleotide and pyruvate sites (Scheme 2). An analogous model has been proposed to account for separate site covalent catalysis by succinyl-CoA synthetase (Vogel & Bridger, 1982; Frey, 1992). A bond rotation model has also been considered for the biotin-dependent transcarboxylase as an alternative to the "swinging arm" model (Fung & Mildvan, 1976; DeTitta, 1980).

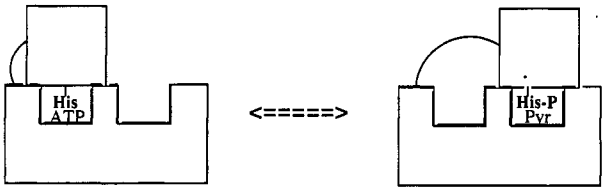
The second model considered in Scheme 2 is what we have termed the "domain movement" model. According to this model, substrate binding domains serve as modules for the movement of the catalytic histidine between ATP and pyruvate binding sites. Thus, global rather than local conformational changes are assumed to occur within the protein during catalysis. As a part of our efforts to characterize the mechanism of separate site catalysis by *Clostridium symbiosum* PPDK (a homodimer of 93-kDa subunits), we have examined the domain structure of this protein using proteolysis techniques. In this paper we report our results which suggest that PPDK (monomer) may be organized into four structural domains and that the catalytic histidine and the ATP and pyruvate binding sites are located on (three) separate domains.

Scheme 2: Illustration of the "Bond Rotation" and "Domain Movement" Models of PPDK Separate Site Catalysis

Bond Rotation Model




Domain Movement Model




## MATERIALS AND METHODS

*Materials.* Buffers were from U.S. Biochemical Corp. Subtilisin Carlsberg, ATP, PMSF, and AMPPNP were from Sigma. [$^{14}$C]AMP and EN$^3$HANCE were purchased from NEN and [$^{14}$C]pyruvate was from Amersham. Autoradiograms were made on Kodak OR-mat film.

*Preparation of PPDK.* PPDK was prepared from *Escherichia coli* JM 101 carrying the plasmid pACYC184D-12 (Pocalyko et al., 1990). The cells were grown in LB media containing 12.5 μg/mL tetracycline at 37 °C until late log phase ($OD^{600}$ ~1.1). The cells were collected by centrifugation and suspended in extraction buffer (10 mL/g cells) containing 20 mM imidazole·HCl, 2.5 mM $Na_2EDTA$, 75 mM KCl, 3 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride (PMSF) at pH 6.8. The PMSF was dissolved in dimethylformamide and added immediately before lysis. The cells were lysed by using a French pressure cell at a pressure of 18 000 psi. PPDK was prepared from the cell free lysate by the procedure of Wang et al. (1988) with the exception that gel filtration chromatography was performed on a 2 cm × 80 cm column of Sephacryl 200 (Pharmacia). This procedure typically yields 30 mg of PPDK/g of cell paste having a specific activity of 15–25 units/mg of protein. This protein is ≥90% pure as judged by SDS–PAGE. The enzyme activity was assayed using the spectrophotometric asay described by Wang et al. (1988).

*Synthesis of oAMP and [$^{14}$C]oAMP.* Synthesis of the dialdehyde derivative of AMP was carried out based on the procedure reported by Lowe et al. (1982). A 1.1-mL aliquot of a 200 mM solution of sodium periodate (0.22 mmol) was added to a 0.9-mL solution containing 200 mM AMP (0.18 mmol) in 50 mM Hepes, pH 7.0. The oxidation reaction was carried out at 4 °C for 1 h in the dark. Unconsumed periodate was quenched with the addition of 3 μL of ethylenediol, and the mixture was chromatographed on a Sephadex G-10 column (1.6 × 78 cm) equilibrated with deionized water at 4 °C. Fractions (1.5 mL) were assayed by measuring their absorbance at 260 nm. The leading half of the nucleotide peak was pooled, lyophilized, and stored at −80 °C. The product (>90% pure) was analyzed by thin layer chromatography on PEI cellulose plates, run in 0.8 M $NH_4HCO_3$ (oAMP at the origin and AMP at an $R_f$ of 0.64).

For synthesis of [$^{14}$C]oAMP, 50 μCi of [$^{14}$C]AMP (594 mCi/mmol; shipped in 1:1 ethanol:water solution) was evaporated to dryness. A 2-mL solution containing 25 mM Hepes, pH 7.0, and 0.165 μmol of cold AMP was added to the [$^{14}$C]AMP to give a final concentration of 125 μM and a specific activity of 200 mCi/mmol. A 10-μL aliquot of a 100 mM solution of $NaIO_4$ was added to the AMP mixture, and the oxidation was allowed to proceed as above. After quenching with ethylenediol, the reaction mixture was loaded onto a Sephadex G-10 column (1 × 45 cm) as before, at 4 °C. Fractions (1 mL) were assayed for radioactivity, and the radioactive peak was pooled and concentrated by rotory evaporation. Thin layer chromatography indicated that the [$^{14}$C]oAMP was 80–90% pure.

*Synthesis of [1-$^{14}$C]Bromopyruvate.* [1-$^{14}$C]Bromopyruvate was synthesized by the method of Barnett et al. (1971) with some modifications. Briefly, 9.4 μmol of commercial sodium [1-$^{14}$C]pyruvate was dissolved in 250 μL of glacial acetic acid. Freshly redistilled bromine (20 μL) was diluted to 10 mL with acetic acid, and 50 μL of this solution was added to the pyruvate–acetic acid solution above. Concentrated $H_2SO_4$ (one drop) was added to the reaction mixture as a catalyst. The reaction was carried out at 56 °C for 3 h in microreflux glassware fitted with a $CaCl_2$ drying tube. The

[2] Alternatively, the histidine could remain essentially stationary and the binding sites moved to, and then away from, it.

[1-$^{14}$C] bromopyruvate reaction mixture was taken to dryness in *vacuo* and stored at −80 °C. The specific activity of the [1-$^{14}$C]bromopyruvate was taken to be that of the sodium [1-$^{14}$C]pyruvate employed for synthesis, which was 32 mCi/mmol. Before use an aliquot was twice dissolved in a small amount of acetone and concentrated *in vacuo* and then finally dissolved in 50 mK K$^+$Hepes (pH 7.0). The pH of the solution was adjusted to 7 with 0.1 M KOH as necessary. The radioactivity of the resulting solution was measured, and the concentration of [1-$^{14}$C]bromopyruvate was calculated using its specific activity.

*Digestion of PPDK with Subtilisin Carlsberg.* All digestions were carried out at 18 ± 2 °C in 100 mM K$^+$Hepes (pH 7.5) unless otherwise stated. A buffered solution of 600 μg of PPDK in a 270-μL volume was incubated in a water bath for 15 min. Subtilisin (30 μL of 10 mg/mL solution in buffer) was added and the resulting solution mixed and returned to the water bath. At the indicated time points, a 25-μL aliquot was removed and the digestion terminated by the addition of 2.5 μL of 50 mM PMSF in dimethylformamide. Half of the aliquot was assayed for enzymatic activity, and the other half was frozen on dry ice and stored at −80 °C until analyzed by SDS–PAGE (Laemmli, 1970). The SDS–PAGE was carried out in a BRL vertical slab gel apparatus using a 5% acrylamide stacking gel and a 12% acrylamide separating gel. Following electrophoresis the gels were stained with Coomassie brilliant blue and dried under vacuum.

In order to locate the domain which contains the active site histidine, the phosphoryl enzyme was formed with either [$^{32}$P]-PEP or [β-$^{32}$P]ATP (Carroll et al., 1989) before digestion. PPDK (100 μg) was incubated with 100 000 cpm [$^{32}$P]PEP in 100 mM K$^+$Hepes (pH 7.5). The labeling reaction was conducted at room temperature for 30 min prior to digestion. The 45-μL [β-$^{32}$P]ATP labeling reaction contained 100 μg of PPDK, 2.5 mM $MgCl_2$, 10 mM $NH_4Cl$, 1 mM $KH_2PO_4$, and 100 000 cpm [β-$^{32}$P]ATP. The labeling reaction was conducted at room temperature for 30 min prior to digestion. After labeling, the digestion was initiated by the addition of 5 μL of 10 mg/mL subtilisin in 100 mM K$^+$Hepes (pH 7.5). Aliquots (10 μL) were taken at the indicated times, and the reaction was stopped by the addition of 1 μL of 50 mM PMSF in DMF. The aliquots were frozen on dry ice and stored at ~80 °C until analyzed by SDS–PAGE.

To test for the ability of the proteolyzed enzyme to phosphorylate itself with [$^{32}$P]PEP or [β-$^{32}$P]ATP/$P_i$, fixed time point assays with varying amounts of subtilisin were conducted. Each 45-μL reaction contained 100 μg of PPDK, 100 mM K$^+$Hepes (pH 7.5), 2.5 mM $MgCl_2$, and 10 mM $NH_4Cl$. The [β-$^{32}$P]ATP labeling reactions also included 1 mM $KH_2PO_4$. All substrates except the radiolabeled substrate were incubated with PPDK for 15 min at room temperature. The appropriate amount of subtilisin (1–50 μg) was added and the mixture incubated at room temperature for 15 min. The digestion was stopped by the addition of 5 μL of 50 mM PMSF in DMF, and then 5 μL of the radiolabeled substrate (100 000 cpm) was added. Each reaction mixture was incubated at room temperature for 5 min and then frozen on dry ice and stored at −80 °C until analyzed by SDS–PAGE.

*Preparation of Proteolytic Fragments for Protein Sequencing.* The location of the protease cleavage sites in the primary sequence was determined by N-terminal sequence analysis of the fragments. The fragments to be sequenced were separated by SDS–PAGE as previously described and then electroeluted onto a poly(vinylidene difluoride) (PVDF) membrane. Gels to be transfer blotted were first soaked in 10 mM Caps (pH 11)/10% methanol (v/v) for 10 min prior to electroelution in a Hoefer Semiphor transfer blot apparatus. The proteolytic fragments were visualized and their N-terminal sequence determined by published procedures (Matsudaira, 1987) using an Applied Biosystems model 470 gas phase protein sequencer. The 35-kDa fragment did not yield interpretable data when sequenced by this procedure, and it proved necessary to isolate this fragment by reverse-phase HPLC for sequencing. The 35-kDa fragment was prepared by digestion of 2.5 mg of PPDK with 1 mg of subtilisin in a 2-mL volume. After a 10-min digestion, the reaction mixture was injected directly onto an Alltech Econosphere C-8 4.6 mm × 15 cm reverse-phase HPLC Column. The column was eluted with 0.15% trifluoroacetic acid (TFA; buffer A) for 10 min at a flow rate of 1 mL/min. After 10 min the column was eluted with a linear gradient of 0–60% of 0.15% TFA in acetonitrile (buffer B). The concentration of buffer B was increased at a rate of 1%/min. The UV absorbance was monitored at 220 nm, and the peaks were collected and dried under vacuum in a speedvac apparatus. The pellets were resuspended in 100 μL of 0.15% TFA in $H_2O$, and the protein concentration was determined with the Bio-Rad microassay system using BSA as a protein standard. Each peak (2–5 μg) was analyzed by SDS–PAGE. The peak containing the 35-kDa fragment eluted from 61.0 to 63.5 min. The N-terminal sequence of the 35-kDa fragment was then determined by automated Edman degradation techniques.

*Digestion of the E-P·Oxalate Complex.* The phosphoryl form of PPDK was prepared by reacting 200 μg of PPDK in an 80-μL reaction containing 1.3 mM $KH_2PO_4$, 1.3 mM ATP, 3.1 mM $MgCl_2$, and 12.5 mM $NH_4Cl$ in 100 mM K$^+$Hepes (pH 7.5). After incubation for 30 min at room temperature, the reaction mixture was divided into 40-μL aliquots. The control reaction received 5 μL of 100 mM K$^+$Hepes (pH 7.5) while the oxalate reaction received 5 μL of 10 mM disodium oxalate in the same buffer. After mixing, both reactions were incubated an additional 5 min at room temperature prior to the addition of 5 μL of 10 mg/mL subtilisin in buffer to each. At the indicated time point a 15-μL aliquot was removed and quenched by the addition of 2 μL of 50 mM PMSF in dimethylformamide, and the aliquots were frozen on dry ice and stored at −80 °C until analyzed by SDS–PAGE.

*Digestion of the $Mg^{2+}$·AMPPNP Complexed Enzyme.* A solution of 100 μg of PPDK in a 45-μL reaction volume containing 1 mM AMPPNP, 2.5 mM $MgCl_2$, 10 mM $NH_4Cl$, 10 mM $KH_2PO_4$, and 100 mM K$^+$Hepes (pH 7.5) was incubated at room temperature for 10 min. Subtilisin (5 μL of a 10 mg/mL solution in 100 mM K$^+$Hepes, pH 7.5) was then added. At the indicated time points a 15-μL aliquot was removed and the digestion stopped by the addition of 2 μL of 50 mM PMSF in dimethylformamide. The aliquots were frozen on dry ice and stored at −80 °C until analyzed by SDS–PAGE.

*Inactivation of PPDK by [1-$^{14}$C]Bromopyruvate.* PPDK (50 μg) was dissolved in 20 μL of 50 mK K$^+$Hepes (pH 7.0) containing 7.5 mM $MgCl_2$, 30 mM $NH_4Cl$, and 500 μM [1-$^{14}$C]bromopyruvate. After incubation at 30 °C for 15 h, the remaining enzyme activity was determined. The control reaction contained 50 μg of PPDK, 7.5 mM $MgCl_2$, 30 mM $NH_4Cl$, and 50 mM K$^+$Hepes (pH 7.0) in a final volume of 20 μL. The reaction was incubated at 30 °C for 15 h before the enzyme activity was examined using the spectrophotometric assay system (Wang et al., 1988).

*Protection of Pyruvate Binding Site from [1-$^{14}$C]Bromopyruvate by PEP.* PPDK (18 μg) was dissolved in 15 μL

of 50 mM K+Hepes (pH 7.0) containing 10 mM $MgCl_2$, 40 mM $NH_4Cl$, and 1.3 mM PEP. After incubation at 30 °C for 15 min, 5 μL of 200 μM [1-$^{14}$C]bromopyruvate ($1.7 \times 10^5$ cpm) in 50 mM K+Hepes (pH 7.0) was added. The reaction mixture was incubated at 30 °C for 25 min before being analyzed by SDS–PAGE. The control experiment was carried out in the same manner but in the absence of PEP.

*Identification of the Pyruvate Binding Domain.* PPDK (36 μg) was incubated in a 13-μL reaction containing 7.5 mM $MgCl_2$, 30 mM $NH_4Cl$, and 35 μM [1-$^{14}$C]bromopyruvate (35 000 cpm) in 50 mM K+Hepes (pH 7.0). After incubation at 30 °C for 25 min, 2 μL of 6 mg/mL subtilisin in 50 mM K+Hepes (pH 7.0) was added to the mixture. The digestion was carried out at 25 °C for 6 min and then stopped by the addition of 2 μL of 50 mM PMSF in dimethylformamide. Six microliters of 200 mM $NaBH_4$ in $H_2O$ was added to the reaction mixture to reduce the labeled enzyme, and the reduction was carried out for 6 min at 25 °C. The reaction was frozen on dry ice and stored at −80 °C until analyzed by SDS–PAGE.

*oAMP Inactivation Studies.* The time course of oAMP inactivation of PPDK in the presence and absence of AMP was carried out essentially as described by Evans et al. (1980). A 500-μL solution containing 1 μM PPDK, 5 mM $MgCl_2$, 3 mM $NaCNBH_3$, 50 mM Na+Hepes, pH 7.0, and 1 mM AMP (if specified) was prepared, and a 10-μL aliquot was assayed for PPDK activity. The inactivation reaction was initiated by adding oAMP to a final concentration of 1 mM. At specific time points, 10-μL aliquots were removed from the reaction mixture and assayed for PPDK activity (Wang et al., 1988).

*Preparation of [$^{14}$C]oAMP-Labeled PPDK for SDS–PAGE Analysis.* Two hundred micrograms of PPDK (20 μM final concentration, based on a molecular mass of 90 kDa) was added to a 100-μL solution containing 5 mM $MgCl_2$, 50 μM [$^{14}$C]oAMP (SA 200 mCi/mmol), and 50 mM K+Hepes, pH 7.0. After 5 min, the reaction was quenched with 4 μl of freshly prepared 250 mM $NaBH_4$ in water. The solution was incubated for 2 min and then centrifuged for 1 min to degas. The labeled PPDK was then subjected to limited subtilisin digestion and SDS–PAGE electrophoresis/autoradiography.

*Analysis of Proteolytic Digests by SDS–PAGE and Autoradiography.* The proteolysis reaction sample was mixed in 2:1 volume ratio to gel loading buffer. The 3× gel loading buffer contained 150 mM Tris·HCl (pH 6.8), 30% glycerol, 3% SDS, 3% β-mercaptoethanol, and 0.8% w/v bromophenol blue. After being heated at 100 °C in water bath for 2 min, the sample was loaded on a 12% acrylamide gel with a 5% stacking gel. The gel was run at 200 V for 3.5 h. The running buffer contained 3 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS in $H_2O$. After electrophoresis the gel was stained with a 0.25% solution of Coomassie blue in 50% methanol, 40% $H_2O$, and 10% acetic acid for 30 min and destained in 50% methanol, 40% $H_2O$, and 10% acetic acid until excess dye had been removed. After destaining, the gel was soaked in the EN$^3$HANCE solution for 45 min and then in cold water for 30 min. The gel was dried in a Hoefer slab gel drier for 45 min at 75 °C and kept under vacuum for an additional 45 min at room temperature. The dried gel was placed against a Kodak X-ray film and exposed at −80 °C.

## RESULTS AND DISCUSSION

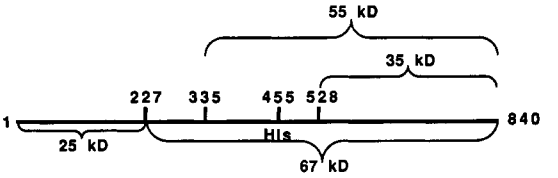
Subtilisin Carlsberg is a nonspecific serine protease which cleaves accessible peptide bonds. It is generally assumed that peptide regions located between protein structural domains are less ordered (solvent exposed) and therefore, are more accessible to proteolysis (Fonda, 1976; Fontana et al., 1986, 1989). Cleavage of these interdomain regions by the protease releases tightly folded domains which are less susceptible to degradation by the protease and, as a result, can be observed to accumulate in the proteolysis reaction. The proteolytic technique has been successfully employed to examine domain structure in many proteins including DNA ligase I, alanine racemase, fatty acid synthetase, β-galactosidase, and FLP recombinase (Tomkinson et al., 1990; Touama et al., 1991; Wong et al., 1981; Edward et al., 1988; Pan et al., 1991; Mattick et al., 1983). In the present study this technique was used to examine the domain structure of PPDK.

Table 1: Amino Acid Sequences of the N-Terminals of the Fragments Generated from PPDK by Limited Proteolysis with Subtilisin[a]

| fragment size (kDa) | N-terminal sequence | location in the primary sequence |
|---|---|---|
| 93 (holoenzyme) | AKWVYKFE | 1–8 |
| 67 | NDIPGD | 227–332 |
| 55 | QT–NGKTAP | 335–444 |
| 35 | ADKE–TLKV | 528–536 |
| 25 | AKWVYKFEEG | 1–10 |

[a] The fragments were separated by SDS–PAGE and transfer blotted onto a PVDF membrane as described under Materials and Methods. All fragments were sequenced by Edman degradation on the membrane except for the 35-kDa fragment which was isolated by HPLC for sequencing. The one-letter amino acid code is used; (–) indicates a blank cycle.

Scheme 3: Sites of Proteolytic Cleavage within the PPDK Primary Structure As Denoted by Amino Acid Number[a]




[a] The full length amino acid sequence of PPDK is reported in Pocalyko et al. (1990).

### *Proteolysis of Native PPDK with Subtilisin Carlsberg*

For the purpose of identifying stable folding domains within the PPDK structure, native PPDK was subjected to limited proteolysis with subtilisin Carlsberg. The proteolysis reaction was examined at varying conversion using SDS–PAGE gel techniques. The time course for the reaction is shown in Figure 1A. At the earliest time point examined, the holoenzyme species (93 kDa) is the predominant protein band on the gel. It is accompanied by two major protein bands corresponding to 67- and 25-kDa sized fragments and two minor bands corresponding to 55- and 35-kDa sized fragments. Cleavage of the holoenzyme to the 67- and 25-kDa fragments represents the major proteolytic pathway.

The N-terminal sequences of the 67- and 25-kDa fragments are presented in Table 1. The 25-kDa fragment has the same N-terminal sequence as does the holoenzyme (93 kDa), indicating that it is derived from the N-terminal portion of the protein (Pocalyko et al., 1990). The 67-kDa fragment begins with Asn 227 thus indicating that the first proteolytic cleavage of the holoenzyme occurs between Met 226 and Asp 227 to generate the 25-kDa (N-terminal) and 67-kDa (C-terminal) pieces (see Scheme 3).

As the proteolysis of PPDK proceeds, the 67-kDa fragment is consumed and the 35-kDa fragment accumulates (Figure 1A). The N-terminal sequence of the 35-kDa fragment (Table 1) indicates that the 67-kDa fragment underwent a second

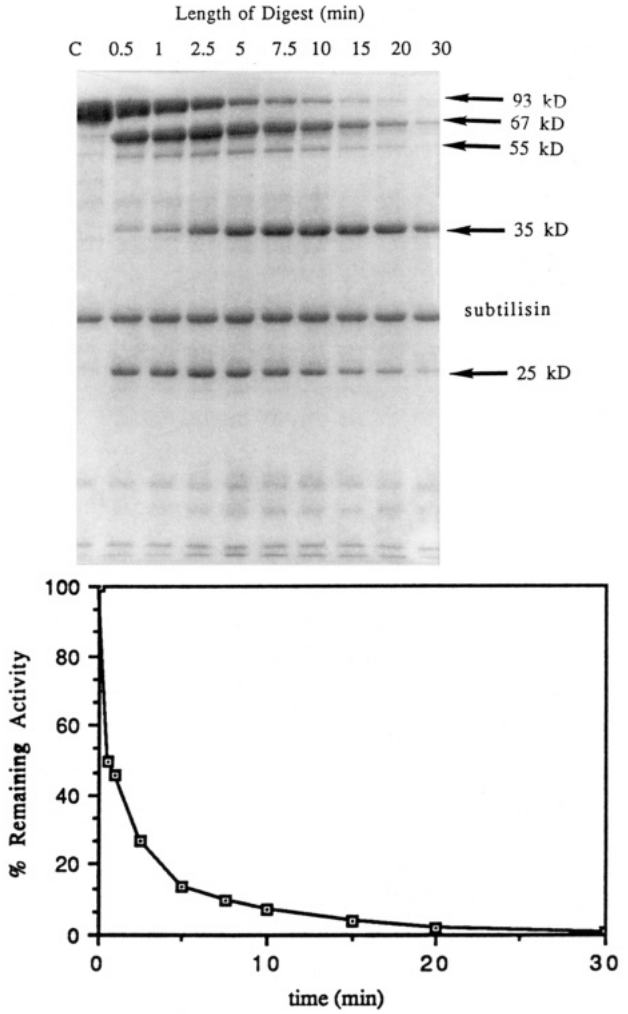



FIGURE 1: (A, top) Coomassie blue stained SDS–PAGE gel of the proteolysis of the native form of PPDK. PPDK was digested with subtilisin at an enzyme to protease ratio of 2:1 (w/w). At the indicated time point, an aliquot was taken and the digestion stopped by the addition of PMSF. A portion, 10 mg, of each aliquot was analyzed by SDS–PAGE on a 12% separating gel with a 5% stacking gel as described under Materials and Methods. (B, bottom) Graph of the activity of the proteolyzed enzyme as a function of the length of digest. PPDK was digested with subtilisin at an enzyme to protease ratio of 2:1 (w/w). At the indicated time point, an aliquot was taken and the digestion stopped. The remaining PPDK activity was assayed using the spectrophotometric assay and then plotted as a function of the length of the subtilisin digestion.

cleavage between Trp 527 and Ala 528 (Scheme 3) to generate a relatively stable 35-kDa (C-terminal) fragment and a relatively unstable (based on our inability to visualize it from the Coomassie blue stained SDS–PAGE gel of Figure 1A) 32-kDa (N-terminal) fragment. The 32-kDa fragment spans amino acid positions 227–527, and therefore this fragment contains the catalytic histidine located at amino acid position 455.

Thus, the major proteolytic pathway consists of initial cleavage of the holoenzyme into a 25-kDa N-terminal piece which is relatively stable to further proteolysis and a 67-kDa C-terminal piece which undergoes further cleavage yielding an unstable 32-kDa fragment (containing the catalytic histidine) from the N-terminal end and a stable 35-kDa C-terminal fragment (Scheme 3).

The minor proteolytic pathway (Scheme 3) involves cleavage of the holoenzyme between Leu 334 and Gln 335 to generate a 37-kDa piece (which presumably is rapidly trimmed to the 25-kDa fragment spanning amino acid position 1–226) and the 55-kDa C-terminal piece observed at the earliest time points of the proteolysis reaction (Figure 1A). The 55-kDa fragment is probably converted next to the 35-kDa fragment by cleavage between Trp 527 and Ala 528 although one can not conclude this directly from Figure 1A. As discussed below, the significance of this minor proteolytic cleavage pathway is that the 55-kDa fragment predominates in the proteolysis of the E-P·oxalate·Mg complex.

### *Proteolysis of the Phosphorylated Form of PPDK with Subtilisin Carlsberg*

The pattern for proteolytic cleavage of PPDK was also examined with the phosphorylated form of the enzyme (E-P). The time course for the proteolysis of E-P shown in Figure 2A was typical for phosphorylated enzyme generated from ATP plus $P_i$ or from PEP. This time course (Figure 2A) is essentially identical to that observed with the native enzyme (Figure 1A). To identify the fragment on the gel containing the catalytic histidine, $^{32}P$-labeled E-P was generated using [$^{32}P$]PEP. Following chromatography on the SDS–PAGE gel, the proteolytic digest was transfered to a PVDF membrane. The membrane was used to make an autoradiogram and then stained with Coomassie blue dye. The autoradiogram made from the membrane (Figure 2B) shows the most predominant bands of radioactivity are at 93 kDa (the holoenzyme) and at 67 kDa. Significantly less intense bands, which correspond to lesser amounts of protein, are seen at 62, 57, and 55 kDa. When a larger exposure (3 days) was made of the same membrane, bands which correspond to 30 and 20 kDa were visible (Figure 2C). We suspect the band seen at 30 kDa is the unstable fragment containing the catalytic histidine which is liberated from the 67-kDa fragment when it is proteolytically cleaved between Trp 527 and Ala 528. The 20-kDa fragment may derive from proteolytic cleavage of the 30-kDa fragment.

### *Determination of the Catalytic Activity of the Proteolyzed PPDK*

The proteolysis of PPDK with subtilisin was monitored by SDS–PAGE gel (Figure 1A) and by an activity assay (Figure 1B). The activity assay was based on the production of pyruvate (plus ATP and $P_i$) from catalysis of PEP, AMP, and $PP_i$. As can be seen by comparing Figure 1 panels A and B, as the proteolysis of PPDK proceeds, catalytic activity (toward the full reaction) is lost.

To test whether proteolytic cleavage of the native PPDK precluded catalysis of the individual partial reactions (i.e., E + ATP + $P_i$ → E-P + $PP_i$ + AMP or E + PEP → E-P + pyruvate), $^{32}P$-labeling of the enzyme with [$^{32}P$]PEP or [$\beta$-$^{32}P$]-ATP/$P_i$ following its cleavage by subtilisin was tested. After the PPDK was incubated with subtilisin for a given period, the subtilisin activity was destroyed by the addition of PMSF (the control lanes of Figure 3 show the effectiveness of PMSF in terminating proteolysis). The [$^{32}P$]PEP or [$\beta$-$^{32}P$]ATP/$P_i$ was then added to the protein mixture for catalyzed $^{32}P$-labeling, and the resulting reaction solution was analyzed by SDS–PAGE (Figure 3). The results obtained with the [$^{32}P$]-PEP and [$\beta$-$^{32}P$]ATP reaction mixtures are essentially identical. Both reactions resulted in the $^{32}P$-labeling of the holoenzyme (93 kDa) and the 67-kDa (major cleavage pathway, Scheme 3) and 55-kDa (minor cleavage pathway, Scheme 3) proteolytic fragments. Thus, cleavage between Met 226 and Asp 227 or between Leu 334 and Gln 335 does not appear to destroy the ability of the enzyme to catalyze either partial reaction. At the current time we do not know,

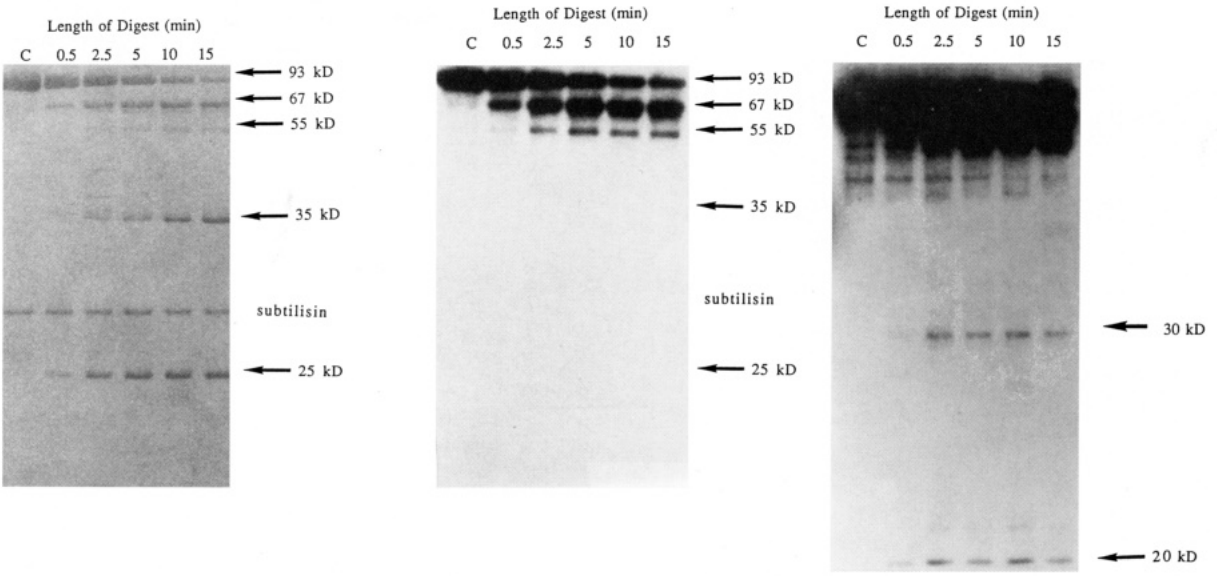



FIGURE 2: (A, left) Coomassie blue stained transfer blot membrane of an SDS–PAGE gel of the subtilisin-digested [$^{32}$P]phosphoryl enzyme. The phosphoryl enzyme was prepared by the reaction of the enzyme with [$^{32}$P]PEP. The enzyme was then digested with subtilisin at an enzyme to protease ratio of 2:1. Aliquots taken at varying conversion were analyzed on a 12% SDS–PAGE gel, and the protein fragments were then transferred to a PVDF membrane and stained as described under Materials and Methods. (B, middle) Autoradiogram of the transfer blot membrane of an SDS–PAGE gel of the subtilisin-digested [$^{32}$P]phosphoryl enzyme. The membrane from panel A was used to make an autoradiogram. The film was exposed overnight at –80 °C with an intensifying screen. (C, right) Autoradiogram of the tranfer blot membrane of an SDS–PAGE gel of the subtilisin-digested [$^{32}$P]phosphoryl enzyme, 3-day exposure. The membrane from panel A was used to make an autoradiogram. The film was exposed 3 days at –80 °C with an intensifying screen.

however, what impact proteolytic cleavage has on the efficiency of catalysis of the partial reactions.

Separation of the PPDK proteolysis mixture by gel filtration chromatography and SDS–PAGE gel analysis of the column fractions (Figure 1 of Supplementary Material) provided evidence that the proteolyzed enzyme tends to stay intact. Specifically, the protein fractions contained all of the expected proteolytic fragments in addition to full length monomer. Only at the end of the PPDK elution region was the 35-kDa fragment alone observed. Catalysis then is probably restricted to the 67- or 55-kDa fragment as it remains associated with the corresponding N-terminal piece. An intact subunit may serve to stabilize a fragmented subunit within the dimeric structure.

### *Location of the ATP and Pyruvate Binding Domains in Native PPDK*

On the basis of the proteolysis experimental experimental results described above, we envision the domain structure of PPDK to roughly correspond to the model presented in Scheme 4. Within this model the 25- and 35-kDa domains are considered to be stable folding units as each shows considerable resistance to subtilisin proteolysis and, in addition, the 35-kDa domain has been separated by gel filtration chromatography (Figure 1 of Supplementary Material). We have subdivided the ~32-kDa fragment containing the catalytic histidine into two subdomains (13 and 18 kDa) based on the relative locations of the cleavage sites leading to the 67- and 55-kDa proteolytic fragments (Scheme 3) and the observation that both a 30- and ~20-kDa fragment labeled with $^{32}$P is observed from the proteolysis of [$^{32}$P]E-P (Figure 2C).

The next issue addressed was where on these four domains are the substrate binding sites located. In view of the existing kinetic evidence for separate site catalysis (Milner & Wood, 1976; Yoshida & Wood, 1978; Evans et al., 1980), it was of great interest to determine the physical proximity of the substrate binding sites relative to the catalytic histidine and relative to one another. For the purpose of identifying the PPDK domain(s) which contain the ATP and pyruvate binding sites, radiolabeled affinity labels were used to modify the protein, and then, following proteolysis, the radiolabeled domain(s) were identified by SDS–PAGE/autoradiography.

*Pyruvate Binding Domain.* Bromopyruvate has been used extensively in the past for studies of the location of pyruvate and/or PEP substrate binding sites within enzymes (Okamoto & Morino, 1974; Barnett et al., 1971; Vlahos & Dekker, 1990; Meloche, 1970; Chang & Hsu, 1977; Satterlee & Hsu, 1991; Huynh, 1991). Previous work, carried out by Wood and coworkers, had shown that bromopyruvate is a competitive inhibitor of PPDK catalysis with respect to PEP and that incubation of the enzyme with relatively low concentrations of bromopyruvate inactivates the enzyme by covalent modification of a Cys residue (Yoshida & Wood, 1978). In order to determine in which PPDK domain this Cys residue is located, we carried out covalent modification of PPDK with [1-$^{14}$C]-bromopyruvate and then subjected the labeled enzyme to partial proteolysis by subtilisin.

First, to test whether our synthetic [1-$^{14}$C]bromopyruvate inactivated PPDK, the enzyme was incubated with a 40-fold molar excess of this reagent. The enzyme was completely inactive after incubation at 30 °C for 15 h. A control reaction in which [1-$^{14}$C]bromopyruvate was absent was carried out concurrently. Ninety-five percent of the enzyme activity was observed to remain after the 15-h incubation period. Next, to test whether PPDK is covalently labeled at the pyruvate binding site, the enzyme was incubated with a 5 molar excess of [1-$^{14}$C]bromopyruvate in the presence (1.0 mM) and absence of PEP. The two reactions were quenched with $NaBH_4$ and then analyzed by SDS–PAGE. The autoradiogram generated from the resulting gel (Figure 4) demonstrates protection of the enzyme from [1-$^{14}$C]bromopyruvate modification afforded by the PEP.

To locate the pyruvate binding domain, PPDK was treated with [1-$^{14}$C]bromopyruvate, reduced with $NaBH_4$, and then subjected to limited proteolysis. The resulting protein fragments were separated on an SDS–PAGE gel and stained with Coomassie blue dye (Figure 5A). The autoradiogram (Figure

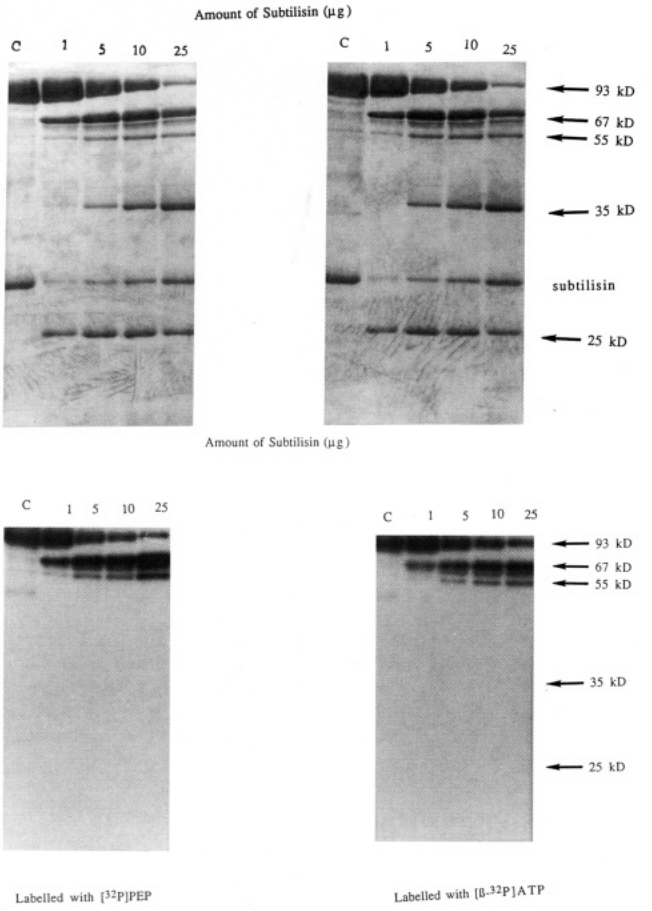
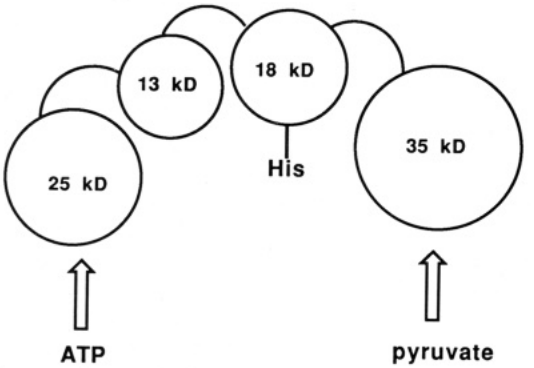



FIGURE 3: (A, top) Coomassie blue stained SDS–PAGE gel of the fragments generated by fixed time point digestion of PPDK. PPDK (100 mg) was incubated with subtilisin (10 mg) at room temperature for 15 min. The reaction was stopped by the addition of PMSF, and an aliquot was analyzed by SDS–PAGE. (B, bottom) Autoradiogram of the SDS–PAGE gel of the fragments generated by fixed time point digestion of PPDK. The fixed time point digestions of panel A were incubated with 100 000 cpm of [$^{32}$P]PEP or with [$\beta$-$^{32}$P]ATP and 1 mM $P_i$ for 5 min in the presence of 2.5 mM $MgCl_2$ and 10 mM $NH_4Cl$. After the labeling reaction, an aliquot was analyzed by SDS–PAGE, and an autoradiogram of the gel was made.

Scheme 4: Proposed Model of the PPDK Structural/Substrate Binding Domains[a]




[a] For convenience, only the proteolytic fragments are represented as spacially separate structural domains. The ATP binding site is shown to be located on the 25-kDa domain, the pyruvate binding site on the 35-kDa domain, and the catalytic histidine on the 18-kDa domain.

5B) made from the gel suggested that the [1-$^{14}$C]bromopyruvate specifically labeled the C-terminal 35-kDa domain. Each of the fragments containing the 35-kDa domain, namely, the 67- and 55-kDa fragments were (in addition to the haloenzyme) also labeled. The 25-kDa fragment, which is located in the sequence end opposite to the 35-kDa domain

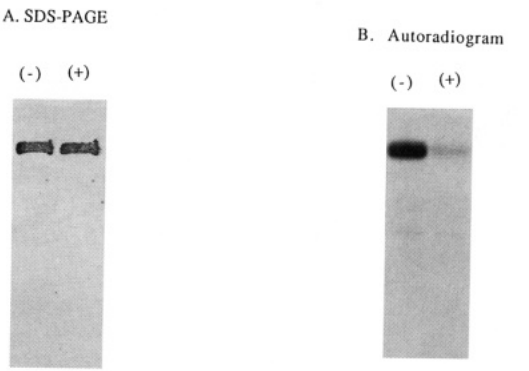



FIGURE 4: Identification of the pyruvate binding domain of PPDK. (A) Coomassie blue stained gel of the fragments generated by proteolysis of the [1-$^{14}$C]bromopyruvate/$NaBH_4$ treated PPDK with subtilisin. (B) Autoradiogram obtained from the gel.

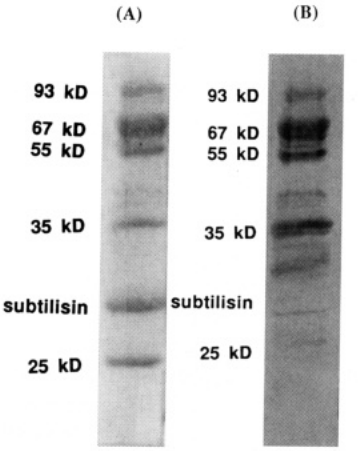



FIGURE 5: Labeling of PPDK with [$^{14}$C]oAMP. After reaction with [$^{14}$C]oAMP, PPDK was subjected to limited subtilisin digestion for 5 min, followed by gel electrophoresis and autoradiography. Lane A shows the SDS–PAGE of the $^{14}$C-labeled and digested sample; lane B shows the corresponding autoradiogram. Molecular weight markers indicate sizes of major domains generated by subtilisin.

(Scheme 3), was not labeled. These results are in accord with an earlier prediction of the location of the pyruvate/PEP based on the regions of sequence identify (located near the C-terminal) shared between PPDK and the PEP binding enzyme, enzyme 1 of the bacterial PEP:sugar phosphotransferase system (Pocalyko et al., 1990).

*ATP Binding Domain.* Next, our attention turned to locating the ATP binding site within the PPDK domain structure. The 2′,3′-dialdehyde derivative of adenosine 5′-phosphate (oAMP) had been reported by Wood and coworkers (Evans et al., 1980) to specifically modify the nucleotide site of PPDK. In general, the use of dialdehyde derivatives of nucleotides as affinity labels is restricted to proteins that contain a lysine at or near the nucleotide binding pocket. One or both of the aldehyde moieties on the oAMP reacts with the E-$NH_2$ of lysines, forming a Schiff base which, in the presence of $NaCNBH_3$ or $NaBH_4$, is reduced to the corresponding amine (Easterbrook-Smith et al., 1976). The observation that PPDK is inactivated by oAMP as well as by pyridoxal phosphate (Phillips et al., 1983) strongly suggest the presence of a lysine residue at the nucleotide binding site.

In our hands, synthetic oAMP (1 mM) inactivated PPDK (1 $\mu$M) in the presence of 5 mM $MgCl_2$, 3 mM $NaCNBH_3$, and 50 mM $K^+$Hepes (pH 7, 25 °C) with $t_{1/2}$ = 5 min. In the presence of 2 mM AMP the observed $t_{1/2}$ was increased to 25 min, thereby evidencing substrate protection and, hence, covalent modification at a common oAMP/AMP binding site

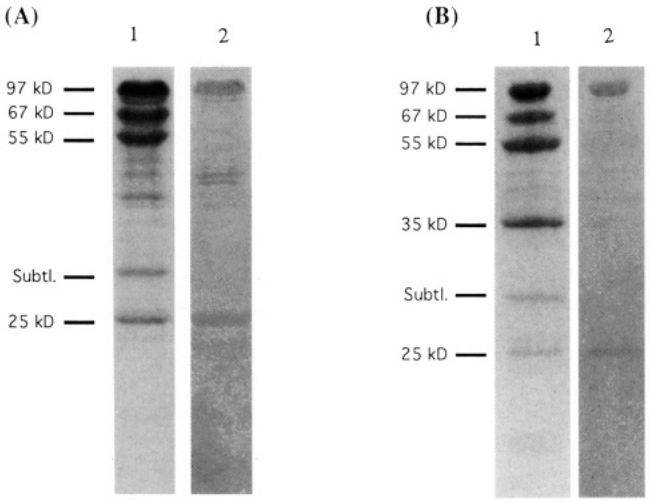



FIGURE 6: Protection of the pyruvate binding site of PPDK from [1-$^{14}$C]bromopyruvate by PEP. (A) Coomassie blue stained SDS–PAGE gel. (B) Autoradiogram made from the gel. (+) indicates that the enzyme was incubated with PEP before [1-$^{14}$C]bromopyruvate was added. (–) indicates that the enzyme was incubated with [1-$^{14}$C]bromopyruvate without preincubation with PEP.

on the enzyme. The location of the site of [$^{14}$C]oAMP covalent modification within the domain structure of PPDK was addressed next. Figure 6 shows the SDS–PAGE gel and corresponding autoradiogram of the subtilisin digest of PPDK subjected to [$^{14}$C]oAMP covalent modification (using conditions found to optimize labeling specificity). These data indicate that the [$^{14}$C]oAMP modifies the 25-kDa domain (the N-terminal domain of the PPDK structure, Scheme 3). While the holoenzyme is also $^{14}$C-labeled, the 67- and 55-kDa fragments (lacking the 25-kDa domain) are not. Other experiments (data not shown) which employed the same labeling conditions but longer subtilisin digestion times (that would allow cleavage of the 67-kDa fragment into the 35-kDa fragment) showed that the 35-kDa domain which is labeled by the [1-$^{14}$C]bromopyruvate is not labeled with the [$^{14}$C]-oAMP. Thus, as illustrated in Scheme 4, the ATP binding site appears to be located on the 25-kDa domain while the pyruvate binding site appears to be located on the 35-kDA domain. Both of these domains are separate from the domain containing the catalytic histidine. While the domain model depicted in Scheme 4 implies four separate structural regions connected by peptide loops, this has not actually been demonstrated.

### *Ligand-Induced Conformational Changes in PPDK Reflected by Proteolysis Cleavage Patterns*

In view of the existing evidence for separate site catalysis in PPDK and for separate ATP and pyruvate binding domains, we were prompted to test for global conformational changes triggered by substrate binding. We were specifically looking for signs of domain movement in response to substrate binding site occupancy.

To probe for a conformational change occuring upon formation of a PPDK complex with ATP, proteolysis of the enzyme was carried out in the presence of the ATP analogue AMPPNP. While AMPPNP is not a substrate for PPDK, it binds to the ATP site with a $K_D$ = 50 $\mu$M (Mehl, 1990). As indicated by the time course for the subtilisin-catalyzed proteolysis shown in Figure 7, the relative distribution of PPDK fragments generated by the subtilisin-catalyzed hydrolysis of the enzyme-AMPPNP complex is the same as that observed for the free enzyme. On the other hand, the overall rate of

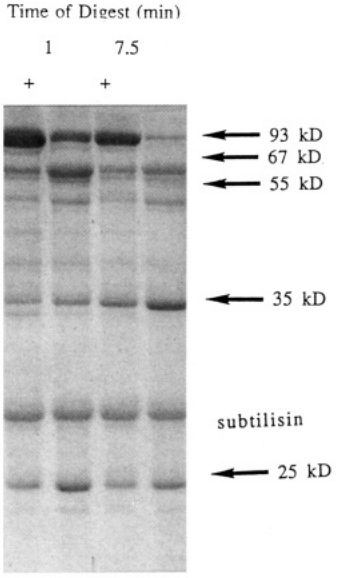



FIGURE 7: Coomassie blue stained SDS–PAGE gel of the fragments generated from the native enzyme by digestion with subtilisin in the presence of AMPPNP. The native enzyme was incubated with AMPPNP, phosphate, and metal cofactors as described under Materials and Methods. The enzyme was digested with subtilisin at an enzyme to protease ratio of 2:1. Aliquots were taken at the indicated times, quenched, and analyzed by SDS–PAGE. Lanes marked with (+) indicate the presence of AMPPNP in the reaction mixture.

the digestion of the enzyme-AMPPNP complex appears to be comparatively slower. In particular, bound AMPPNP appears to slow down cleavage of the holoenzyme to the 67- and 25-kDa fragments and, hence, the 67-kDa fragment to the 35-kDa fragment. This observation suggests a possible conformational change in the native PPDK induced by nucleotide binding. AMPPNP binding also induces a change in the fluorescence spectrum of native PPDK consistent with the proposed change in protein conformation (McQuire and Dunaway-Mariano, unpublished data).

To test for a conformation change in E-P induced by oxalate binding, subtilisin proteolysis of the phosphorylenzyme was carried out in the presence and absence of oxalate. Previous studies have shown that oxalate binds to E-P in the presence of divalent metal ions to form a very tight complex in which the phosphoryl group of the catalytic histidine is coordinated to the metal ion cofactor and it in turn to the bound oxalate (Kofron et al., 1988). In fact, kinetic experiments have shown that while the ATP, $P_i$, and pyruvate reaction sites on the enzyme are nonoverlapping, the conformation that is adopted in the E-P·oxalate·Mg complex precludes catalysis at the AMP/$PP_i$ reaction site (Thrall & Dunaway-Mariano, 1994). Presumably this is the same conformation that the enzyme assumes while catalyzing the E-P·pyruvate·Mg $\rightleftharpoons$ E·PEP·Mg partial reaction. To determine whether this conformation is significantly different from that of E-P or native PPDK, we carried out proteolysis on the E-P·oxalate·Mg complex. The results, shown in Figure 8, indicate a clear change in cleavage patterns. The minor cleavage pathway observed for E-P (and native PPDK) which produces the 55-kDa fragment is now, with the E-P·oxalate·Mg complex, a predominant pathway. Appearance of the 35-kDa fragment from proteolysis of the E-P·oxalate·Mg complex lags well behind that from proteolysis of the E-P·Mg complex. Thus, the oxalate appears to induce a conformational change in the phosphorylated form of the protein which allows cleavage of the ATP binding domain from the rest of the protein but inhibits removal of the pyruvate binding domain (Scheme 4).

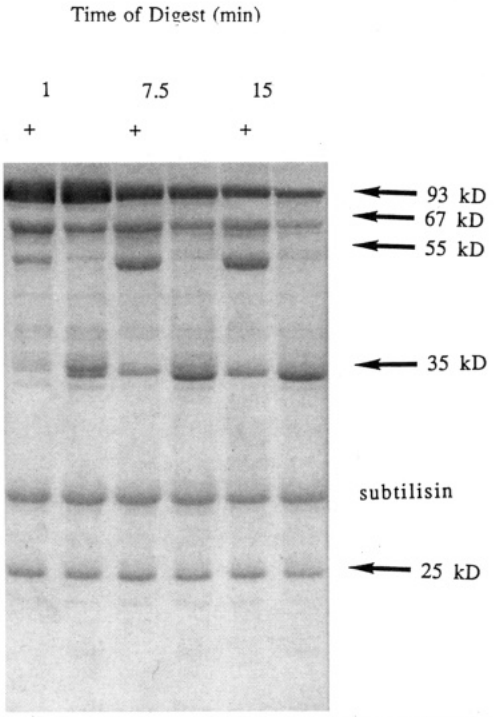



FIGURE 8: Coomassie blue stained SDS–PAGE gel of the fragments generated from the phosphoryl enzyme by digestion with subtilisin in the presence of oxalate. The phosphoryl enzyme was made by incubation with ATP and phosphate in the presence of metal cofactors. Sodium oxalate was added and the enzyme digested with subtilisin at an enzyme to subtilisin ratio of 2:1 (w/w). At the indicated time points, aliquots (10 mg of PPDK) were taken, quenched, and analyzed by SDS–PAGE. (+) Indicates oxalate was include in the digestion.

*Conclusion.* Subtilisin-catalyzed proteolysis of PPDK cleaves this protein into relatively stable 25-kDa N-terminal and 35-kDa C-terminal fragments which contain the ATP and pyruvate binding sites, respectively. The internal fragment, which is not stable, contains the catalytic histidine. Cleavage patterns indicate that this region of the protein may contain 13- and 18-kDa subdomains (Scheme 4). These results do not allow us at this time to unequivocally distinguish between the models of PPDK catalysis illustrated in Schemes 2 and 3. On the other hand, they do fuel the imagination and prompt us to look further into the PPDK domain structure and its possible role in catalysis.

## SUPPLEMENTARY MATERIAL AVAILABLE

One figure showing a Coomassie blue stained SDS–PAGE gel of the fractions produced by gel filtration chromatography of subtilisin-digested PPDK (2 pages). Ordering information is given on any current masthead page.

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