Investigation of the Catalytic Site within the ATP-Grasp Domain of Clostridium symbiosum Pyruvate Phosphate Dikinase*

Dongmei Ye‡, Min Wei§, Marielena McGuire§, Kui Huang¶, Geeta Kapadia¶, Osnat Herzberg¶, Brian M. Martin**, and Debra Dunaway-Mariano‡

From the ‡Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, the §Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, the ¶Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, Maryland 20850, and **Clinical Neuroscience, National Institutes of Health, Bethesda, Maryland 20892

Pyruvate phosphate dikinase (PPDK) catalyzes the interconversion of ATP, P₆, and pyruvate with AMP, PP₃, and phosphoenolpyruvate (PEP) in three partial reactions as follows: 1) E-His + ATP → E-His-PP-AMP; 2) E-His-PP-AMP + P₁ → E-His-P-AMP-PP₃; and 3) E-His + pyruvate → E-PEP using His-455 as the carrier of the transferred phosphoryl groups. The crystal structure of the Clostridium symbiosum PPDK (in the unbound state) reveals a three-domain structure consisting of consecutive N-terminal, central His-455, and C-terminal domains. The N-terminal and central His-455 domains catalyze partial reactions 1 and 2, whereas the C-terminal and central His-455 domains catalyze partial reaction 3. Attempts to obtain a crystal structure of the enzyme with substrate ligands bound at the nucleotide binding domain have been unsuccessful. The object of the present study is to measure Mg(II) activation of catalysis with substrate ligands bound at the nucleotide binding site.

Pyruvate phosphate dikinase (PPDK) catalyzes the interconversion of ATP, P₆, and pyruvate with AMP, PP₃, and PEP in three partial reactions as follows: 1) E-His + ATP → E-His-PP-AMP; 2) E-His-PP-AMP + P₁ → E-His-P-AMP-PP₃; and 3) E-His + pyruvate → E-PEP using His-455 as the carrier of the transferred phosphoryl groups. The crystal structure of the Clostridium symbiosum PPDK (in the unbound state) reveals a three-domain structure consisting of consecutive N-terminal, central His-455, and C-terminal domains. The N-terminal and central His-455 domains catalyze partial reactions 1 and 2, whereas the C-terminal and central His-455 domains catalyze partial reaction 3. Attempts to obtain a crystal structure of the enzyme with substrate ligands bound at the nucleotide binding domain have been unsuccessful. The object of the present study is to measure Mg(II) activation of catalysis with substrate ligands bound at the nucleotide binding site.

Pyruvate phosphate dikinase (PPDK) catalyzes the interconversion of ATP, P₆, and pyruvate with AMP, PP₃, and PEP in three partial reactions as follows: 1) E-His + ATP → E-His-PP-AMP; 2) E-His-PP-AMP + P₁ → E-His-P-AMP-PP₃; and 3) E-His + pyruvate → E-PEP using His-455 as the carrier of the transferred phosphoryl groups. The crystal structure of the Clostridium symbiosum PPDK (in the unbound state) reveals a three-domain structure consisting of consecutive N-terminal, central His-455, and C-terminal domains. The N-terminal and central His-455 domains catalyze partial reactions 1 and 2, whereas the C-terminal and central His-455 domains catalyze partial reaction 3. Attempts to obtain a crystal structure of the enzyme with substrate ligands bound at the nucleotide binding domain have been unsuccessful. The object of the present study is to measure Mg(II) activation of catalysis with substrate ligands bound at the nucleotide binding site.
activation of catalysis at the ATP/Pi active site, to identify the residues that contribute to catalysis at this site, and to derive probable functional roles for these residues based on their positions within the active site scaffold.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutants**—Mutant genes were prepared from the plasmid pACYC184-D12 (9) using a polymerase chain reaction-based procedure analogous to that described previously (6). Mutagenic primers, 18–22 base pairs in length, were synthesized by Life Technologies, Inc. BglII and BstXI restriction sites were employed in the construction of the T253A, E279A, Q240A, R92A, R92K, K22A, and K22R PPDK mutants, whereas BstXI and KpnI restriction sites were used in the construction of the E323D, E323L, D321N, and Q335A mutants. The sequence analyses of the mutant genes were carried out by the Center for Agricultural Biotechnology at the University of Maryland. Wild-type and mutant PPDK genes were expressed in Escherichia coli JM101 cells, and the protein products were purified to homogeneity as described previously (6). The mutant gene, encoding residues 1–533 in PPDK, was used in the construction of the E323D, E323L, D321N, and Q335A mutants. The fragment analyses of the mutant genes were carried out by the Center for Agricultural Biotechnology at the University of Maryland. Wild-type and mutant PPDK genes were expressed in Escherichia coli JM101 cells, and the protein products were purified to homogeneity as described previously (6). The mutant gene, encoding residues 1–533 in PPDK, was used in the construction of the E323D, E323L, D321N, and Q335A mutants. The fragment analyses of the mutant genes were carried out by the Center for Agricultural Biotechnology at the University of Maryland. Wild-type and mutant PPDK genes were expressed in Escherichia coli JM101 cells, and the protein products were purified to homogeneity as described previously (6).

**N-terminal Domain-Central Domain PPDK Mutant Protein (Tem533)**—The mutant gene, encoding residues 1–533 in PPDK, was prepared and expressed as described above. The protein was purified in a yield of 4 mg/g cell. Its N-terminal sequence, determined by automated protein sequencing, is identical to that of the wild-type PPDK. The molecular mass of Tem533 was determined by matrix-assisted laser desorption ionization-mass spectrometry at the University of Michigan Protein and Carbohydrate Structure Facility to be 58.6 ± 0.2 kDa. This value compares well with the theoretical molecular mass of 58,654 Da. The molecular mass of the native protein was determined by gel column chromatography to be ~51 kDa. Thus, the mutant protein is monomeric.

**Spectrophotometric Assay**—Initial velocities were measured as a function of the concentration of the varied substrate (in a range of 0.5–10 mM) at fixed, saturating concentrations of cosubstrates (0.5 mM AMP, 0.5 mM PEP, 1 mM PPi) and metal ion cofactors (5 mM MgCl2 and 40 mM NH4Cl) in 20 mM imidazole (pH 6.8, 25 °C). The initial velocity data were analyzed using Equation 1 and the computer programs of Cleland (10). The $k_{cat}$ value was calculated from $V_{max}$/[E].

$$ V_v = V_{max}[S]/(K_s + [S]) $$

(Eq. 1)

where $V_v$ is the initial velocity; [E] is the enzyme concentration; [S] is the substrate concentration; $V_{max}$ is the maximum velocity, and $K_s$ is the Michaelis constant.

The $K_v$ values for the competitive inhibitor AMPPNP were determined from initial velocity data obtained at varying AMP concentrations and fixed, saturating concentrations of cosubstrates and cofactors. The initial velocity data were analyzed using Equation 2 where $K_v$ is the inhibition constant and [I] is the inhibitor concentration.

$$ V_v = V_{max}[S]/(K_v(1 + [I]/K_i) + [S]) $$

(Eq. 2)

**Radioisotopic Assay**—Reaction solutions (100 μl) initially containing 30 μg enzyme, 1 mM [14C]AMP, 1 mM Pi, 1 mM PEP, 5 mM MgCl2, 40 mM NH4Cl, 0.2 mM NADH, 20 units/ml lactate dehydrogenase in 50 mM K+ Hepes (pH 7.0, 25 °C) were quenched at specific times with 390 μl of 0.6 M HCl. The protein was removed by centrifugal filtration (5000 g, 5 min). The [14C]AMP and [14C]ATP were separated from the supernatant by HPLC using a 4.6-mm × 25-cm Beckman ultrasphere C18 reversed-phase analytical column and 25 mM KH2PO4, 2.5% triethylamine, and 5% methanol as eluant (1 ml/min flow rate). Peaks fractions were quantified by scintillation counting.

**ATP + P_1 + Pyruvate → AMP + PP + PEP Reaction**—Reaction solutions (100 μl) initially containing 0.1–20 μg enzyme, 5 mM pyruvate, 5 mM P1, 5 mM MgCl2, 40 mM NH4Cl, and 20 units/ml inorganic pyrophosphatase (to drive the reaction to completion by catalyzing PPi, hydrolysis) in 50 mM K+ Hepes (pH 7.0, 25 °C) were quenched at specific times with 300 μl of 0.6 M HCl. The resulting solutions were filtered and analyzed by HPLC as described above.

**Mg(II) Activation of Tem533**—The time courses ([14C]AMP concentration versus reaction time) for wild-type and Tem533 PPDK-catalyzed single turnover reactions of E → [14C]ATP → E-PP-AMP and E → [14C]ATP + P1 → E-PP + P1 were carried out as described previously (6). The time courses ([14C]AMP concentration versus reaction time) used for the determination of the $k_{cat}$ versus [Mg(II)] plot were measured with 60-μl reaction solutions initially containing 40 μM [14C]ATP, 40 μM Tem533 PPDK, and variable MgCl2 concentration (10–5000 μM) in 40 mM NH4Cl and 50 mM K+ Hepes (pH 7.0, 25 °C). The reactions were terminated and analyzed for [14C]AMP and [14C]ATP as described previously (6). The $k_{cat}$ was calculated from the time course data using Equation 3, where $P_1$ is the concentration of [14C]AMP at time $t$, and $Pog$ is the concentration of [14C]AMP at equilibrium.

$$ \log P/P_0 = k_{cat}(t) $$

(Eq. 3)

**X-ray Crystallographic Analysis of K22A PPDK**—The crystallization conditions used for the C. symbiosum K22A PPDK mutant were the same as those used for the wild-type enzyme (4). Single crystals were obtained at 30 °C by vapor diffusion in hanging drops. Protein drops

**FIG. 1.** A, conformer 1, ribbon stereodиagram of the C. symbiosum PPDK monomer generated from the x-ray coordinates of the apoPPDK structure reported previously (4) using INSIGHTII. Catalysis of the E + ATP + P1 → E-PP + AMP + PP, partial reactions takes place at the N-terminal domain (green) active site conformer 1. B, conformer 2, ribbon stereodиagram of the C. symbiosum PPDK monomer modeled from the structure shown in A. Catalysis of the E-PP + pyruvate → E + PEP partial reaction takes place at the C-terminal domain (blue) active site in the modeled conformer 2. Movement of the catalytic His-455 residue (black) between the two active sites is perceived to occur through the interconversion of conformers 1 and 2 via the swivel of the central domain (yellow) about its helical linkers (red) (4).
were equilibrated against reservoir solutions containing 50–55% saturated ammonium sulfate and 100 mM Hepes buffer, pH 7.0. The drops consisted of protein at 10 mg/ml, 20 mM imidazole buffer, pH 6.5, 100 mM KCl, 0.1 mM EDTA, and 1 mM mercaptoethanol diluted by an equal volume of reservoir solution. The crystals belong to the space group P21, and within the accuracy of the data the unit cell dimensions are the same as those of the wild-type protein crystals (Table IV). X-ray diffraction data were collected on a Siemens area detector mounted on a 3-circle goniostat, with monochromated CuKα x-ray supplied by a Rigaku Rotaflex RU200BH rotating anode generator. Data were processed with the XENGEN package (11). Data collection statistics are provided in Table IV. Initial structure refinement was performed with the program X-PLOR (12). The simulated annealing slow-cooling protocol at 3000 K was followed by positional refinement cycles. Data between 8.0 and 2.8 Å for which $F_\alpha^2/F_\sigma^2$ were included. Adjustments to the model were made on a Silicon Graphics INDIGO II computer graphics work station using the program TURBO-FRODO (13). The later stages of refinement were performed with the program TNT (14) including data between 10 and 2.8 Å for which $F \geq 2\sigma (F)$. The refinement results are summarized in Table IV.

RESULTS

Mg(II) Activation—The N-terminal domain of PPDK has a fold similar to the folds of the ATP binding domains of the ATP-Grasp enzyme superfamily. The members of this superfamily bind, and activate, ATP for γ-P phosphoryl transfer to a variety of cosubstrates (15). PPDK, which catalyzes pyrophosphoryl transfer by displacement at the ATP $\beta$-P, represents a point of chemical divergence within the family. Aside from PPDK, each member of the ATP-Grasp family is known to bind one or more divalent metal ions at the catalytic site, and all but synapsin (which binds a single Ca(II) ion) utilizes Mg(II) as the physiological cofactor. PPDK requires Mg(II) for catalysis (1).

Based on previous studies of the Mg(II) dependence of PPDK catalyzed isotope exchange, there is evidence that suggests that Mg(II) is a required cofactor at both active sites (16, 17).

The object of this study was to measure Mg(II) activation at the N-terminal domain active site. For this purpose, the truncation mutant Tem533 (PPDK residues 1–533), in which the C-terminal domain has been deleted, was used in place of native PPDK. Since the Tem533 does not contain the PEP/pyruvate active site, Mg(II) binding at the ATP/Pi site of Tem533 may be studied without the interference from Mg(II) binding at the PEP/pyruvate site.

The Tem533 PPDK mutant gene was constructed by site-directed mutagenesis and expressed in E. coli cells to provide 4 mg of purified protein/g of cells. The efficiency of catalysis of the $E \rightarrow E$-PP-AMP and $E + ATP + P_i \rightarrow E-P + AMP + PP_i$ partial reactions was measured in the presence of saturating Mg(II) (5 mM). The rate data are plotted in Fig. 2. The $k_{obs}$ values, determined by fitting the time course data to a single exponential equation (Equation 3) (Table I), indicate that Tem533 PPDK has retained 10% of the activity of the native enzyme. At the present, the reason behind the 10-fold reduction in catalytic efficiency is not known. We suspect, however, that substrate binding and product release from the

![Fig. 2.](image-url)
N-terminal domain active site require that the central domain is docked at the C-terminal domain. Nonetheless, the Tem533 mutant was sufficiently active to carry out the Mg(II) activation studies as planned.

The $E + \text{ATP} + P_1 \rightarrow E-P + \text{AMP} + PP_i$ partial reaction, and not the $E + \text{ATP} \rightarrow E-PP$-AMP partial reaction, was chosen to measure Mg(II) activation. This is because the level of product formation in the $E + \text{ATP} \rightarrow E-PP$-AMP partial reaction is small (5% (17)) when Mg(II) is used as cofactor (note that the Co(II) used in the reaction reported in Fig. 2A serves to increase product formation to 25% owing to tighter ATP binding and greater $E-PP$-AMP stability (17)). The $E + \text{ATP} + P_1 \rightarrow E-P + \text{AMP} + PP_i$ partial reaction, on the other hand, is driven to 65% completion (Fig. 2B) by $P_1$, independent of the cofactor used. The higher the level of the product formation, the more accurate is the product determination. The rate ($k_{obs}$) of the $E + \text{ATP} + P_1 \rightarrow E-P + \text{AMP} + PP_i$ partial reaction was measured using 40 $\mu$M enzyme to react with 40 $\mu$M $[^{14}\text{C}]$ATP in the presence of 11 mM $P_i$. A similar data set was obtained using 1 mM $P_i$. The Mg(II) concentrations used in the reaction ranged from 0 to 5 mM. The plot of $k_{obs}$ versus [Mg(II)], shown in Fig. 3A, deviates from the hyperbolic curve predicted for single site Mg(II) binding. Several determinations of $k_{obs}$ were carried out for reactions containing between 0 and 100 $\mu$M Mg(II) in order to define an apparent “lag” in enzyme activation observed at the low concentration range (Fig. 3B).

The presence of a lag in the $k_{obs}$ versus [Mg(II)] of plot (Fig. 3) may be explained by a two-site Mg(II) binding model. Specifically, one site on the enzyme binds Mg(II) with high affinity, whereas the second site binds Mg(II) with significantly lower affinity. The absence of product formation at Mg(II) concentrations below ~80 $\mu$M, followed by a “hyperbolic” response between the rate of product formation and the Mg(II) concentration in the 100–5000 $\mu$M range, suggests that a high affinity site is being titrated at low Mg(II) concentration (i.e. on the order of the enzyme concentration = 40 $\mu$M), and once this first site is filled Mg(II) is available to bind to the second site. Only when both sites are occupied is the enzyme competent to catalyze the reaction.

# Table I

The observed rate constants ($k_{obs}$) derived from single turnover reactions catalyzed by wild-type (WT) and Tem533 PPDK

<table>
<thead>
<tr>
<th>Reaction</th>
<th>WT $k_{obs}$</th>
<th>Tem533 $k_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E + ^{14}\text{C}[\text{ATP}]$</td>
<td>300 s$^{-1}$</td>
<td>25 s$^{-1}$</td>
</tr>
<tr>
<td>$E + ^{14}\text{C}[\text{ATP}] + P_1$</td>
<td>6.5 s$^{-1}$</td>
<td>0.4 s$^{-1}$</td>
</tr>
</tbody>
</table>

---

---

Kinetic Properties of Active Site Mutants—The amino acid residues whose polar side chains project into the ATP/Pi active site crevice include Gln-240, Arg-92, Lys-22, Thr-253, Asp-321, Glu-279, and Asp-280 (Fig. 4). There are also two loops (the 101–103 loop and the 278–281 loop) that contribute to the active site. The 101–103 loop (Gly-101 to Met-102 to Met-103), which is reminiscent of the classical phosphate binding loop observed in other nucleotide-dependent enzymes, is located to one side of the crevice entrance. The 278–281 loop (containing residues Glu-279 and Asp-280 whose side chains project into the active site) will be referred to as the “$\omega$-loop.” This loop forms the roof of the active site crevice, but in the apoPPDK crystal structure the $\omega$-loop is seen to protrude into the active site, where Glu-279 forms an ion pair with active site residue Arg-92. Substrate binding must displace the loop, thus allowing Glu-279 or Asp-280 to favorably interact with Arg-135 (which may function to hold the loop over the active site).
instead of with Arg-92 (which appears to draw the loop into the active site).

Amino acid replacements were made at Gln-240, Arg-92, Lys-22, Thr-253, Asp-321, Glu-323, Gln-335, Arg-337, Glu-279, and Asp-280 in order to determine if these residues contribute to catalysis at the ATP/Pi active site. The mutant enzymes were purified to homogeneity and then evaluated for catalytic activity by measuring their $k_{cat}$ and $K_m$ values using steady-state kinetic techniques. Although the $k_{cat}$ value is determined by the rate at which catalysis occurs at the C-terminal domain active site (Scheme 1, partial reaction 3) as well as by the rate at which catalysis occurs at the N-terminal domain active site (partial reactions 1 and 2), previous studies have shown that these two rates are comparable in magnitude. Thus, a mutation that significantly slows down catalysis at the N-terminal domain active site will result in a significant reduction in the $k_{cat}$ value measured for the complete reaction.

The mutants were first tested for catalysis of AMP + PPi + PEP → ATP + Pi + pyruvate reaction using a continuous spectrophotometric assay. Although applicable only to mutants having >0.1% of the wild-type activity, the assay allows easy determination of substrate $k_{cat}$ and $K_m$ values as well as the $K_i$ values of the inert ATP analog AMPPNP (Table II). Less active mutants (R92A, R92K, K22A, D321N, E323D, Q335A, and E279A/D280A) were analyzed using a fixed time, radioisotopic assay. Here, the $k_{cat}$ was determined from the initial velocity measured at fixed substrate concentrations, which for the wild-type enzyme are saturating (Table II). Since amino acid replacement may significantly alter the substrate binding affinity, the assumption that the fixed substrates saturate the mutant enzymes may not hold. Thus, it is possible that the $k_{cat}$ values measured for some of these mutants under-estimate the true $k_{cat}$ values.

Catalysis was also measured in the ATP + P_i + pyruvate → AMP + PP_i + PEP reaction direction using a fixed time, radioisotopic assay (Table III). Only the more active mutants (R135A, D280A, and E279A) were subjected to an initial velocity study to evaluate $k_{cat}$ and $ATP_K_i$.

Based on the kinetic values shown in Tables II and III, it can be seen that amino acid replacement at Lys-22, Arg-92, Asp-321, Glu-323, and Gln-335 severely inhibits catalytic turnover.

The activities of these mutants are too low for the measurement of $K_m$ or $K_i$ values. Thus, all that can be said about these mutants is that they are inactive.

Mutation at Arg-337 inhibits but does not preclude catalysis (6). The R337A mutant is 18-fold less active in the ATP-forming direction and 520-fold less active in the AMP-forming direction (Tables II and III). The 13-fold increase observed in the $PP_i/K_m$ value suggests that PP_i binding may be seriously impaired. Consistent with this deduction is the observation, from transient kinetic studies, that the rate of the $E + ATP \rightarrow E$-PPAMP step is reduced only 5-fold in the R337A mutant, whereas the rate of the $E$-PP-AMP + P_i → E-P + PP_i + P step is reduced 140-fold (6).

The Q240A and T253A mutants have $k_{cat}$ values that are 80 and 50% that of the wild-type enzyme. This suggests that these two residues do not play significant roles in substrate activation. On the other hand, in both cases, the Ala substitution does have some inhibitory effect, as indicated by the 2–10-fold increase seen for $AMPK_m$ and $AMPPNP/K_i$ values.

Substitution of the $\omega$-loop residues Glu-279 and Asp-280 and, the putative loop docking site residue, Arg-135 resulted in significant but not severe reductions in catalytic efficiency. The double-loop mutant E279A/D280A, however, is devoid of activity. Measured in the AMP-forming direction, the R135A and E280A mutants showed an 8- and 5-fold reduction in $k_{cat}$, whereas the E279A mutant showed a 55-fold reduction (Table III). In the ATP-forming direction, $k_{cat}$ is 4-fold smaller in the R135A and E280A mutants and 10-fold smaller in the E279A mutant (Table II). Interestingly, the $AMPK_m$ and $AMPPNP/K_i$ values increased 10-fold in the R135A mutant and 2-fold in the D280A mutant. Perhaps these three residues affect catalysis and substrate binding by modulating loop closure and, hence, active site desolvation.

**Electronic and Structural Changes in the Active Site Mutants**—The replacement of a charged active site amino acid residue with an uncharged one will necessarily perturb the local electrostatic environment, in addition to removing the potential for favorable ligand interaction (viz. hydrogen bond formation with substrate or coordination bond formation with Mg(II)). Given that bonding is sensitive to orientation effects, it is sometimes possible to distinguish the role of a charged residue in bonding from that of maintaining an electrochemical
balance, by comparing the kinetic properties of neutral versus charged mutants. Here, we examine the three positively charged active site residues Lys-22, Arg-92, and Arg-337. In Tables II and III, the kinetic properties of K22A versus K22R PPDK, R92A versus R92K PPDK, and R337A versus R337K PPDK are compared. In the cases of Lys-22 and Arg-92, the charge-conserving mutants are not significantly more active than are the Ala mutants, thus suggesting that the side chains of the Lys-22 and Arg-92 residues must not only be charged but must also be precisely oriented for substrate activation. Substitution of Arg-337 with Lys results in a 10-fold smaller reduction in catalysis than does substitution with Ala. However, the activity gain with the Lys residue is offset by the loss of chemical specificity. With the R337K mutant, but not the R337A mutant, phosphoryl transfer to water competes with the normal reaction pathway (6). Thus, precise orientation of the charged group at position 337 is also required for efficient catalysis.

The possibility that the replacement of one amino acid with another will trigger a change in active site structure is always a concern. For this reason we attempted to obtain x-ray crystal structures of each of the site-directed mutants. So far the findings are suggestive of direct binding interaction between the AMP analogs, IMP and GMP, having modified base, ribose, or phosphate moieties, were tested as substrates and competitive inhibitors. The $k_{cat}$ and $K_m$ values obtained from steady-state kinetic measurements are listed in Table V. The AMP analogs, IMP and GMP, are not active substrates nor are they effective inhibitors. Thus, at least with these two mutants, we can reasonably attribute the alterations observed in their kinetic properties solely to electronic effects and, more precisely, to the positioning of the charged side chain.

**Kinetic Properties of Substrate Analogs**—Nucleotide analogs, having modified base, ribose, or phosphate moieties, were tested as substrates and competitive inhibitors. The $k_{cat}$ and $K_m$ values obtained from steady-state kinetic measurements are listed in Table V. The AMP analogs, IMP and GMP, are not active substrates nor are they effective inhibitors. Thus, at least with these two mutants, we can reasonably attribute the alterations observed in their kinetic properties solely to electronic effects and, more precisely, to the positioning of the charged side chain.
AMP, and 2',3'-dideoxy-ATP. These results indicate that the ribose 2'-hydroxy group, but not the 3'-hydroxy group, is important for productive binding.

**TABLE IV**

Statistics of x-ray crystallographic data collection for K22A PPDK

<table>
<thead>
<tr>
<th>Data collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell parameters</td>
<td>a = 89.9 Å; b = 58.6 Å; c = 102.3 Å</td>
</tr>
<tr>
<td>Maximum resolution (Å)</td>
<td>2.8</td>
</tr>
<tr>
<td>No. of crystals used</td>
<td>1</td>
</tr>
<tr>
<td>No. of observations</td>
<td>58,293</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>25,068</td>
</tr>
<tr>
<td>Rmerge a</td>
<td>0.07</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98</td>
</tr>
<tr>
<td>Structure refinement</td>
<td>53 (2.9–2.8 Å)</td>
</tr>
<tr>
<td>R-factor b</td>
<td>0.193</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>20,093 (10–2.8 Å, F ≥ 2σ(F))</td>
</tr>
<tr>
<td>Root mean square deviation from</td>
<td>standard</td>
</tr>
<tr>
<td>structure chemistry</td>
<td>0.019</td>
</tr>
<tr>
<td>Bond length (Å)</td>
<td>2.7</td>
</tr>
<tr>
<td>Planar group (Å)</td>
<td>0.019</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>6719</td>
</tr>
<tr>
<td>No. of solvent</td>
<td>43 (2 sulfate ions)</td>
</tr>
<tr>
<td>Root mean square deviation from</td>
<td>wild-type PPDK</td>
</tr>
<tr>
<td>(1DK) on Cu atoms (Å)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

a Rmerge = \( \sum_{i=1}^{n} |\langle F_i \rangle - (\langle F_i \rangle|/\sum_{i=1}^{n} |\langle F_i \rangle| \), for symmetry equivalent reflections.  
b R-factor = \( \sum_{i=1}^{n} |F_i - \langle F_i \rangle|/\sum_{i=1}^{n} |\langle F_i \rangle| \).

**TABLE V**

Kinetic constants measured for ATP/AMP analogs as alternate substrates or as competitive inhibitors of PPDK versus AMP

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( k_{cat} )</th>
<th>( K_m )</th>
<th>( K_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>8.3 ( \mu )</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>2'-dATP</td>
<td>( 1.1 \times 10^{-1} )</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>3'-dATP</td>
<td>6.7</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>2',3'-didATP ( a,b )</td>
<td>( 4.4 \times 10^{-2} )</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>25</td>
<td>9</td>
<td>50 ( d )</td>
</tr>
<tr>
<td>2'-dAMP</td>
<td>1.6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>AMPPNP ( b,e )</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPS ( b,e )</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdeS ( b,e )</td>
<td>( &gt;1 \times 10^4 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade ( e )</td>
<td>320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMP ( e,f )</td>
<td>8700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>( &gt;1 \times 10^4 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Reactions initially containing varying nucleotide concentration, 5 mM Pᵢ, 5 mM pyruvate, 5 mM MgCl₂ and 40 mM NH₄Cl in 50 mM K Hepes (pH 7.0, 25 °C) were monitored using the HPLC-based assay for nucleotide substrate and product.

b 2',3'-didATP, 2',3'-dideoxyadenosine 5'-triphosphate; AMPS, adenosine 5'-mono-O-phosphorothioate; AdeS, adenosine 5'-sulfate.

c Reactions initially containing varying nucleotide concentration, 1.0 mM PP, 0.5 mM PEP, 5 mM MgCl₂, and 40 mM NH₄Cl in 20 mM imidazole HCl (pH 6.8, 25 °C) were monitored using the spectrophotometric coupled assay for pyruvate.

d \( K_i \) values were determined from a Scatchard plot of equilibrium dialysis data (17).

e Reactions initially containing varying nucleotide concentration, 1.0 mM AMP, 0.5 mM PEP, 5.0 mM MgCl₂, and 40 mM NH₄Cl in 20 mM imidazole HCl (pH 6.8, 25 °C) at fixed inhibitor concentration were monitored using the spectrophotometric coupled assay for pyruvate.

f Noncompetitive inhibition was observed: \( K_i = 8.7 \) mM and \( K_m = 25 \) mM.
DISCUSSION

Essential Groups—Based on the substrate analog results (Table V), we propose that the adenine ring, ribose 2'-hydroxy group, and α-phosphate group of ATP participate in productive nucleotide binding. The results from the Mg(II) activation studies (Fig. 3) suggest that a minimum of two Mg(II) cofactors participate in substrate activation. Mutagenesis studies show that the active site residues Gln-240 and Thr-253 play no significant role in catalysis. The α-loop residues Asp-280 and Glu-279, along with Arg-135 which resides outside of the active site crevice, contribute to catalytic efficiency but are not critical for product formation. Gln-335, Asp-321, Glu-323, Lys-22, and Arg-92 are essential for catalysis and are thus, directly (through ion pair formation; Lys-22 and Arg-92) or indirectly (through Mg(II) coordination; Gln-335, Asp-321, and Glu-323), required for ATP binding/activation. From previous studies (6) it is known that Arg-337 contributes modestly to ATP activation (5-fold rate contribution) and more significantly (140-fold rate contribution) to Pγ activation.

Active Site Model—The results summarized above are used, in conjunction with analogies drawn with the ligand binding modes observed for other ATP-Grasp enzymes, to construct a model for ATP, Pγ, and Mg(II) binding within the PPDK N-terminal domain active site. The ATP-Grasp family includes PPDK, α-Ala-α-Ala ligase (18, 28), glutathione synthetase (19), carbamoyl-phosphate synthetase (20), succinyl-CoA synthetase (21), synapsin (22), glycaminde ribonucleotide synthetase (23), PurP-encoded glycaminde ribonucleotide transformylase (24), and biotin carboxylase (25, 29). Common to each of these enzymes is the nucleotide binding crevice formed between a 4-stranded antiparallel β-sheet of one subdomain and a 5-stranded antiparallel β-sheet of the other (see the synapsin structure shown in Fig. 6A for a clear example of the ATP Grasp-motif). The side chains and backbone amide groups that function in ATP, cosubstrate, and Mg(II) binding are stationed along these 9 β-strands and along the connecting loops. Despite the low degree of sequence homology existing between these enzymes,6 the conservation of the active site scaffold and the positioning of polar groups for substrate/cofactor binding on this scaffold is remarkably high. This fact has been noted by others (4, 18–25, 28, 29).

In Fig. 6B, a generalized topological diagram is provided for the purpose of illustrating the use of common stations for positioning binding/catalytic groups within the ATP-Grasp active sites of the family members. It is based on this common station usage that one can generate an active site model, from the structure of the unbound form of a new family member, with reasonable certainty. However, in drawing an analogy between the ATP-Grasp active sites observed in the family members listed above and that of PPDK, it is necessary to keep in mind the difference in the chemistries involved. Specifically, whereas most members catalyze γ-phosphoryl transfer from ATP to a cosubstrate, PPDK catalyzes two transfers at this active site: first the transfer of the βPγP unit by attack of the carrier His-455 at the β-P of ATP and then the γ-P transfer from the His-455-βPγP to the cosubstrate, Pγ. Thus, one might anticipate that some modification of the basic ATP-Grasp active site would be manifested in PPDK. The active site model derived for PPDK is shown in a three-dimensional structural format (derived from the apoPPDK x-ray coordinates (4)) in Fig. 7A and in a schematic format in Fig. 7B.

Stations for Binding the Adenine Ring—In each ATP-Grasp enzyme, the substrate adenine ring binds (in an anti-conformation) at the apex of the crevice formed between β-sheet 1 (strands 1–4) and β-sheet 2 (strand 5–9) (see synapsin-ATP

6 The identity is too low, in fact, for useful sequence alignments to be made. Instead, common sites for substrate and cofactor-binding residues have been identified by superpositioning the three-dimensional structures (18–25, 28, 29).
The PPDK Leu-107, Met-242, and Leu-334 side chains surround the adenine ring, thus providing the hydrophobic component of substrate binding observed with the other ATP-Grasp enzyme-nucleotide complexes and suggested by lack of substrate activity and the decreased binding affinity observed for the polar adenine ring analogs GMP and IMP.

**Stations for Binding the Ribose Ring**—In each ATP-Grasp fold enzyme, the substrate ribose ring is bound in a 2'-endo, 3'-exo conformation (see ribose of ATP ligand bound to synapsin shown in Fig. 6A). In some, but not all, ATP-Grasp enzymes, the side chain of an active site Asp or Glu residue, located at the N terminus of β-strand 5, interacts with the 2'-OH and/or 3'-OH (see topological diagram in Fig. 6B). In PPDK, this position is occupied by Thr-253. Two family members, β-Ala-β-Ala ligase and glutathione synthetase, also use backbone amide groups of the loop connecting β-strand 6 with β-strand 7 (corresponding to the α-loop in PPDK) to bind the ribose moiety. The ATP ribose was modeled into the PPDK active site in the 2'-endo, 3'-exo conformation, to obtain a good fit (i.e. if one ignores the misplaced α-loop) (Fig. 7A). The ribose 2'-OH resides in the vicinity of Thr-253 of β-strand 5, but it is not optimally positioned to engage in hydrogen bond interaction. Indeed, mutation of Thr-253 to Ala resulted in only a modest reduction in catalytic efficiency. The ribose of the modeled ATP occupies the region that is filled by the backbone amide NHs of Gly-101 and Met-103 hydrogen bond with γ-P, and the β4–β5 loop (gold; backbone amide NH of Val-243 hydrogen bonds with N(1)). B, schematic illustration of the interactions between PPDK active site residues, substrates, and Mg(II) ions.

**Stations for Binding the Mg(II) Cofactors**—Within the active sites of the ATP-Grasp enzymes, the polyphosphate moiety of the bound ATP interacts with one, two, or three divalent metal ion cofactors (18–25, 28, 29). The metal ion-binding residues are, in turn, located on β-strands 8 and 9 (Fig. 6B). In PPDK, the three essential residues Glu-323, Asp-321, and Gln-335 are positioned on these strands. These three residues, along with the ATP polyphosphate moiety and P_i, are probable ligands for the Mg(II) cofactors, as we have indicated in the PPDK active site model of Fig. 7A. Whereas the Mg(II) sites shown in this model meet spatial restrictions, they are at this stage highly speculative. Moreover, the positions of the Mg(II) ions in partial reaction 1 may change for partial reaction 2. Nevertheless, for catalysis of partial reaction 1, we assume that one Mg(II) is positioned to coordinate with Glu-323 and Gln-335 of the enzyme and with the β-P and γ-P of the ATP ligand. The second Mg(II) is shown to bridge the Asp-321 carboxylate and the P_i ligand.

**Stations for Binding the Polyphosphate Chain**—Fig. 6B shows that the phosphoryl groups of nucleotides bound to ATP-Grasp domains interact with a Lys or Arg located on β-strand 2 (α-P), with the backbone amide NHs of a segment of the loop connecting β-strand 2 with β-strand 3 (β-P) and, in some cases, with the side chain of a Lys or Arg residue located on this same loop. In some of the ATP-Grasp domains, the α-helical segment leading into the N terminus of β-strand 1 is additionally used to position an interacting Lys or Arg side chain. The polyphosphate moiety of the modeled ATP in PPDK interacts with the side chains of Arg-92 (β-strand 2; α-P) and Lys-22 (α-helical segment leading N terminus of β-strand 1; γ-P) and with the backbone amide NHs of Gly-101 and Met-103 (the 101–103 loop connecting β-strand 2 with β-strand 3; γ-P) (Fig. 7A). Amino acid replacement at position Lys-22 or Arg-92 precludes reac-

**Fig. 7.** A, stereodiagram of the proposed PPDK N-terminal domain active site model. ATP (blue), P_i (red), and two Mg(II) ions (green) are shown with the side chains (black) as well as with the β6–β7 α-loop (red; contains Glu-279 and Asp-280), the β2–β3 101–103 loop (gray; backbone amide NHs of Gly-101 and Met-103 hydrogen bond with γ-P), and the β4–β5 loop (gold; backbone amide NH of Val-243 hydrogen bonds with N(1)). B, schematic illustration of the interactions between PPDK active site residues, substrates, and Mg(II) ions.
tion with the ATP, suggestive of their important roles in ATP orientation/activation. The Gly-101—Met-102—Met-103 loop is conserved in all 17 known PPDK sequences, attesting to the importance of this loop in PPDK catalysis. The loop is somewhat more open in the PPDK structure than are the analogous loops observed in the other family members. The alteration in loop conformation seen in PPDK provides for interaction with the γ-P which has been rotated into a unique position. This switch in function, namely using the 101–103 loop in binding the ATP γ-P rather than β-P, is necessary to accommodate the change in location of the transfer reaction from the γ-P to the β-P. The position occupied by the γ-P in the typical ATP-Grasp active site (see for instance, the synapsin structure of Fig. 6A) is proposed to be filled by the cosubstrate Pγ (Fig. 7) in PPDK. Thus, the use of a station on β-strand 9 (Arg-337), for cosubstrate (P) binding, is also unique to PPDK. In carbamoyl-phosphate synthetase for example, this position is filled with an Arg residue that interacts with the ATP γ-P (19).

Conclusions—Presently, our search for conditions that will facilitate the co-crystallization of PPDK with substrate and cofactor ligands for x-ray structure determination continues. We offer the active site model represented in Fig. 7, A and B, as a reasonable estimate of the mode of substrate and cofactor binding within the ATP/Pγ active site. From this model it is evident that PPDK catalysis evolved with minimal alteration of the basic ATP-Grasp active site scaffold.

Acknowledgments—We thank Dr. Fraser and Dr. Wolodoko for early release of the succinyl-CoA synthetase coordinates.

REFERENCES