Identification of Domain-Domain Docking Sites within 
Clostridium symbiosum Pyruvate Phosphate Dikinase by
Amino Acid Replacement*§

Min Wei‡, Zhong Li§, Dongmei Ye‡, Osnat Herzberg§, and Debra Dunaway-Mariano‡‡

From the ‡Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131 and the §Center for
Advanced Research in Biotechnology, Rockville, Maryland 20850

Potential domain-domain docking residues, identified from the x-ray structure of the Clostridium symbiosum
apoPPDK, were replaced by site-directed mutagenesis. The steady-state and transient kinetic properties of the
mutant enzymes were determined as a way of evaluating docking efficiency. PPDK mutants, in which one of two
stringently conserved docking residues located on the N-terminal domain (Arg219 and Glu271) was substituted,
displayed largely unimpaired catalysis of the phosphoenolpyruvate partial reaction at the C-terminal do-
main, but significantly impaired catalysis (>10⁴) of the ATP pyrophosphorylation of His⁴⁵⁵ at the N-terminal
domain. In contrast, alanine mutants of two potential docking residues located on the N-terminal domain
(Ser262 and Lys149), which are not conserved among the PPDKs, exhibited essentially normal catalytic turnover.
Arg219 and Glu271 were thus proposed to play an important role in guiding the central domain and, hence, the
catalytic His⁴⁵⁵ into position for catalysis. Substitution of central domain residues Glu⁴³⁹/Glu⁴⁴⁷ and Thr⁴⁵³
the respective docking partners of Arg219 and Glu271, resulted in mutants impaired in catalysis at the ATP
active site. The x-ray crystal structure of the apo-T453A
PPDK mutant was determined to test for possible mis-
alignment of residues at the N-terminal domain-central
domain interface that might result from loss of the
Thr⁴⁵³-Glu²⁷¹ binding interaction. With the exception of
the mutation site, the structure of T453A PPDK was
found to be identical to that of the wild-type enzyme. It
is hypothesized that the two Glu²⁷¹ interfacial binding
sites that remain in the T453A PPDK mutant, Thr⁴⁵³
backbone NH and Met⁴⁵⁶ backbone NH, are sufficient to
stabilize the native conformation as observed in the
crystalline state but may be less effective in populating
the reactive conformation in solution.

Pyruvate phosphate dikinase (PPDK)¹ catalyzes the inter-
conversion of ATP, P, and pyruvate with AMP, PP, and PEP
(1) using two separate active sites linked by a mobile domain
containing the phosphoryl group carrier His⁴⁵⁵ (2). At the first
active site, to which ATP, P, and Mg(II) are bound, His⁴⁵⁵
attacks the ρ-P of ATP, forming a pyrophosphorylhistidine
enzyme intermediate (E-PP) and AMP (Scheme 1). The P, li-
gand then reacts with the terminal phosphoryl group of E-PP to
form a phosphorylhistidine intermediate (E-P) and PP.
The phosphorylhistidine of E-P then moves to the second active site
where pyruvate, Mg(II), and a monovalent cation (K⁺ or NH₄⁺)
are bound. Upon transfer of the phosphoryl group to pyruvate
to form the final product PEP, the His⁴⁵⁵ residue returns to
the first active site to initiate a new catalytic cycle.

The x-ray crystal structure (2) of the three-domain, 96-kDa
subunit of the apo-PPDK homodimer from Clostridium symbio-
sum is represented at the top of Fig. 1. A Mg(II) cofactor and the
ATP and P, substrate ligands have been modeled into the
structure at the N-terminal “ATP grasp” domain (residues
2–430). The locations of the ligand binding sites (comprising
active site 1) within the N-terminal domain are supported by
the results from earlier affinity labeling and mutagenesis stud-
ies (4, 5). As His⁴⁵⁵ is located in close proximity to active site 1,
the PPDK crystal structure, with some local adjustments, cor-
responds to a conformer competent to catalyze the ATP/P partial reaction (Scheme 1). Henceforth, this conformer will
be referred to as “conformer 1.”

Affinity labeling and mutagenesis studies have shown that
the pyruvate ligand binds at a second active site, located on the
a/b barrel C-terminal domain (residues 534–874) (6, 7). A model
of an enzyme conformer (illustrated at the bottom of Fig. 1;
henceforth referred to as “conformer 2”) judged to be effective
in catalysis of the pyruvate partial reaction, was generated
from the original structure by transferring the central domain
(residues 390–504) from its binding site at the concave surface
of the N-terminal domain to the concave surface of the C-
termal domain (2). Mg(II) and PEP were modeled into active
site 2 of conformer 2 (as pictured in Fig. 1 (bottom)). In this
model, the PEP phosphoryl group is aligned for nucleophilic
attack by the catalytic His⁴⁵⁵ residue.

We anticipate that the rotation about the two interdomain
linkers (residues 341–389 and 505–533), as required by the
conformer 1 ↔ conformer 2 model of Fig. 1, occurs freely in
solution, thus allowing the central domain to “swivel” between
the two active sites. Full catalytic turnover on the enzyme will

* This work was supported by National Institutes of Health Grant
GM 36280 (to D. D. M.) and National Science Foundation Grant
DMSB9119340 (to O. H.). The costs of publication of this article were
defrayed in part by the payment of page charges. This article must
therefore be hereby marked “advertisement” in accordance with 18
U.S.C. Section 1734 solely to indicate this fact.

S The on-line version of this article (available at http://www.jbc.org)
contains Supplementary Figure 1, which shows the alignment of 17
known PPDK sequences generated using CLUSTALW program sup-
ported by the GCG Wisconsin package.

The atomic coordinates and structure factors (code 1ggo) have been
deposited in the Protein Data Bank, Research Collaboratory for Struc-
tural Bioinformatics, Rutgers University, New Brunswick, NJ (http://
www.rcsb.org/).

† To whom correspondence should be addressed Tel.: 505-277-3383;
Fax: 505-277-2699; E-mail: dd39@unm.edu.

² The abbreviations used are: PPDK, pyruvate phosphate dikinase;
AMPNP, adenylylimidodiphosphate; PNP, imidodiphosphate; PEP,
phosphoenolpyruvate; NADH, dihydronicotinamide adenine dinucle-
otide; HPLC, high performance liquid chromatography.

Published, JBC Papers in Press, September 19, 2000, DOI 10.1074/jbc.M006149200
**ATP/Pi, Partial Reaction at Active Site 1 on N-terminal Domain:**

1. \( E + ATP + P_i \rightarrow E\cdotATP\cdotP_i \rightarrow E\cdotPP\cdotAMP\cdotP_i \)
2. \( E\cdotPP\cdotAMP\cdotP_i \rightarrow E\cdotP\cdotAMP\cdotPP_i \rightarrow EP + AMP + PP_i \)

**Pyruvate Partial Reaction at Active Site 2 on C-terminal Domain:**

3. \( E\cdotP + pyruvate \rightarrow E\cdotP\cdotpyruvate \rightarrow E\cdotP\cdotPEP \rightarrow E + PEP \)

**Scheme 1.** The ATP/Pi and pyruvate partial reactions of pyruvate phosphate catalysis (1, 3).

---

Thus require that ATP, \( P_i \), and Mg(II) first bind to active site 1 of conformer 2 (see Scheme 2). The central domain must then dissociate from the C-terminal domain and bind with the N-terminal domain to form conformer 1. Despite the fact that the rotation about the linkers is intrinsically fast (ns time scale), the central domain must remain docked long enough for catalysis at active site 1 to be completed (millisecond time scale (Ref. 3)), but not so long as to impede the ensuing reaction of His(P) of pyruvate at active site 2. The transition from conformation 1 to conformation 2 allows dissociation of the AMP and \( P_i \) product ligands from active site 1 as well prepares the enzyme for catalysis at active site 2, provided that this active site was "pre-loaded" with pyruvate and the Mg(II)/NH₄⁺ cofactors while the enzyme was in conformation 1. Following catalytic turnover at active site 2 in conformer 2, the central domain must dissociate from the C-terminal domain to allow release of the PEP product from conformer 1. If active site 1 contains ATP and \( P_i \), the next catalytic cycle can directly follow from this conformer.

The separate site catalysis of PPDK thus requires the transient formation of precisely oriented domain-domain complexes. An alignment made of the 17 known PPDK sequences (see Fig. 1 in supplemental materials, available in the on-line version of the journal) shows that a majority of the residues defining the proposed binding surfaces on the N- and C-terminal domains are stringently conserved as are many of the surface residues surrounding the catalytic His₄⁵⁵ on the central domain (see Fig. 2). In contrast, the surface residues on the opposite, solvent-exposed faces of the three domains are variable. The conservation of residues at the respective domain-domain interfaces of the observed and modeled conformers has been necessary throughout evolution for productive domain docking and accurate targeting of the catalytic His₄⁵⁵.

Through inspection of the domain-domain interface region of the x-ray structure of apo-PPDK and of the alignment made of the PPDK sequences, two stringent conserved residues of the N-terminal domain (namely Arg²¹⁹ and Glu²⁷¹), whose side chains are positioned for favorable polar interaction with side chain and/or backbone atoms of the central domain, were identified. As is shown in Fig. 3, the guanidinium group of Arg²¹⁹ is within interaction distance of the carboxylate groups of the central domain residues Glu⁴³⁴ (Glu or Asp conserved) and Glu⁴³⁷ (not conserved). The carboxylate group of Glu²⁷¹ is within interaction distance of the hydroxyl group of the central domain Thr⁴⁵³ and the backbone amide NHs of Thr⁴⁵³ and Met⁴⁵². In addition, N-terminal domain residues Ser²⁶² and Lys¹⁴⁹, which are not conserved among all of the PPDK sequences, are also positioned for favorable interaction with the central domain. The hydroxyl group of Ser²⁶² is within H-bonding distance of the backbone C=O of central domain residue Ala³⁹⁸. The side-chain ammonium group of Lys¹⁴⁹ is located 4.4 Å from the central domain Glu⁴³⁴ carboxylate, which is too far for significant interaction. However, if the side chain of the Lys¹⁴⁹ is free to rotate from the conformation observed in the crystal, it can move within 3.5 Å of the carboxylate, close enough for favorable interaction to occur.

Since productive domain-domain binding is an integral component of PPDK catalysis (Scheme 2), an examination of the roles of Arg²¹⁹, Glu²⁷¹, Ser²⁶², and Lys¹⁴⁹ in domain docking can be made by replacing these residues with amino acids that can not interact favorably and evaluating the catalytic efficiencies of the corresponding mutant enzymes. In the text that follows, we report on the steady-state and transient kinetic properties of the C. symbiosum PPDK mutants R219A, R219E, E434A/E437A, E271A, T453A, S262A, S262W, and K149A. The rate constants obtained for the mutants in catalysis of the full reaction and the \( E + ATP \rightarrow E\cdotPP\cdotAMP \), \( E + ATP + P_i \rightarrow E\cdotPP\cdotAMP\cdotPP_i \), and \( E + PEP \rightarrow E\cdotPP\cdotPEP \) partial reactions are compared with those determined in an earlier kinetic analysis of the wild-type PPDK (8). The results suggest that the productive docking of the central domain is controlled, in part, through the interaction of N-terminal domain residues Arg²¹⁹.
and Glu271 with central domain residues Glu434/Glu437 and Thr453/Met452, respectively. The N-terminal domain Ser262 and Lys149 residues, on the other hand, do not appear to play a vital role in orienting or stabilizing the domain-domain complex.

EXPERIMENTAL PROCEDURES

Preparation of *C. symbiosum* PPDK Mutants—Mutant genes were prepared from the plasmid pACYC184-D12 (9) using polymerase chain reaction techniques analogous to those described in (4). 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 Lys 149, Arg 219, Ser 262, and Glu 271 mutants, whereas BstXI and KpnI sites were used for the Glu 434/Glu 437 and Thr 453 mutants. The sequences of the isolated mutant genes were determined at 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 (judged by SDS-polyacrylamide gel electrophoresis analysis) in yields of 20–25 mg/g cell as described in Ref. 4. The chromatographic properties of the mutant proteins as well as their stability to storage in buffered solutions at 4°C were observed to be very similar to the chromatographic properties and stability of the wild-type enzyme.

Steady-state Kinetic Analysis—The spectrophotometric assay described in Ref. 10 was used to monitor the initial velocity of the PPDK-catalyzed reaction of AMP, PPi, and PEP to ATP, Pi, and pyruvate. Initial velocities were measured as a function of the concentration of the varied substrate (in a range of 0.5–10-fold *Km*) 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 (11). The *kcat* was calculated from:

\[ \frac{v_0}{v_{\text{max}}} = \frac{[S]}{[S] + [K_m]} \]

(Eq. 1)

where *v*0 is the initial velocity, [E] is the enzyme concentration, [S] is the substrate concentration, *v*max is the maximum velocity, and *Km* is the Michaelis constant. The *Ki* values for the competitive inhibitors AMP-PNP and PNP were determined from initial velocity data obtained at varying AMP or PPi concentrations and fixed, saturating concentrations of cosubstrates and cofactors. The initial velocity data were analyzed using Equation 2, where *K* is the inhibition constant and [I] the inhibitor concentration:

\[ \frac{v_0}{v_{\text{max}}} = \frac{[S]}{[S] + [K_m] + [I]} \]

(Eq. 2)

**Rapid Quench Analysis of PPDK-catalyzed Single-turnover Reactions of[^32P]PEP**—[^32P]PEP was prepared from [*γ-^32P*]ATP according to the procedure described in Carroll et al. (3). [*β-^32P*]ATP (specific activity >
forms of PPDK conformer 1 (E1 binds & releases pyruvate or PEP but reacts only with bound ATP, E2-P) and supernatant were analyzed for 32P by scintillation with the ligands that these enzyme forms bind and ratio of the radioactivity in the pellet to the total radioactivity in the [32P]PEP, 2.5 mM MgCl2, and 10 mM NH4Cl in 50 mM K-Hepes (pH 7.0) was custom-synthesized by PerkinElmer Life Sciences. The reaction contained 0.5 mM AMP, 0.5 mM PEP.

**Table I**

| Sample          | $k_{in}$ (mM) | $k_{cat}$ (s$^{-1}$) | $K_i$ (mM) |
|-----------------|---------------|----------------------|------------|
| Wild-type       | 0.009 ± 0.002 | 22 ± 2               | 0.07 ± 0.01 (AMPPNP) |
| Vary AMP$^a$    | 0.058 ± 0.005 | 28 ± 1               | 0.031 ± 0.02 (PNP) |
| Vary PP$^b$     | 0.04 ± 0.005  | 25 ± 1               |            |
| R219E (0.045–0.21 μM) | 0.010 ± 0.002 | 3.2 ± 0.2           | 0.09 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.02 ± 0.001  | 3.8 ± 0.2           | 0.71 ± 0.06 (PNP) |
| Vary PP$^p$     | 0.0055 ± 0.0003 | 4.2 ± 0.5   |            |
| R219A (0.055–0.21 μM) | 0.0044 ± 0.0005 | 1.83 ± 0.06 | 0.08 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.158 ± 0.006 | 2.8 ± 0.1           | >3 (PNP)   |
| Vary PP$^p$     | 0.010 ± 0.001 | 2.14 ± 0.04         |            |
| E434A/E437A (0.055–0.24 μM) | 0.004 ± 0.001 | 2.21 ± 0.02 | 0.03 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.091 ± 0.004 | 2.72 ± 0.06         | >4 (PNP) |
| Vary PP$^p$     | 0.0048 ± 0.0003 | 2.64 ± 0.05 |            |
| E271A (10 μM)   |               |                     | <0.005     |
| T453A (0.11–0.42 μM) | 0.0029 ± 0.0002 | 1.14 ± 0.02   | 0.11 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.078 ± 0.002 | 1.52 ± 0.02         | >4 (PNP) |
| Vary PP$^p$     | 0.0044 ± 0.0002 | 1.34 ± 0.02 |            |
| S262A (0.004–0.017 μM) | 0.0033 ± 0.0002 | 21 ± 1         | 0.11 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.007 ± 0.0005 | 25 ± 1             | 0.03 ± 0.02 (PNP) |
| Vary PP$^p$     | 0.0037 ± 0.0002 | 20 ± 1         |            |
| S262W (1.2–2.3 μM) | 0.0075 ± 0.0003 | 0.15 ± 0.01   | 0.32 ± 0.02 (AMPPNP) |
| Vary AMP$^p$    | 0.187 ± 0.006 | 0.27 ± 0.1          | >4 (PNP) |
| Vary PP$^p$     | 0.0092 ± 0.0003 | 0.45 ± 0.03 |            |
| K149A (0.013–0.031 μM) | 0.0103 ± 0.0008 | 14.4 ± 0.6    | 0.30 ± 0.01 (AMPPNP) |
| Vary AMP$^p$    | 0.082 ± 0.005 | 15.6 ± 0.5          | 0.011 ± 0.006 (PNP) |
| Vary PP$^p$     | 0.0043 ± 0.0003 | 15.4 ± 0.8      |            |

$^a$ Reactions contained 1.0 mM PP, 0.5 mM PEP.

$^b$ Reactions contained 0.5 mM AMP, 0.5 mM PEP.

$^c$ Reactions contained 1.0 mM PP, 0.5 mM AMP.

(Eq 3) using the KaleidaGraph computer program to yield the reported rate constants.

\[
[B] = [B]_0 \times (1 - \exp(k_{obs} \times t))
\]

$[B]_0$ is the product concentration at time $t$, $[B]_0$ is the product concentration at equilibrium, and $k_{obs}$ is the observed rate constant for the reaction.

**Rapid Quench Analysis of PPDK-catalyzed Single-turnover Reactions of [14C]ATP in the Absence or Presence of Pi**—[14C]ATP was synthesized from [14C]AMP (PerkinElmer Life Sciences) as follows. A 1-mL reaction containing 0.08 μM [14C]AMP (50 μCi), 50 μM PP, 0.5 mM PEP, 5 mM MgCl2, 40 mM NH4Cl, 6 units of PPDK in 50 mM K-Hepes (pH 7.0) was carried out at 25 °C for 1 h. The reaction mixture was then chromatographed on a 9-ml DEAE-Sepharose column using a linear gradient (0.25–1.1 M) of triethylamine bicarbonate (pH 7) as eluant. The fractions containing [14C]ATP were pooled and concentrated in vacuo (rotary evaporation) at room temperature. Excess triethylamine bicarbonate was removed by repeated water dilution followed by evaporation. The resulting [14C]ATP was stored frozen in 50 mM K-Hepes buffer (pH 7.0) at −30 °C.

**Scheme 2.** An illustration of the free and phosphorylated forms of PPDK conformer 1 ($E_1$, $E_1$-P) and conformer 2 ($E_2$, $E_2$-P) (see Fig. 1) with the ligands that these enzyme forms bind and convert to products..

1 Ci/mmol was custom-synthesized by PerkinElmer Life Sciences. The single-turnover reactions of 40 μM wild-type or mutant PPDK, 5 μM [32P]PEP, 2.5 mM MgCl2, and 10 mM NH4Cl in 50 mM K- Heps (pH 7.0, 25 °C) were carried out in a rapid quench apparatus from KinTek Instruments. Reactions were initiated by mixing 32 μl of buffered enzyme/cofactors with 32 μl of buffered substrate and then terminated at varying conversion with 182 μl of 0.6 M HCl. The protein was precipitated from quenched solutions by vigorous mixing with CCl4 and separated by centrifugation. The protein pellet (dissolved in 500 μl of boiling 10 N H2SO4) and supernatant were analyzed for [32P] by scintillation counting. The percentage of conversion was deduced from the ratio of the radioactivity in the pellet to the total radioactivity in the pellet and supernatant. The concentration of [32P]PEP was calculated from 40 μCi X% conversion and plotted as a function of reaction time. The resulting time course was fit to a single exponential equation (Equation 3) using the KaleidaGraph computer program to yield the reported rate constants.

$[B] = [B]_0 \times (1 - \exp(k_{obs} \times t))$
Fig. 4. The time courses for \( ^{32}P\)E-P formation in the single-turnover reaction of 40 \( \mu \)M wild-type or mutant PPDK, 5 \( \mu \)M \( ^{32}P\)PEP, 2.5 mM MgCl\(_2\), and 10 mM NH\(_4\)Cl in 50 mM K-Hepes (pH 7.0, 25 °C). A, wild-type (●), R219A (○), R219E (□), and E434A/E437A (▲). B, wild-type (●), E271A (○), S262W (□), and T453A (▲). The curves were generated by computer fitting the data to a first order rate equation using the computer program KaleidaGraph. The \( k_{\text{cat}} \) values calculated from these curves are reported in Table II.

### RESULTS AND DISCUSSION

**Steady-state Kinetic Properties of PPDK Mutants**—The PPDK mutants were first screened by measuring \( k_{\text{cat}} \) and \( K_m \) values for substrates and \( k_{\text{cat}} \) values for competitive inhibitors. The steady-state kinetic constants were determined for the AMP + PP\(_i\) + PEP \( \rightleftharpoons \) ATP + Pi + pyruvate reaction (with Mg\(^{2+}\) and NH\(_4\)\(^+\) serving as cofactors) using a continuous spectrophotometric assay to monitor pyruvate formation. PPDK adheres to an ordered, nonclassical bi (ATP, P\(_i\)) bi (PP\(_i\), AMP) uni (pyruvate) uni (PEP) kinetic mechanism (10). For convenience, the \( K_m \) (and \( V_{\text{max}} \)) for each substrate was evaluated from initial velocity data measured with the concentrations of the two co-substrates held constant at saturating levels, while the concentration of the third substrate was varied at 0.5–10-fold the \( K_m \) value. The \( K_m \) values of the ATP analog AMPPNP (versus AMP) and PP\(_i\) analog PNP (versus PP\(_i\)) were determined from the initial velocity data measured in the absence and presence of the inhibitor. The \( K_m \), \( K_{\text{in}} \), and \( k_{\text{cat}} \) values obtained for wild-type PPDK and the PPDK mutants at pH 6.8 and 25 °C are listed in Table I. Except for S262A and K149A, each of the mutants display significantly reduced catalytic efficiency compared with that of the wild-type PPDK. The \( k_{\text{cat}} \) is decreased 10-fold for the R219A, R219E, and E434A/E437A mutants, 20-fold for T453A, 100-fold for S262W, and >5000-fold for E271A. As will be described in the section that follows, the reduction in \( k_{\text{cat}} \) derives from a reduction in the rate of the nucleotide partial reaction.

The abilities of the mutant enzymes to bind ATP and PP\(_i\), are reflected by the \( K_m \) values measured for the inert analogs AMPPNP and PNP. AMPPNP competes with AMP for the same binding site on conformer 1 of the free and phosphorylated forms of the enzyme (see Scheme 2). Likewise, PNP competes with PP\(_i\), for the same site on the AMP complex of the free or phosphorylated enzyme in conformation 1. An increase in the \( K_m \) value may signify that the structure or electronic environment of the binding site has been altered by the mutation. Alternatively, if the ligand binding site is changed by domain-domain association, a mutation that affects domain binding can affect the \( K_m \) of the inhibitor. Thus, an increase in the \( K_m \) values may also indicate impaired domain-domain docking.

Based on the AMPPNP \( K_m \) values listed Table I, it is evident that the ATP binding affinity is not altered in the R219E, R219A, E434A/E437A, S262W, and T453A mutants, despite the fact that all but the S262A mutant has a significantly lower turnover rate. On the other hand, the AMPPNP \( K_m \) values measured for the S262W and K149A mutants are 4-fold larger than that measured for wild-type PPDK. The exact cause of reduced ATP binding in these mutants is presently unknown; however, since the Ser\(^{262}\) and Lys\(^{49}\) residues do not appear to function as docking residues (i.e., the \( k_{\text{cat}} \) values of the Ala mutants are normal), we have not pursued the issue further.
The PPi binding affinity was found to be severely weakened in the R219E, R219A, E434A/E437A, S262W, and T453A PPDK mutants, as indicated by the comparatively large PNP Ki values observed for the mutant enzymes (≈20–100-fold larger than that of the wild-type PPDK). Only the K149A and S262A mutants, both of which display normal kcat values (and thus, unimpaired domain-domain docking), were unaffected. The PPi binding site lies outside of the regions mutated and, therefore, may not be directly effected by the amino acid replacements made. On the other hand, PPi binds at the entrance of active site 1, which becomes desolvated by the region of the central domain surrounding (and including) the His(P)455. A likely scenario is that the reduced PNP binding affinity observed with the mutant enzymes is a reflection of altered domain-domain binding.

Catalysis at the PEP/Pyruvate Active Site—The E-PEP → E-P- pyruvate partial reaction is catalyzed by PPDK in conformation 2 (Fig. 1; Scheme 2). Thus, mutations made at the N-terminal domain are not expected to inhibit catalysis at the C-terminal domain. This was tested by measuring the kobs for single-turnover reactions of R219A, R219E, E271A, and S262W mutant PPDK using rapid quench techniques. The time courses for these reactions are shown in Fig. 4, whereas the kobs values calculated from the rate data are reported in Table II. All four mutants displayed normal catalysis of the E-PEP → E-Pyruvate partial reaction. The fact that the kobs values for the four mutants are not noticeably higher than that measured for the wild-type enzyme suggests that the population of conformer 2 has not been significantly increased and/or that the rate of conformer equilibration is not limiting.

[In contrast to the ATP binding site, the Pi binding site is located at the entrance to the active site crevice where Arg337, and possibly Mg(II), are the probable binding residues (4) D. Ye, M. Wei, M. V. McGuire, K. Huang, G. Kapadia, O. Herzberg, and D. Dunaway-Mariano, unpublished data). The affinity of Pi for this site is quite low (Kd; 6m M) and the binding rate, slow (k on; 50.04 M⁻¹ s⁻¹; k off; 200 s⁻¹) (8). Since ATP binds beneath the Pi, substrate binding is ordered, with ATP first and Pi second (10). When the central domain binds to the N-terminal domain, the active site is closed and the dissociation of the two substrate ligands is prevented. The Pi ligand is covered by the central domain region surrounding the catalytic His455 (see Fig. 1). Whether or not direct binding interaction occurs between the bound Pi ligand and the central domain, cannot be discerned on the basis of the data on hand. Nevertheless, it is reasonable to expect that the domain docking produces significant alterations in the Pi environment. The PPi ligand displays a higher binding affinity for the E-P-AMP complex (Kd; 50 μM) than does Pi for the enzyme-ATP complex (8, 10). PPi binding (k on; 2 μM⁻¹ s⁻¹; k off; 100 s⁻¹ (Ref. 8)) is followed by phosphohistidine 455 binding. It is likely that the environment of the PPi ligand is altered by domain-domain association and that the PPi binding constant is affected by mutations that impair the domain-domain docking reaction.

---

**Fig. 5.** The time courses for [14C]AMP formation in the single-turnover reactions of 40 μM (O) or 80 μM (●) enzyme, 10 μM [14C]ATP, 5 mM MgCl₂, and 10 mM NH₄Cl in 50 mM K-Hepes (pH 7.0, 25 °C) catalyzed by wild-type, R219E, R219A, E434A/E437A, T453A, and S262A PPDK. The plots are generated by fitting the time point data to a first order rate equation.
The E434A/E437A and T453A mutations modify the central domain. Since the central domain must interface with the C-terminal domain in order to catalyze the $E_\text{z}$PEP$^7$E-P$^z$pyruvate partial reaction, it was anticipated that the central domain mutants would display reduced catalytic efficiency at both active sites. However, the time course measured for the $E_1$[32P]PEP$^3$[32P]$E_\text{z}$-P$^z$pyruvate single-turnover catalyzed by the E434A/E437A mutant is essentially the same as that catalyzed by wild-type PPDK, whereas that measured for the T453A mutant reflects a 2-fold reduction in $k_{\text{obs}}$ and a 30% reduction in the position of the reaction equilibrium ($[E_\text{z}$-P$^z$pyruvate]/[E$\text{z}$PEP]), where for wild-type PPDK the $K_d$ values for E$\text{z}$-P$^z$pyruvate and E$\text{z}$PEP complexes are 75 and 170 mM, respectively, and the internal $K_{eq}$ = $[E$-P$^z$pyruvate]/[E-PEP] = 1 (Ref. 8) (Fig. 4; Table II). The Glu$^{434}$ and Glu$^{437}$ residues of the central domain thus do not appear to function as docking sites in the association of the central and C-terminal domains. On the other hand, the Thr$^{453}$ residue appears to play a minor role in promoting catalysis at the C-terminal active site.

Catalysis at the ATP/\textit{P}_i Active Site—Catalysis at the N-terminal domain consists of two reaction steps: the pyrophosphorylation of His$^{455}$ by ATP, followed by the transfer of a phosphorlyl group from the pyrophosphorylated His$^{455}$ to phosphate (see Scheme 1). Both reaction steps require activation of the enzyme by a divalent metal ion (Mg(II) is the physiological cofactor, but Co(II) and Mn(II) are also effective activators) (1). The reaction of His$^{455}$ with ATP does not require \textit{P}_i and, thus, can be studied in isolation. The single-turnover reaction of excess PPDK with [14C]ATP to produce E$\text{z}$-PP$^z$AMP is monitored using a rapid quench instrument in conjunction with HPLC techniques to separate the [14C]AMP released from the quenched enzyme (8). Previous studies of the wild-type PPDK have shown that the amount of AMP formed under the conditions of the single-turnover reaction is governed by the level of enzyme-bound versus unbound ATP and by the E$\text{z}$-PP$^z$AMP/ E$\text{z}$ATP equilibrium (8). Substitution of Co(II) for Mg(II) as cofactor in the reaction increases both ATP binding to the enzyme and the internal equilibrium, so that the amount of E$\text{z}$-PP$^z$AMP produced from reaction of 40 mM PPDK with 10 mM ATP increases from ~5% for the Mg(II)-activated enzyme to ~25% for the Co(II)-activated enzyme. For this reason, the

![Fig. 6. The time courses for [14C]AMP formation in the single-turnover reactions of 5 mM [14C]ATP, 11 mM P$_i$, 5 mM MgCl$_2$ and 10 mM NH$_4$Cl in 50 mM K$^+$Hepes (pH 7.0, 25 °C) catalyzed by 20 mM enzyme wild-type, R219E, R219A, E434A/E437A, E271A, T453A, S262A, and S262W PPDK. The plots are generated by fitting the time point data to a first order rate equation.](image-url)
abilities of the PPDK mutants to catalyze the \( E +[^{14}C]ATP \rightarrow E-PP[^{14}C]AMP \) partial reaction were evaluated using Co(II) rather than Mg(II) as co-factor.

On the other hand, Mg(II) is the superior choice for kinetic analysis of the single-turnover reaction of \( E (20 \mu M) + ATP (5 \mu M) + P_i (11 \text{mM}) \rightarrow E-PP[^{14}C]AMP \). For this reaction, it is advantageous to minimize the level of AMP formed in the initial \( E-ATP \rightarrow E-PP[^{14}C]AMP \) step so that the total amount of AMP formed will coincide with the amount of AMP produced as the result of the second partial reaction: \( E-PP[^{14}C]AMP \rightarrow E-PP[^{14}C]AMP-PP_i \), which, by mass action effect, drives the first reaction forward. For wild-type PPDK, the \( k_{obs} \) for the first step is \( \sim 300 \text{ s}^{-1} \) while the \( k_{obs} \) for the second step is \( \sim 6 \text{ s}^{-1} \) (4). If catalysis of the first step is impaired in the PPDK mutant, this will be reflected by the time course for \( [^{14}C]AMP \) formation in the \( E-ATP \rightarrow E-PP[^{14}C]AMP \) reaction, as well as by the time course for \( [^{14}C]AMP \) formation in the \( E-PP[^{14}C]AMP \rightarrow E-PP[^{14}C]AMP-PP_i \) reaction. If, on the other hand, the first step is not severely inhibited, then the degree to which the second step is inhibited can be discerned from the time course for \( [^{14}C]AMP \) formation in the \( E-PP[^{14}C]AMP \rightarrow E-PP[^{14}C]AMP-PP_i \) reaction (see for example, Ref. 4).

The time courses for \( [^{14}C]AMP \) formation in the \( E-PP[^{14}C]AMP \rightarrow E-PP[^{14}C]AMP-PP_i \) reaction exhibit a 20-fold (as is demonstrated for the T453A mutant by x-ray crystallographic analysis) rate increase due to decreased binding affinity and large reduction in catalytic efficiency. We suspect that the large Trp side chain causes conformational changes effecting both the ATP binding site and the domain-domain docking process. Here, we argue that the N-terminal domain active sites in the PPDK mutants are intact, because catalysis of the \( E-ATP \rightarrow E-PP[^{14}C]AMP \) partial reaction is not affected by the mutations made in the PPDK mutants. However, the first step is rate-limiting in the single-turnover reaction. The results, reported in Table V, indicate that catalysis and not ATP binding is rate-limiting for each of these enzymes.

The \( k_{obs} \) values measured for the \( E-PP[^{14}C]AMP \rightarrow E-PP[^{14}C]AMP-PP_i \) reaction are summarized in Table V. The marked error was the difference between the \( k_{obs} \) values obtained from the two trials.

### Table III

| Enzyme | \( k_{obs} \) for trial 1 | \( k_{obs} \) for trial 2 | \( k_{obs} \) for trial 1 | \( k_{obs} \) for trial 2 |
|--------|-----------------|-----------------|-----------------|-----------------|
| Wild-type | \( \sim 300 \pm 70^a \) | \( 3.3 \pm 0.03^b \) | \( 0.008 \pm 0.005^b \) | \( 0.5 \pm 0.004^b \) |
| R219E | 0.05 \pm 0.03 | \( <0.05^b \) | \( 0.0069 \) | \( 0.046 \pm 0.003 \) |
| E434A/E437A | 0.08 \pm 0.05 | \( 0.04 \) | \( 0.015 \pm 0.001 \) | \( 0.021 \pm 0.002 \) |
| E271A | \( <0.05^b \) | \( 0.0069 \) | \( 0.017 \pm 0.008 \) | \( 0.00029 \pm 0.00003 \) |
| T453A | \( 5.7 \pm 0.3 \) | \( 0.071 \pm 0.005 \) | \( 0.04 \pm 0.01 \) | \( 0.0069 \) |
Lys177, Glu168, Glu170, and Glu175 PPDK mutants examined) (5). Although it can be argued that these sites are not located at the domain-domain interface, we point out that two of the domain-domain interface residues examined in the present study, Ser262 and Lys419, have been replaced by an Ala residue without loss of catalytic activity.

Second, we note that mutation of Arg219 to Glu has the same impact on catalysis at active site 1 that mutation of Arg219 to Ala has. Thus, the loss of binding interaction between Arg219 and the Glu434/Glu437 residues is what is critical to catalysis and not the type of charge alteration made at position 219. Moreover, which partner of the paired docking residues, Arg219 or Glu434/Glu437, is replaced in the mutagenesis experiment does not effect the outcome; the catalytic properties of the Glu434/Glu437 mutant and the Arg219 mutant are similar.

Finally, we note that disruption of the Glu271-Thr453/Met452 docking site has more impact on catalysis than does disruption of the Arg219-Glu434/Glu437 docking site. Unlike the Arg219-Glu434/Glu437 docking site, the Glu271-Thr453/Met452 docking site is close to the His455 residue (see Fig. 3). The His455 side chain is positioned on the N terminus of a short helix, just down stream from the Glu271 docking residues Thr453 and Met452. The backbone amide NHs of both of these residues as well as the side chain of the Thr453 residue bind with the Glu271 carboxylate group, thereby drawing the His455 into the active site region. Consequently, the Glu271-Thr453/Met452 docking site might play a more dominant role in the productive domain-domain binding than the Arg219-Glu434/Glu437 site, which is further removed from the His455. Alternatively, the differential effects of mutating Arg219 and Glu271 may be attributed to the difference in the number of interdomain electrostatic interactions lost. Specifically, the present data indicate that the removal of a single interaction, regardless of the docking site involved, results in a $-10^4$-fold reduction in $k_{obs}$. This may be a simple coincidence, or it may suggest that the contributions of the electrostatic interactions are additive, in which case one can rationalize the greater loss in activity for the E271A PPDK mutant in terms of the loss of three rather than one interaction.

**TABLE VI**

| Statistics of x-ray crystallographic data collection for T453A PPDK |
|---------------------------------------------------------------|
| **Space group**     | $P_2_1$ |
| **Cell parameters** | $a = 89.9 \AA$, $b = 58.7 \AA$, $c = 102.1 \AA$ |
| **Maximum resolution (Å)** | 2.6 |
| **No. of crystals used** | 1 |
| **No. of observations** | 43,302 |
| **No. of unique reflections** | 27,567 |
| **$R_{merge}$** | 0.076 |
| **Completeness (%)** | 83 (68)$^a$ |
| **$I/\sigma(I)$** | 11.7 (2.1)$^a$ |

$^a$ $R_{merge} = \sum_{hkl} (|I_{hkl}| - \langle |I| \rangle) / \sum_{hkl} |I_{hkl}|$.

$^b$ The values in the parentheses correspond to the highest resolution shell between 2.76 and 2.60 Å.

Hydrophobic interactions deriving from the desolvation of nonpolar residues are often present at protein-protein interfaces (17, 18). Inspection of the domain-domain interface within the PPDK structure reveals two matched sets of hydrophobic regions which may contribute to the intrinsic binding energy. The electrostatic interaction between Arg219 and Glu434/Glu437 and between Glu271 and Thr453/Met452(NH) may also contribute to the domain-domain binding energy as well as provide specificity to the domain-domain interactions. A steering mechanism, based on the pairing of specific residues at the domain-domain interface, could reduce the number of collisions required to achieve productive binding of the catalytic His455 in the N-terminal domain active site.

Proof that the electrostatic interaction between Arg219 and Glu434/Glu437 and between Glu271 and Thr453/Met452(NH) is used for domain-domain steering is not easily obtained. Cryst-
talization experiments of these mutant enzymes, which follow-
ing structure determination would reveal if the relative orient-
ation of the two domains has been altered, have been unsuccess-
ful. Thus far, only the x-ray structure of the T453A mutant has been determined (see Fig. 7, Table VI, and “Exper-
imental Procedures” for details). Within the accuracy of the structure determination, the overall structure is the same as
that of the wild-type protein (2). The $C_\text{a}$ atomic coordinates of
the mutant and wild-type molecules superimpose with a root-
mean-square deviation of 0.5 Å. The electron density map at
the mutation site is consistent with the replacement of a thre-
onine by an alanine residue at position 453. The interaction
between the side chain of Glu$^{271}$ and the hydroxyl group of
Thr$^{453}$ is therefore eliminated, but the remaining two interac-
tions with the two NH groups of residues 452 and 453 are
maintained (Fig. 7). In addition, the side chains of Met$^{452}$ and
Leu$^{396}$ shift concertedly so that the Met$^{452}$ side chain occupies
space that was partially occupied in the wild-type protein by
the methyl group of Thr$^{453}$, and the Leu$^{396}$ side chain shifts
toward the position occupied by Met$^{452}$ in the wild-type protein.
These changes are compensatory and therefore are not ex-
pected to impact significantly on the catalytic efficiency of the
enzyme. Thus, although the structure of T453A PPDK provides
evidence that the reduction in catalytic efficiency is
not caused
by alteration in active site conformation, it does not provide
any indication of misalignment of the domain-domain surfaces.
This static picture may or may not provide an accurate view of
the dynamics between protein species in solution. The absence of
a particular conformer in the crystal does not mean that it is
also absent in solution.

CONCLUSIONS

The current model of PPDK catalysis involves the swivel-
type movement of a central, phosphoryl carrier domain be-
tween the ATP/$P_i$ active site of the N-terminal domain and the
pyruvate active site of the C-terminal domain. We hypothesize
that the stringently conserved residues at the respective do-
main-domain interfaces play an active role in guiding the cata-
lytic His$^{455}$ of the central domain into the active sites and
holding it there long enough for catalysis to be completed. The loss of catalytic activity observed for the mutants examined in
the present study supports the proposed roles of the N-terminal
residues Arg$^{219}$ and Glu$^{271}$ in the productive binding of the central domain for catalysis of the ATP/$P_i$ partial reaction.

REFERENCES

1. Wood, H. G., O’Brien, W. E., and Micheales, G. (1977) Adv. Enzymol. Relat.
Areas Mol. Biol. 45, 85–155
2. Herzberg, O., Chen, C. C., Kapadia, G., McGuire, M., Carroll, L. J., Noh, S. J.,
and Dunaway-Mariano, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2652–2657
3. Carroll, L. J., Mehli, A. F., and Dunaway-Mariano, D. (1989) J. Am. Chem. Soc.
111, 5965–5967
4. McGuire, M., Huang, K., Kapadia, G., Herzberg, O., and Dunaway-Mariano, D.
(1998) Biochemistry 37, 13463–13474
5. McGuire, M., Carroll, L. J., Yankie, L., Thrall, S. H., Dunaway-Mariano, D.,
Herzberg, O., Jayaram, B., and Haley, B. H. (1996) Biochemistry 35, 8544–8552
6. Xu, Y., Yankie, L., Shen, L., Jung, Y. S., Mariano, P. S., Dunaway-Mariano, D.,
and Martin, B. M. (1995) Biochemistry 34, 2181–2187
7. Yankie, L., Xu, Y., and Dunaway-Mariano, D. (1995) Biochemistry 34, 2188–2194
8. Mehli, A., Xu, Y., and Dunaway-Mariano, D. (1994) Biochemistry 33, 1093–1102
9. Pocalyko, D. J., Carroll, L. J., Martin, B. M., Bailey, P. C., and Dunaway-
Mariano, D. (1990) Biochemistry 29, 10757–10765
10. Wang, H. C., Cischanik, L., Dunaway-Mariano, D., von der Saal, W., and
Villafranca, J. J. (1988) Biochemistry 27, 625–633
11. Cleland, W. W. (1979) Methods Enzymol. 63, 500–513
12. Howard, A. J., Gililand, G. L., Finzel, B. C., Poulos, T. L., Ohlendorf, D. H.,
and Salemme, F. R. (1987) J. Appl. Crystallogr. 20, 383–387
13. Brunger, A. T. (1992) X-PLOR Version 3.1: A System for X-ray Crystallography
and NMR, Yale University, New Haven, CT
14. Roussel, A., and Cambillu, C. (1989) TURBO FRODO; Silicon Graphics
Geometry Partner Directory, Silicon Graphics, Mountain View, CA
15. Deleted in proof
16. Stites, W. E. (1997) Chem. Rev. 97, 1253–1250
17. Privalov, P. L., and Gill, S. J. (1988) Adv. Protein Chem. 39, 191–234
18. Spolar, R. S., Ha, J. H., and Record, M. T. (1989) Proc. Natl. Acad. Sci. U. S. A.
86, 8382–8385
19. Engh, R. A., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 392–400