Ligand-induced Effects on Pyruvate Dehydrogenase Kinase Isoform 2

Received for publication, December 19, 2005, and in revised form, March 3, 2006. Published, JBC Papers in Press, March 3, 2006, DOI 10.1074/jbc.M513514200

Yasuaki Hiromasa, Liangyan Hu, and Thomas E. Roche†

From the Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506

Tryptophan fluorescence was used to analyze binding of ligands to human pyruvate dehydrogenase isoform 2 (PDHK2) and to demonstrate effects of ligand binding on distal structure of PDHK2 that is required for binding to the inner lipoyl domain (L2) of the dihydrolipoyl acetyltransferase. Ligand-altered binding of PDHK2 to L2 and effects of specific ligands on PDHK2 oligomeric state were characterized by analytical ultracentrifugation. ATP, ADP, and pyruvate markedly quenched the tryptophan fluorescence of PDHK2 and gave maximal quenching/L0.5 estimates: ~53%/3 μM for ATP; ~49%/15 μM for ADP; and ~71%/~590 μM for pyruvate. The conversion of Trp-383 to phenylalanine completely removed ATP- and ADP-induced quenching and ≥80% of the absolute decrease in fluorescence due to pyruvate. The W383F-PDHK2 mutant retained high catalytic activity. Pyruvate, added after ADP, quenched Trp fluorescence with an L0.5 of 3.4 μM pyruvate, ≥150-fold lower concentration than needed with pyruvate alone. ADP-enhanced binding of pyruvate was maintained with W383F-PDHK2. Binding of PDHK2 dimer to L2 is enhanced when L2 are housed in oligomeric structures, including the glutathione S-transferase (GST)-L2 dimer, and further strengthened by reduction of the lipoyl groups (GST-L2red) (Hiromasa and Roche (2003) J. Biol. Chem. 278, 33681–33693). Binding of PDHK2 to GST-L2red was modestly hindered by 200 μM level of ATP or ADP or 50 μM pyruvate; a marked change to nearly complete prevention of binding was observed with ATP or ADP plus pyruvate at only 100 μM levels, and these conditions caused PDHK2 dimer to associate to a tetramer. These changes should make major contributions to synergistic inhibition of PDHK2 activity by ADP and pyruvate. Ligand-induced changes that interfere with PDHK2 binding to GST-L2red may involve release of an interdomain cross arm between PDHK2 subunits in which Trp-383 plays a critical anchoring role.

Because this results in animals in the irreversible reduction of body carbohydrate reserves, PDC activity is tightly regulated by dedicated regulatory enzymes (1, 2). The pyruvate dehydrogenase kinase isozymes (PDHK1, PDHK2, PDHK3, and PDHK4) and pyruvate dehydrogenase phosphatase isozymes (PDHP1 and PDHP2) control the fractional activity of PDC in a tissue-specific manner. Phosphorylation of the pyruvate dehydrogenase (E1) component by the PDHKs results in inactivation and dephosphorylation by the PDHPs results in reactivation of E1.

The components required in the mammalian complex for the five-step PDC reaction are E1, the dihydrolipoyl acetyltransferase (E2), the dihydrolipoyl dehydrogenase (E3), and the E3-binding protein (E3BP) (1, 2). E2 and E3BP associate via their C-terminal domains to form the pentagonal-dodecahedron shaped core of the complex, a 60-subunit structure with a stoichiometry estimated to be E2/E3BP (3). Via specific binding domains, E2 binds E1 and E3BP binds E3 (1, 2). Two lipoyl domains (L1 and L2) of E2 and one lipoyl domain in E3BP act to shuttle reaction intermediates between active sites of the E1, E2, and E3 components.

PDHK and PDHP1 also bind to lipoyl domains, primarily the L2 domain of E2 (4–17). The functional properties and allosteric regulation of the PDHK and PDHP1 are markedly affected by their effector-modulated interactions with the L2 domain and its lipoyl group (4–17). Feedback inhibition of PDC due to increases in the NADH/NAD+ and acetyl-CoA/CoA ratio is primarily achieved by increasing PDHK activity, particularly that of the PDHK2 isoform (4–17). Feedback inhibition requires NADH and acetyl-CoA to react in the reverse of the E3 and E2 reactions to reduce and acetylate the lipoyl groups (9, 21–25). Specifically, human PDHK2 is stimulated by reduction (~1.8-fold) and then acetylation (~2.3-fold) of the lipoyl group of the L2 domain of E2 (12, 20, 21). Reduction and then acetylation also progressively increase the affinity of PDHK2 for the L2 domain (15, 21). Other effectors (ATP/ADP) decrease the binding of PDHK2 to E2 (15, 17).

Attenuation of PDHK2 activity is primarily by ADP (an indicator of low energy state) and pyruvate (an indicator of substrate sufficiency). These effectors act synergistically to inhibit PDHK activity (26, 27). Recently, we presented evidence that PDHK2 catalysis is limited by ADP dissociation and that having ADP bound favors the development of inhibition by pyruvate binding to the PDHK2-ADP intermediate (27). Furthermore, stimulation by reductive acetylation of lipoyl groups was linked to an increase in speed in the dissociation of ADP (21). The capacity to characterize the E2-enhanced physiological PDHK2 reaction by kinetic studies is limited by the nonequilibrium nature of the catalytic steps. The first substrate (ATP) dissociates slower than kcat (27). The second substrate, E1, is delivered within the confines of complex, in part, by nondissociative mechanisms (2, 7, 8, 15), and is converted through a two-step series to becoming alternative substrate 1 and then alternative substrate 2. Even as a highly phosphorylated product phosphorylated-E1 is not a significant inhibitor of kinase activity (28). It seems likely that post dissociation conformational changes alter E1 at each step. To gain insights into the linkage between regulatory sites and
to begin to relate major structural differences observed in crystal structures with ligands bound, biophysical studies are needed to analyze the effects of ligand binding on intersite communication, PDHK2 structure, and protein interactions.

The PDHKs and branched chain dehydrogenase kinase are serine kinases unrelated to the major class of cytoplasmic Ser/Thr/Tyr kinases (2, 29–33). The three-dimensional structure of rat PDHK2 with ADP bound has been described previously (34). PDHK2 is a two-domain dimer formed by association of its C-terminal domain. The C-terminal domain contains the ATP/ADP binding site and is a member of the GHKL superfamily (31–34). Further x-ray crystallography studies (35) established three ligand binding sites in the N-terminal domain, including the DCA/pyruvate binding site and the binding site of the tight binding 3,3,3-trifluoro-2-hydroxy-2-methylpropanoyl-containing inhibitors (such as Nov3r (36)). The Nov3r binding site is the same site at which the lipoyl group binds in PDHK3-L2 domain complex (37). A cross arm between subunits of PDHK2 dimer (35) was shown to participate in binding the L2 domain in PDHK3-L2 complex (37). A key anchoring role at the end of the cross arm involves insertion of Trp-383 into the other subunit (details under "Discussion").

Here, we find that the binding interactions of ATP, ADP, or pyruvate (or the pyruvate analog dichloroacetate (DCA)) have pronounced effects in quenching tryptophan fluorescence with PDHK2. We establish by mutation that Trp-383, which is far removed from ATP/ADP binding site, is the specific tryptophan residue undergoing marked fluorescence quenching by ATP and ADP as well as the majority of quenching by pyruvate. Related effects of ligands in hindering binding of PDHK2 to the L2 domain are described. We consider whether the Tryptophan anchoring role at the end of the cross arm involves insertion of Trp-383 into the other subunit (details under "Discussion").

EXPERIMENTAL PROCEDURES

Materials—Recombinant human PDHK2 (12), E2 (3), GST-L2 (38), and E3 (39) were prepared as previously described. The modified expression cDNA for preparation of PDHK2 in which Trp-383 is substituted by a phenylalanine was developed by a two-step PCR procedure in which primers flanking the cDNA (routinely used for sequencing PDHK2-cDNA) were paired with matched primers introducing the mutation. Residue numbering for PDHK2 and PDHK3 (under “Discussion”) are based on the sequences of the mature polypeptides (see supplemental data Ref. 35). These initial PCR products were purified and then used as primers in the second round to produce a double-stranded DNA containing the mutated cDNA (with a His tag and PreScission protease site encoded to be expressed at the N terminus). The cDNA was excised by XbaI and HindIII restriction enzymes and inserted into PET28a (Novagen) expression plasmid. The sequence of the cDNA expressing this PDHK2 mutant was confirmed by DNA sequencing. Expression and purification of W383F-PDHK2 were performed as described for native PDHK2 (12).

Fluorescence Quenching—Steady-state fluorescence spectra were recorded with a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.) at 20 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 2.0 mM MgCl2 (buffer A). The concentration of PDHK2 in these (and AUC studies below) was based on the extinction coefficient at 280 nm of 92,600 M−1 cm−1 (15). Experimental error (i.e. precision of measurements) was estimated to be ±3% (so a concentration of 0.105 μM is being reported to nearest 0.005 μM). Initial fluorescence measurements (F0) for a given concentration were within ±1.5% of the average value (n = 12) at 0.105 μM and 1.53 μM PDHK2. In the case of W383F-PDHK2, protein concentration was based on BCA protein assay, performed as previously described (38), using native PDHK2 as a standard. Excitation was at 290 nm (slit routinely 5 nm but 2.5 nm in some studies) and fluorescence spectra were recorded from 320 to 420 nm (slit usually 10 nm but 5 nm in some studies). Most studies were conducted using a standard quartz (1 × 1 cm) cuvette and a 2.0-ml initial volume with varied ligand added in 2- to 10-μl increments. Some studies with more concentrated PDHK2 were conducted with a 0.5-× 0.5-cm quartz cuvette. In the analysis of the fluorescence changes due to these titrations, the concentration of PDHK2 was continuously corrected for the slight dilution due to the addition of the concentrated ligand. Most studies were conducted with PDHK2 in the range of 0.10–1.6 μM with the majority of the data shown being from studies in the range of 0.1–0.35 μM. Although full 320–420 nm scans were taken, the change in fluorescence at 350 nm was used to assess fluorescence quenching. When a peak center is reported, it is for the upper third of a peak.

Our analysis assumed, at least initially, that the fraction of bound ligand (Y) is equal to the ratio of the fluorescence quenching (Q = F0 − Fq) to maximum quenching (Qmax = F0 − Fqmax), where F0, Fq, and Fqmax are fluorescence at 350 nm measured in the absence of added ligand, the quenched fluorescence in the presence of ligand, and the projected maximum fluorescence quenching at a saturating level of ligand, respectively. Therefore, Y = Q/Qmax or Y = %Q/Qmax, where %Q = 100%[F0 − Fq)/F0] and %Qmax = 100%[F0 − Fqmax/F0]. L is the free concentration of varied ligand. The concentration that gave a half-maximal effect, L0.5 = (Lq)(1 − Y)/Y = (Lq)(%Qmax−%Q)/%Q or Q = %Qmax/Lq(Lq + L). %Qmax is therefore calculated using a double reciprocal plot and then using this first approximation of Qmax, free ligand concentrations (Lq) were calculated, correcting the added concentration for bound ligand assuming that binding was at two equivalent sites in the PDHK2 dimer (Lq = Lq − 2Ye). Generally, at the lower concentrations of PDHK2 used in most studies, this involved very small corrections. The parameters derived from those analyses at low PDHK2 concentrations, for which these corrections were very small, were compared with parameters obtained at higher concentrations of kinase for which the corrections of free ligand were substantial, particularly at the lower concentrations of tight binding ligand. The finding, that estimates of L0.5 values were equal within experimental error over this range of PDHK2 concentrations, was interpreted as supporting the assumption of two equivalent binding sites per PDHK2 dimer. The corrected data were fit by hyperbolic curve (%Q versus free) using Sigma Plot 8.02 software and by a double reciprocal plot (1/%Q versus 1/free ligand, a Klotz plot under the assumption that binding is proportional to Q/Qmax) using Origin 7.0 software (linear least squares).

The concentrations of ATP and ADP were evaluated based on their absorbance (εs09 nm = 1.54 × 104 M−1 cm−1). Titration of ATP and ADP gave hyperbolic changes in fluorescence quenching and equivalent L0.5 at different concentrations of PDHK2. The L0.5 values are then considered to provide apparent equilibrium binding constants. However, we know these constants reflect multiple equilibria in which specific binding of K+ and phosphate have significant effects. The marked and involved influence of these ions on ligand binding/fluorescence quenching will be described in a subsequent study.3 With 50 mM potas-
sium phosphate buffer (pH 7.5, at 25 °C; 83.3 mM K+), these ions are present at near saturating levels.

In general, quenching studies that included pyruvate or DCA, whether added alone or in combination with ATP or ADP, yielded non-hyperbolic profiles for the decrease in fluorescence with increasing concentration of the varied ligand. In those cases, $Q_{\text{max}}$ values were estimated from a double reciprocal plot using the higher concentrations and the data plotted in Hill plots (log($Y_{1}-Y$) versus log($L_0$)) or plots of log($Y$) versus log($L_0$) with the latter made and analyzed by the Sigma Plot program. A Hill plot yielded $n_H$ from the slope estimated in the range log($Y_{1}-Y$) usually between $-0.5$ and $+0.5$ ($-0.5$ and $+1.0$ when $n_H < 1$). $L_{0.5}$ was estimated from log($L_0$) when log($Y_{1}-Y$) = 0 in the Hill plot. In the Sigma Plot analyses of log($Y$) versus log($L_0$) plots, the maximum ($Y_{\text{max}} \approx -1$) and minimum ($Y_{\text{min}} \approx -\infty$) were independently re-evaluated in fitting the data and $L_{0.5}$ obtained when log($Y'$) = 0.5 where $Y'$ is half way between the revised minimum and maximum; $n_H$ was then estimated from the slope of the Hill plot created by the Sigma Plot program using those values. For simplicity, the above analyses invoke the limiting assumptions of binding at two sites with the change in fluorescence quenching being proportional to binding of the varied ligand. However, that cannot be meaningfully assessed in the present work. Again, complex equilibria are involved in which binding of K+ and phosphate occurs by a variety of pathways, particularly when there is binding of pyruvate/DCA along with ATP or ADP. Our studies provide condition-dependent $L_{0.5}$ values that demonstrate large changes in the concentration dependence that result from major changes in binding affinities due to the binding of a second ligand.

The effects of all these ligands on the fluorescence of N-acetyltryptophanamide (NATA) were analyzed to evaluate effects due to chemical quenching and self-absorption (see data under Supplemental Data, Fig. 15) (40–42). Additionally, the effects of ATP and ADP on PDHK2 fluorescence were analyzed in the absence of Mg++, which is required for binding of these ligands to PDHK2 (27, 43). Ligands were added only at levels in which they did not measurably interfere with the fluorescence of NATA. We also evaluated the chemical quenching effects of acrylamide (40, 41) on the fluorescence of PDHK2 (see Supplemental Data, Fig. 25).

PDHK2 Activity Assays—PDHK2 activity assays were conducted using methods previously described (9, 12, 24, 27). In short, PDHK2 activity was measured in duplicate or triplicate using the Sigma Plot program using those values. For simplicity, the above analyses invoke the limiting assumptions of binding at two sites with the change in fluorescence quenching being proportional to binding of the varied ligand. However, that cannot be meaningfully assessed in the present work. Again, complex equilibria are involved in which binding of K+ and phosphate occurs by a variety of pathways, particularly when there is binding of pyruvate/DCA along with ATP or ADP. Our studies provide condition-dependent $L_{0.5}$ values that demonstrate large changes in the concentration dependence that result from major changes in binding affinities due to the binding of a second ligand.

The effects of all these ligands on the fluorescence of N-acetyltryptophanamide (NATA) were analyzed to evaluate effects due to chemical quenching and self-absorption (see data under Supplemental Data, Fig. 15) (40–42). Additionally, the effects of ATP and ADP on PDHK2 fluorescence were analyzed in the absence of Mg++, which is required for binding of these ligands to PDHK2 (27, 43). Ligands were added only at levels in which they did not measurably interfere with the fluorescence of NATA. We also evaluated the chemical quenching effects of acrylamide (40, 41) on the fluorescence of PDHK2 (see Supplemental Data, Fig. 25).

**RESULTS**

**PDHK2 Cross Arm and Trp Residues**—The bulk of human PDHK2 structure resides in two domains (Fig. 1), the N-terminal regulatory (R) domain (residues 6–169, green in Fig. 1) and the ATP/ADP-binding C-terminal catalytic (C) domains (residues 178–364, blue or red in Fig. 1). The Cα domains associate via an interface primarily formed by the offset interaction of equivalent mixed β-sheet regions (34, 35). From
this subunit association base, the subunits rise in opposed directions. Toward the outside, the interface between the R and Cat domains forms an extended cavity with the ATP/ADP binding site in the Cat domain at one end. The γ-phosphate of ATP held next to the central groove of the domain interface at Gly-319 (Fig. 1, A and D) (35). These outward facing cavities (thick wedge-shaped channel opening, Fig. 1) housing the active site will be referred to as the “front side” of the subunits/domains. The “back sides” of the subunits form a large opening with the appearance of a twisted trough with equivalent structure at diagonal positions on each side of the trough (open trough, Fig. 1D).

The central base of the trough is formed by association of the Cat domains, and the upper sides of the trough are formed by the back side of the opposite facing R domains. In the apo structure, an additional lobe is formed by a C-terminal segment (residues 365–384) that departs from the dimer interface, positions along the back side of each Cat domain, and then spans across the trough region to the back side of the other PDHK2 subunit (35). Fig. 1 (A and C) shows the cross arm interactions formed by each of these lobes in the apo structure (purple when departing from the blue Cat domain and rust-colored for the red Cat domain). A similar cross arm has been recently reported in PDHK3-L2 structures and shown to play a critical role in the binding of the L2 domain by PDHK3 (37).

Fig. 1 also shows the location of the three tryptophan residues within PDHK2. Trp-371 and Trp-383, are located in the cross arms (Fig. 1, A–C). Trp-371 is on the outer surface of the cross arm with one side fully exposed to solvent, and its fluorescence is probably low (solvent quenched). The Trp-371 equivalent (Trp-369) in PDHK3 does not interact with the L2 domain (37). Trp-383 is at the end of the cross arm and is inserted into the other subunit at the back-side interface between the R and Cat domains next to where the opposing cross arm begins. Trp-383 from one subunit sits between Arg-362 and Arg-149 of the other subunit (Fig. 1B, and supplemental Fig. 8S) and interacts with a set of aliphatic residues (Leu-152, Leu-363, and Pro-364) from the other subunit (Fig. 8S). The carboxylate group of Asp-382 forms an electrostatic interaction with Arg-149 and an electrostatic interaction with the NH of the indole ring of Trp-383 (Fig. 8S). In PDHK2, Trp-79 is located in the R domain above the His-115 side chain, which, in turn, makes a major contribution on its opposite side toward forming the roof in the DCA/pyruvate binding site (35). Trp-79 makes a significant contribution to the structure of the R domain.

Changes in the Intrinsic Fluorescence of PDHK2 Caused by ATP and ADP—ATP and ADP were shown to interfere with binding of PDHK2 to the L2 domain (15). We initiated these studies to evaluate whether ligands (ATP, ADP, and additionally pyruvate) would cause Trp quenching, which would be predicted if Trp-383 was reversibly dislodged from its anchoring position as found in x-ray crystal structures of PDHK2 with ADP or ADP and DCA bound (34, 35). We also evaluated the changes in binding of PDHK2 to the L2 domain by AUC studies. We first describe the effects of ligands on PDHK2 fluorescence.

PDHK2 has a typical UV absorbance spectrum with no indication of UV light absorbing nonprotein organic ligands. With excitation at 290 nm, the intrinsic fluorescence of PDHK2 was measured between 320 and 420 nm in buffer A. A broad peak was observed with a peak center at 353 nm (Fig. 2A). Given that the incident light was at 290 nm, the observed fluorescence at 350 nm and higher wavelengths is due primarily if not exclusively to tryptophan fluorescence. 150 μM ATP or ADP quenched this fluorescence (Fig. 2A) with ATP causing a greater decrease (53% versus 45%). ATP and ADP also caused a small blue shift for the λmax of the emission spectrum from 353 to 350 nm. Mg²⁺ is required for detectable binding of ATP to PDHK2 (27, 43). When MgCl₂ was not included, there was no change in the fluorescence spectra of PDHK2 (data not shown) with <1.0 mM adenine nucleotide indicating that free ATP or ADP did not contribute to the decreased fluorescence. AMP, which has no effect on PDHK activity (43), did not cause any quenching of PDHK2 fluorescence in the presence of Mg²⁺ (data not shown). Therefore, the observed quenching of fluorescence is due to...
Fluorescence Quenching by the Combination of Pyruvate and Adenine Nucleotides—Steady-state kinetic studies indicated that pyruvate/DCA bind effectively to the PDHK2/ADP intermediate while also binding to the PDHK2/ATP intermediate (27). Therefore, it was of particular inter-

Specific binding of ATP or ADP by PDHK2. The results below further support that conclusion.

Binding of ATP and ADP—The increases in quenching of the 350 nm fluorescence of 0.105 μM PDHK2 with the concentrations of ATP or ADP (Fig. 2, B and C) were fit well by simple equilibrium binding at a single class of sites (i.e. by a hyperbolic fit). In these analyses, quenching was assumed to be proportional to ligand binding (see “Experimental Procedures”) with maximum quenching reflecting binding at two sites based on previous binding studies (21, 27). With corrections of free ATP and ADP, proportionality was supported by the hyperbolic fits and estimates of $L_{0.5}$ (below) being unchanged within experimental error at the higher PDHK2 levels (0.32, 0.95, and 1.53 μM, data not shown). At 0.105 μM PDHK2, the maximum quenching estimated by the hyperbolic fit was 53.5% for ATP and 48.8% for ADP (error ranges Table 1). There was a small decrease in the estimated maximum quenching with increasing PDHK2 concentration. For instance, there was a 9% decrease with ATP varied when comparing the $Q_{\text{max}}$ values estimated at 0.105 and 0.95 μM PDHK2. This small incremental decrease with increasing protein concentration might reflect a small increase in the fluorescence of a tryptophan residue not undergoing quenching. Extrapolation to zero concentration predicts maximum quenching of 54.1 ± 0.4% by ATP and 49.9 ± 0.5% by ADP. With 0.105 μM (Table 1) to 1.53 μM PDHK2, average $L_{0.5}$ values of 3.0 ± 0.2 μM ($n = 6$) for binding of ATP and 16.7 ± 0.9 μM ($n = 6$) for ADP were estimated. With 0.105 μM PDHK2, Hill plots gave $n_H$ values of 0.97 for ATP and 1.11 for ADP and $L_{0.5}$ values within experimental error of those estimated from hyperbolic fits. The $L_{0.5}$ estimated for ATP agrees well with previous $K_d$ estimates (21, 27).

**Effects of Pyruvate and DCA on PDHK2 Fluorescence**—Direct binding of pyruvate to PDHK2 is too weak to detect using labeled pyruvate with workable levels of PDHK2 (>6.0 mg/ml). We have found that addition of pyruvate or DCA also quenched Trp fluorescence (Fig. 3 and Table 1). The quenching effects of pyruvate were assessed at levels of <1.0 mM, because pyruvate quenching of the fluorescence of NATA was observed at >1.0 mM pyruvate with ~20% quenching due to 10 mM pyruvate (see Fig. 1S). Again, assuming quenching was proportional to binding of pyruvate, the data fit (Fig. 3A) curved off in a double reciprocal plot suggesting negative cooperativity or two classes of binding sites. An $L_{0.5}$ of 590 μM was estimated from the reciprocal plot and a Hill plot (data not shown); the Hill coefficient, $n_H$ was 0.87. At 0.105 μM PDHK2, maximum quenching was estimated as ~71%. The maximal quenching decreased with the concentration of PDHK2 but invariably exceeded that by ATP or ADP at all PDHK2 concentrations. Nevertheless, nearly equivalent $L_{0.5}$ values were estimated from studies at higher PDHK2 levels (e.g. ~590 μM at 1.53 μM PDHK2). These data constitute the first evidence that pyruvate can bind to free PDHK2. Although binding at the same site as pyruvate, DCA gave less maximal quenching (37.3% at 0.105 μM PDHK2) than pyruvate (Fig. 3B and Table 1), but half-maximal quenching was achieved at a lower concentration of ~130 μM with the quenching showing a simple hyperbolic change with increasing DCA. Modest additional quenching occurred at much higher DCA levels. However, this was in the range in which chemical quenching of NATA (Fig. 1S) was observed. Therefore, compared with pyruvate, DCA appears to bind tighter to free PDHK2 but have a smaller quenching effect. In contrast, pyruvate is a more potent inhibitor of PDHK2 activity (12, 27).

**Table 1** Parameters derived from hyperbolic fit of ligand binding to 0.105 μM PDHK2 based on the quenching of 350 nm fluorescence

| Ligand   | $L_{0.5}$ (μM) | Error range | Maximum quenching | Error range |
|----------|---------------|-------------|-------------------|-------------|
| ATP      | 2.8 ± 0.2     | 2.55–3.1    | 53.5              | 51.9–55.0   |
| ADP      | 15.3 ± 1.1    | 14.2–16.8   | 48.8              | 47.3–51.3   |
| Pyruvate | 590 ± 20      | 500–640     | 71.5              | 69–75       |
| DCA      | 130 ± 5       | 115–145     | 37.3              | 36.0–38.6   |

$^a$ 95% confidence interval.

$^b$ Hyperbolic fit.

* The $L_{0.5}$ was estimated from the Hill plot with the maximum quenching used in developing the Hill plot obtained from the Klotz plot as described under “Experimental Procedures”; the value was rounded to the nearest 5 μM interval.

**FIGURE 2. Quenching of the intrinsic PDHK2 fluorescence at 350 nm due to ATP and ADP.** All data were obtained with 0.105 μM PDHK2 using excitation at 290 nm. A, scans exhibiting the effects of these ligands on PDHK2 fluorescence measured between 320 to 420 nm. B and C, incremental increases in quenching at 350 nm with increasing ATP and ADP concentration, respectively. Fluorescence measurements were made, and then data were fit assuming a single class of independent binding sites, using the approaches described under “Experimental Procedures.”
est to evaluate how ATP and ADP changed the binding by these ligands as assessed from the quenching of Trp fluorescence. In the presence of 100 μM ADP or ATP, the level of pyruvate required to quench Trp fluorescence was reduced and markedly so with ADP (supplemental Fig. 3S and Table 2). With ATP, the response to varied pyruvate exhibited apparent negative cooperativity, but positive cooperativity was found with ADP included (Fig. 3S (A and B); Hill coefficients, Table 2). Using the $Q_{\text{max}}$ estimated from these double reciprocal plots, Hill plots gave an $L_{0.5}$ for pyruvate of 3.4 μM with 100 μM ADP but 10-fold higher pyruvate was required for half-maximal quenching with 100 μM ATP (Table 2). Thus, with ATP and ADP, the $L_{0.5}$ for quenching by pyruvate was decreased with ≥150-fold decrease with 100 μM ADP.

As a further indication of the tight linkage between ADP and pyruvate binding, addition of only 2 μM ADP reduced the $L_{0.5}$ for pyruvate to 14.6 μM and introduction of 20 μM ADP decreased the $L_{0.5}$ for pyruvate to 4.7 μM. There was not a significant change in the $L_{0.5}$ at levels of ADP ≥ 50 μM. ~96% of maximum quenching was reached with 100 μM pyruvate on top of 100 μM ADP; whereas, with pyruvate alone, ~100-fold higher level of pyruvate is projected to be required to achieve that level of fluorescent quenching. Hill plots analyzing pyruvate-induced quenching gave $n_H = 0.87 ± 0.04$ from a set of experiments in which pyruvate was titrated after addition of ADP at 20, 50, 100, 200, or 1000 μM. Although DCA, alone, quenched Trp fluorescence with a lower concentration dependence than pyruvate, somewhat higher levels of DCA were required in the presence of the adenine nucleotides. Indeed, DCA was less effective than pyruvate (higher $L_{0.5}$, lower $Q_{\text{max}}$) in the presence of 100 μM ADP or ATP (Table 2). Nevertheless, as with pyruvate, ADP and ATP did enhance the binding of DCA. These results support the conclusion that formation of PDHK2-ADP and PDHK2-ATP complexes induces conformational changes that enhance binding of pyruvate (27).

With an $L_{0.5}$ of ~550 μM for pyruvate, alone, only a 0.15 portion of the pyruvate binding site is predicted to be occupied upon adding 100 μM pyruvate. However, as expected for equilibrium binding, in the presence of 100 μM pyruvate, half-maximal quenching by ADP was reached at a significantly lower ADP concentration (~8-fold) but that was not the case with ATP (Table 1 versus Table 2). However, both reciprocal plots (supplemental Fig. 3S (C and D)) and Hill plots exhibited strong positive cooperativity with $n_H$ of 1.95 for ADP and 2.2 for ATP (Table 2). Use of higher pyruvate levels, including 1.0 mM, did not significantly reduce the $L_{0.5}$ for ADP (~1.6 μM), although that led to a substantially narrowed range for observing the additional quenching (beyond pyruvate) by ADP.

These results are consistent with a synergistic process resulting from a two-step mechanism as proposed based on kinetic studies (27) with minimal dissociation of ADP from the PDHK2-ADP-pyruvate complex (see “Discussion”). These studies strongly support sequential conformational changes being induced by the combination of adenine nucleotide and pyruvate. The AUC studies below additionally indicate that these ligands affect the interaction of PDHK2 with the L2 domain of E2 and

### TABLE 2

| Ligand 1 at 100 μM (or as indicated) | Titrated ligand 2 | $L_{0.5}$ μM | Hill coefficient, $n_H$ | Maximum quenching of ligand 2 | Total quenching maximum |
|-------------------------------------|-------------------|-------------|------------------------|-----------------------------|--------------------------|
| ADP                                | Pyruvate          | 3.4 ± 0.18  | 0.87 ± 0.04            | 36.5 ± 1.0                  | ~68                      |
| ATP                                | Pyruvate          | 42 ± 6      | 1.4 ± 0.15             | 26.4 ± 0.8                 | ~69.5                    |
| Pyruvate                           | ADP               | 2.0 ± 0.15  | 1.95 ± 0.2             | 61 ± 1.5                   | ~70                      |
| Pyruvate                           | ATP               | 3.2 ± 0.2   | 2.2 ± 0.2              | 58 ± 0.5                   | ~66.5                    |
| ADP                                | DCA               | 11.9 ± 0.3  | 1.1 ± 0.03             | 31.0 ± 1.0                 | ~59                      |
| ATP                                | DCA               | 57 ± 2      | 1.2 ± 0.03             | 17.5 ± 0.7                 | ~62                      |
| DCA (200 μM)                       | ADP               | 2.7 ± 0.1   | 2.1 ± 0.1              | 49 ± 1.0                   | ~55                      |
| DCA (200 μM)                       | ATP               | 1.65 ± 0.1  | 2.05 ± 0.1             | 48 ± 1.0                   | ~56                      |

* Rounded to the nearest 0.5%; error range was ±3.0%.

FIGURE 3. Quenching of PDHK2 fluorescence by pyruvate or DCA. The main figures in the panels show the best hyperbolic fits for the increase in fluorescence quenching with increasing free pyruvate (A) or DCA (B) concentrations. The insets show double reciprocal plots of those data. Changes in the intrinsic steady-state fluorescence of 0.105 μM PDHK2 were measured at 350 nm. Other experimental conditions and analyses were as described under “Experimental Procedures.”
that the combination of ligands affects the oligomeric state of PDHK2.
However, fluorescence quenching occurs at PDHK2 concentrations well below those at which there is significant self-association of PDHK2.
Finally, these studies were performed in potassium phosphate buffer. As will be described elsewhere, potassium and phosphate influence equilibrium binding. These effects were nearly saturated with the buffer used in this study.

Properties of PDHK2 Mutated at Trp-383—As indicated above, among the three tryptophan residues (79, 371, and 383), Trp-383 at the end of intersubunit cross arm, wedges between residues of the other subunit (Fig. 8S). The lack of this cross arm in PDHK2 structures with ADP (34) or ADP plus DCA bound (35) suggested that the fluorescent quenching upon ligand binding might result from changing the electronic environment around Trp-383. Indeed, under some conditions, this might involve disrupting the intersubunit cross arm interaction, thereby exposing Trp-383 to solvent. A key role of Trp-383 is supported by the far-UV CD spectra of this mutant PDHK2 being essentially different with a much larger decrease in fluorescence at a temperature somewhat below that of wild-type PDHK2 (supplemental Fig. 5S). The transition in Trp fluorescence with increasing temperature was very different with a much larger decrease in fluorescence at a temperature somewhat below the melting transition for native PDHK2 undergoing the same treatment.

Changes in Fluorescence Quenching of W383F-PDHK2—Compared with native PDHK2, there was a reduction of ~60% in the Trp fluorescence of W383F-PDHK2 at 350 nm in the absence of added ligands (supplemental Fig. 4S). This indicates that Trp-383 makes a disproportionately large contribution to the 350 nm fluorescence of native PDHK2. Addition of ATP no longer quenched Trp fluorescence at 350 nm (Fig. 4A). Similarly, ADP had only a very small effect on Trp fluorescence (Fig. 4B). These results indicate that the binding of adenine nucleotides markedly quenches the fluorescence of Trp-383 in native PDHK2. Addition of 100 μM pyruvate, after addition of ATP or ADP, caused a small but definite fluorescence quenching and yielded an absolute residual fluorescence similar to that of native PDHK2 after these treatments (Figs. 4A and 6B). Just introducing pyruvate to W383F-PDHK2, Fig. 4C (△), gave much less quenching than with wild-type PDHK2. Analysis of the quenching profile yielded an L0.5 of ~350 μM, which is close to but apparently somewhat lower than the L0.5 of 590 μM observed with wild-type PDHK2. Therefore, the majority of the extensive quenching of 350 nm fluorescence by pyruvate, alone, is due to quenching of Trp-383 fluorescence. The small amount of residual quenching caused by pyruvate is probably due to changes in PDHK2 conformation altering the interactions/environment of the indole ring of Trp-79, which sits next to the pyruvate binding site (see “Discussion”).

Even though the extent of quenching of 350 nm fluorescence was greatly reduced with W383F-PDHK2, the quenching by pyruvate on top of ATP or ADP (Fig. 4C) gave low L0.5 values for pyruvate of ~25 or ~9 μM, respectively. Therefore, ATP and ADP still greatly enhanced pyruvate binding. Therefore, ineffective ATP and ADP binding to PDHK2 is not the cause of loss of the quenching of the fluorescence of W383F-PDHK2 by these ligands. In agreement with this, ATP and ADP did quench the 350 nm fluorescence when added after low levels of pyruvate. With 100 μM pyruvate, near-maximal quenching was achieved with 15 μM ATP and 10 μM ADP, yielding an absolute residual fluorescence similar to that of native PDHK2 undergoing the same treatment. Therefore, with W383F-PDHK2, even though ATP and ADP, alone, no longer caused significant quenching of Trp fluorescence, the binding of these ligands appears to be little changed, and, upon binding, these ligands still increase the affinity of this mutant kinase for pyruvate.
The fluorescence spectra of native PDHK2, W383F-PDHK2, and the difference between these spectra are shown in supplemental Fig. 4S. Given that W383F mutation did not greatly alter PDHK2 function or ligand binding, the difference spectra is likely due almost entirely to Trp-383. The estimated fluorescence intensity (Fig. 4S) of the native enzyme is ~2.5 times stronger than the intensity of W383F-PDHK2, suggesting ~60% of the fluorescence intensity is due to Trp-383. This deconvolution gives a peak center at ~357 nm for the “difference” Trp-383 peak (Fig. 4S), which is red-shifted 12 nm from the peak center of the W383F-PDHK2. As indicated in Figs. 1 and 8S, Trp-383 is mostly buried in the apo-PDHK2 crystal structure. The intensity attributed to Trp-383 is high based on comparisons to other Trp-containing proteins, including one with a single buried Trp. The high quantum yield and red-shifted fluorescence spectra are apparently predictable for Trp-383 (51–53) based on the Trp-383 environment in apo-PDHK2.

**Sedimentation Velocity and Equilibrium Studies**—As indicated in the introduction, PDHK2 activity is greatly enhanced by PDHK2 binding to the L2 domain of the E2 60-mer. It is only with E2-activated PDHK2 that pyruvate significantly inhibits kinase activity (12). Based on kinetic and other studies, this gain in pyruvate inhibition was interpreted as being linked to ADP dissociation becoming the rate-limiting step when access to the E1 substrate is a facilitated process on the surface of the E2 60-mer (27). Additionally, it is possible that binding of pyruvate to PDHK2 may alter the binding of pyruvate to PDHK2 may alter the binding of PDHK2 to the L2 domain of E2. ATP and ADP were found to reduce binding of PDHK2 to E2 (15). PDHK2 binding to monomeric L2 is weak (15). PDHK2 binds more tightly to dimeric GST-L2 and several-fold tighter yet after reducing the lipoyl groups of GST-L2 (i.e. GST-L2,red). Therefore, we evaluated the effects of ligands on PDHK2 binding to GST-L2,red. As described under “Experimental Procedures,” the lipoyl groups of GST-L2 dimer were reduced by treatment with dithiothreitol (21). Fig. 5A shows the g(s)(*) profiles derived from an experiment in which the sedimentation of 3.7 μM PDHK2, 7.5 μM GST-L2,red, and the combination were simultaneously monitored. Binding of PDHK2 to GST-L2,red gave a faster sedimenting peak due to complex formation. Pyruvate at 100 μM had a minimal effect, whereas 5 mM pyruvate decreased the rate of sedimentation of the fast-moving peak and increased the signal of the trailing GST-L2,red (Fig. 5B). As previously reported using the E2 60-mer, ATP and ADP also caused some dissociation of PDHK2 from GST-L2,red (Fig. 5, C and D). From simulations of the profiles using 7.35 S for the complex (15), we estimated a Kd for the complex of 0.75 ± 0.2 μM for the complex increased ~2-fold due to 200 μM ATP and somewhat more due to 200 μM ADP. At 5 mM pyruvate caused about a 5-fold decrease in binding affinity. The combinations of ATP or ADP (100 μM) and pyruvate markedly increased the size of the peak sedimenting in the trailing region of GST-L2,red (Fig. 5, C and D). Thus, the sedimentation data support ATP or ADP along with low pyruvate causing extensive dissociation of PDHK2 from GST-L2,red.

However, when pyruvate along with ATP or ADP is included (Fig. 5, C and D), the sedimentation profiles did not exhibit simple increases in the region that the PDHK2 dimer sediments. These data suggest that dissociated PDHK2 sediments at a rate faster than the PDHK2 dimer. Fig. 5E shows the sedimentation profiles of GST-L2,red and PDHK2 alone and the combination in the presence of 100 μM ATP and 100 μM pyruvate. Indeed, PDHK2 sedimented faster with these ligands included than it did in the absence of these ligands (panel A). The results indicated that there was minimal but some binding of PDHK2 to GST-L2,red and that PDHK2 was undergoing self-association. From the difference between GST-L2 and the peak for the combination (panel E), ≤3% of GST-L2 (≤5% of PDHK2) was estimated to be bound, indicating an increase in the Kd to >125 μM (>130-fold decrease in binding affinity).

Based on the apparent self-association of PDHK2 (Fig. 5E), we further investigated the effects of various ligands on the sedimentation of PDHK2. Fig. 6 shows the sedimentation velocity patterns with 100 μM ATP, ADP, or pyruvate present (panels A–C) or with pyruvate added in combination with ATP (panel A) or ADP (panel B). None of these ligands, alone, significantly altered the sedimentation pattern of
PDHK2. However, the combination of pyruvate and either ATP or ADP led to a faster sedimenting species. The change suggested self-association of the PDHK2 dimer to a stable tetramer.

Fig. 7A shows the concentration-dependent change in g(s) profiles for sedimentation velocity studies performed using a series of PDHK2 concentrations in the presence of 100 μM ADP and 100 μM pyruvate. The s_{20,w} value of the faster sedimenting species was estimated to be 7.1 S from extrapolating a plot at high concentrations (C) of the observed S versus 1/C to the y-intercept. Based on the N^{2/3} rule, an extrapolated s_{20,w} value of 8.27 S is predicted for a tetramer that is equally compact as the dimer. To be fit by a tetramer, the fractional coefficient ratio (f/f_0) would have to increase from 1.39 for the PDHK2 dimer to 1.61 for the tetramer. This suggests a looser structure (see “Discussion”). Treating this association with a monomer-dimer equilibria using SEDPHAT (i.e. the PDHK2 dimer is treated as a monomer, see “Experimental Procedures”), the sedimentation profiles were not fit well assuming rapid equilibration using the estimated s_{20,w} of 7.1 S for the tetramer. However, good fits were obtained using a slow dissociation step \( f/f_0 = 10^{-3} \) s\(^{-1}\). A global fit (Fig. 7B) gave a \( K_d \) of 6.1 μM with an error range from 5.5 to 6.75 μM based on the F statistic for 95% confidence interval. With a faster equilibrium, the closest fits were obtained when the s_{20,w} value of the tetramer was allowed to increase. Such fits gave higher \( K_d \) values (i.e. with a tetramer s_{20,w} = 7.5 S, \( K_d = 9.65 \) μM; but this fit had an expanded error range of 7.1–12.2 μM).

Sedimentation equilibrium studies were conducted to further evaluate whether the faster moving species is a tetramer and to estimate its binding affinity. Fig. 7C shows profiles for equilibria achieved at three speeds for three concentrations. The data were globally fit (solid lines) using the monomer-dimer model. The fit gave a \( K_d \) of 8.6 μM (error range, 8.05–9.1 μM; variance of 2.8 × 10\(^{-13}\)). The error was much larger when the data were fit by a dimer-trimer model. Thus, sedimentation velocity and sedimentation equilibrium results support pyruvate plus ADP causing reversible association of PDHK2 to a tetramer with an equilibrium dissociation constant of \( \sim 7.5 \pm 2 \) μM.

**Fluorescence Quenching at Elevated PDHK2 Concentrations**—Most fluorescence studies with the combination of pyruvate and ADP were conducted with 0.105 and 1.53 μM PDHK2. ~23% of possible PDHK2 tetramer (\([\text{tetramer}] / ([0.5 \text{ dimer total}]) \) are predicted be formed at the higher concentration. As noted above, there was a trend of decreasing maximum fluorescence quenching with increasing PDHK2 concentration. Beyond nonspecific interference that reduced quenching upon addition of ligands, it is possible that tetramer formation, induced by ADP plus pyruvate, might further reduce Trp quenching by creating an interaction that partially protects Trp-383 and/or Trp-371 from exposure to quenching by solvent. With 30.9 μM PDHK2, ADP and pyruvate caused ~70% dimer (analysis of profile, Fig. 7A). Even at 30.9 μM PDHK2, neither ATP nor ADP alone caused detectable tetramer formation (data not shown).

Fluorescence studies were conducted with 31.9 μM PDHK2 using 295 nm for excitation (2.5 nm bandwidth) with the value from the emission scans at 350 nm still reported. As shown in Fig. 7A (supplemental materials), the profile for variation in % quenching by ADP was similar (\( I_{0.5} = \sim 16.8 \) μM) to that with a 300-fold lower PDHK2 concentration (Fig. 2C). However, the estimated \( Q_{\text{max}} \) was reduced from ~48 to ~38%. Fig. 7B shows the quenching profile when pyruvate was added in the presence of 150 μM ADP; the added pyruvate concentration is plotted, because the level of free pyruvate is too small to accurately assess. There was <1% decrease in the projected maximal quenching by the combination of ligands. Although ADP, alone, does not induce tetramer formation, a major portion of the decrease in \( Q_{\text{max}} \) with increasing kinase level was observed with just ADP added to 31.9 μM PDHK2. Thus, it would appear that tetramer formation makes no more than a small contribution to the reductions in \( Q_{\text{max}} \) values with increasing PDHK2 concentration. With ligand combinations (ATP/pyruvate or ADP/pyruvate), the estimated \( I_{0.5} \) decreased with increasing PDHK2 (range, 0.1–3 μM PDHK2).

**DISCUSSION**

We have found that Trp fluorescence reports the binding of adenine nucleotides and pyruvate/DCA and confirmed a marked effect of ADP on pyruvate binding. Pyruvate plus ADP also markedly decreased binding of PDHK2 to GST-L2 dimer and caused the PDHK2 dimer to associate to form a loose tetramer. By potently interfering with E2 enhancement of PDHK2 activity, these changes make an important mechanistic contribution to the synergistic inhibition of PDHK2 activity by ADP and pyruvate. We analyze these results in a context of the different PDHK2 (34, 35) and PDHK3-L2 crystal structures, and the locations of Trp residues (see beginning of "Results").

**Altered Structures with Ligands Bound**—Opposite the active site cavities on the outward facing front sides of the subunits of a PDHK dimer is a large trough formed between the back sides of the subunits. The trough is spanned in apo-PDHK2 (35) and PDHK3-L2 structures (37) by a C-terminal cross arm with Trp-383 from one PDHK2 subunit inserting into the back side of the other subunit of the dimer (Figs. 1 and 8S). The PDHK2 crystal structures with ADP bound (34) or ADP plus DCA bound (35) do not exhibit the cross arm, so that Trp-383 is no longer lodged in the back side of the other subunit. When the cross arm is not resolved and elevated \( K^* \) is included (34), the spacing of the trough region between the back sides of the regulatory domains in PDHK2 dimer increases (Fig. 1D) and the wedge spacing between the R and Cat domains on the front side decreases (35, 37). This displacement results from movement of the R domain relative to the Cat domain (35, 37). This suggests that the larger (more open) trough region and less open wedge-active site region are favored in the absence of the cross arm interaction (35). Structural differences with ligands bound are proposed to reflect ligand-induced changes and to have important functional con-
sequences (35) with the focus here being the means by which ligands reduce binding to the L2 domain (below).

There was a large loss in fluorescence and nearly complete removal of the quenching by ADP and ATP with the W383F-PDHK2 mutant. This indicates ATP and ADP markedly quench the fluorescence of Trp-383 of the other subunit that is held 20 Å from the ATP/ADP binding site.

The quenching of the fluorescence of W383F-PDHK2 by pyruvate constitutes a much smaller change than with native PDHK2. This pyruvate-induced decrease in fluorescence is probably due to quenching of Trp-79 within the PDHK2 subunit structure when ADP and DCA are bound (35). It is known that interaction of tryptophan with the histidine side chain can cause fluorescence quenching (42, 56). The small extent of quenching limited the accuracy of the estimated binding affinities. Nevertheless, pyruvate binding to W383F-PDHK2 was found to occur with a weak affinity similar to that of the native enzyme, and adenine nucleotides fostered a similar marked increase in affinity (decrease in the $L_0.5$) for pyruvate binding.

With Trp-371 being exposed to solvent interactions on the surface of the cross arm (Fig. 1C), it is very difficult to imagine any mechanism that could cause major quenching of Trp-371 by ligands. Following addition

**FIGURE 7.** Sedimentation velocity (A and B) and sedimentation equilibrium (C) profiles of different levels of PDHK2 in the presence of ADP and pyruvate. A, change in the sedimentation velocity patterns, $g(s*)$ with the increasing concentrations of PDHK2 (solid lines) when sedimentation was conducted in the presence of 100 µM ADP and 100 µM pyruvate. The dotted lines show the sedimentation pattern for 5.7 µM and 30.9 µM PDHK2 in the absence of added ligands. Although sedimentation was monitored at 290 nm for the concentrations of PDHK2 517.0 µM (see “Experimental Procedures”), A shows profiles for these levels based on their calculated relative $A_{280}$, which was used to monitor the lower concentrations. As previously described for another monomer-dimer equilibria (16), B shows examples, for the scans at 2.3 and 5.7 µM, of the global fits obtained with SEDPHAT (solid lines) using the monomer-dimer model. This fit by SEDPHAT was made using a slow dissociation step as described under “Results.” Using the same concentrations of ADP and pyruvate, C shows equilibrium sedimentation patterns obtained when sedimentation equilibrium was attained at 10,000, 12,000, and 14,000 rpm with 1.7, 2.43, and 3.06 µM PDHK2. The solid lines show global fit of the data; the data analyses were made as described under “Experimental Procedures.”
of ligands, the residual fluorescence is low and fits three strongly quenched Trp residues (two due to ligand-induced changes and one continuously due to sustained solvent exposure). The roughly matching fluorescence quenching caused by ADP plus pyruvate to that due to guanidine-unfolding PDHK2 (Fig. 2S) is consistent with our assignments, including the proposal that Trp-371 is already in a highly quenched state. This is also supported by the very low fluorescence of W383F-PDHK2 relative to native PDHK2.

There are important similarities and differences between PDHK2 and PDHK3 structures and interactions with the L2 domain. Trp-381 of PDHK3 aligns with Trp-383 in PDHK2 and locates in the same position in the PDHK3 structure. Phe-31 (colored with cyan in Fig. 1) acts as a lid at the entrance to the Nov3r binding site in PDHK2 (35). Nov3r prevents binding of PDHK2 to GST-L2red or E2.5 Furthermore, selective mutation within the Nov3r site of PDHK2 prevents stimulation by ATP/ADP to quench fluorescence. As with fluorescence, higher DCA levels along with ADP are required to hinder binding of PDHK2 to GST-L2red (data not shown). With its carbonyl group, pyruvate is capable of forming interactions that cannot be made by DCA. Further studies will be needed to assess these differences between DCA and pyruvate.

Combination of Ligands—Kinetic and binding studies indicate that ADP dissociation is a slow step in PDHK2 catalysis, which favors binding of pyruvate by PDHK2-ADP (27). The binding to PDHK2 of the combination of an adenine nucleotide and pyruvate caused maximum quenching, strongly hindered binding of PDHK2 to GST-L2red and induced self-association of the PDHK2 dimer to form a tetramer. The marked hindering of binding of PDHK2 to the L2 domain of E2 by the combination of ADP and pyruvate likely makes a major contribution to the potent inhibition by these effectors. The strong interference by ADP and pyruvate with the binding of PDHK2 to GST-L2red and pronounced quenching of the fluorescence of Trp-383 may, in part, reflect these ligands disrupting the cross arm connection that is required for binding of PDHK2 to the L2 domain. This is in agreement with the lack of a cross arm in the PDHK2-ADP-DCA structure (35). The binding of ATP/ADP is greatly enhanced by K+ and, with PDHK2-ADP-K+, pyruvate binding is enforced by phosphate anion.3 These effects were nearly saturated in this study, and the resulting equilibrium effects contribute to the apparent positive cooperativity when ADP and ATP were added last (Table 2).

We further found that ADP plus pyruvate support PDHK2 tetramer formation, which probably contributes to greatly decreased binding to the L2 domain. The combination of ATP/ADP plus pyruvate must cause a structural change not favored by the individual ligands, because these do not induce tetramer formation. ADP plus pyruvate may foster a persistent disruption of the cross arm structure which, in turn, contributes to tetramer formation. Indeed the cross arms have structural features that in a dislodged state could support tetramer formation.7

The PDHK2 tetramer has a high fictional coefficient (f/f0 = 1.61), which is consistent with a somewhat extended and flexible association. Further studies will be needed to establish whether the released cross arms directly participate in tetramer formation. The conversion of PDHK2 to a tetramer that binds poorly to E2 would also contribute to the inhibition of PDHK activity, in vivo.

With the combination of pyruvate and adenine nucleotides, use of only 100 μM of the fixed ligand supported a near maximal effect when the other ligand was varied. Increasing the concentration of the first ligand did not alter the maximal quenching or significantly decrease the observed I0.5 for the second ligand (data not shown). Given the weak binding by pyruvate, only a small fraction of pyruvate should be bound prior to adding ADP. Based on our kinetic studies (27), we suggested not only that binding of ADP aids binding of pyruvate but that pyruvate binding hinders ADP dissociation. This raises the possibility that movement in and out of the ADP binding site is hindered when pyruvate is bound. A mechanistic basis for the trapping of the complex, in part

---

5 L. Hu, Y. Hiromasa, and T. E. Roche, manuscript in preparation.
6 Y. Hiromasa, T. Peng, and T. E. Roche, manuscript in preparation. This work involves AUC studies of the binding of PDHK3 to different lipoyl domain constructs and establishes that L2 stabilizes PDHK3 as a dimer, that binding of PDHK3 to L2 domain has an equilibrium binding constant below 50 nM, and that there is tighter binding of PDHK3 by E2 or dimeric GST-L2, much weaker binding by GST-L1, and yet much weaker binding by the L1 monomer.

7 A persistently freed C-terminal lobe may form interacting pairs with complementary electrostatic interactions between the acidic ends (Glu-379 and Asp-382) with the positively charged beginnings of these C-terminal lobes (Lys-368, Arg-372, and His-373). The exposed Trp-371 and Ile-377 also seem positioned to pair (Trp to Ile) between the interacting arms of each dimer.

**PDHK2 Intersubunit Cross Arms**
involving optimum K+ ion binding behind ADP, has been suggested based on the PDHK2-ADP-DCA structure (35). As indicated above, both K+ and phosphate have major impacts favoring complex formation, particularly in the case of the PDHK2-ADP-pyruvate complex.3

In summary, we have presented evidence that binding of ATP, ADP, or pyruvate induce substantial conformational changes in PDHK2 as reported by changes in Trp fluorescence, primarily involving Trp-383. Consistent with ADP enhancing pyruvate binding, the apparent affinity for pyruvate \( (I_{0.5}) \) is reduced 150-fold in the presence of ADP. Associated with these changes, there is a marked reduction in bifunctional binding of PDHK2 to the L2 domain of E2 caused by ADP and pyruvate. Furthermore, these ligands cause the PDHK2 dimer to associate as a tetramer. Both changes would act to greatly hinder PDHK2 activity, helping to explain the potent synergistic effects of these inhibitors. Our results are consistent with maintenance of the cross arms contributing to PDHK2 binding to the L2 domain. Trp-383 plays a critical role in cross arm anchoring. A disengaged cross arm may directly contribute to formation of the loose tetramer. Studies on the fluorescence lifetimes of PDHK2 tryptophan residues and changes in these lifetimes with variation in ligands should provide further insights into the changes in the conformational states of PDHK2.

Acknowledgments—We thank Shane A. Kasten for preparing some of the E1 Callis for his general analysis of the chemical shift and quantum yield of Trp-383.

REFERENCES

1. Patel, M. S., and Roche, T. E. (1990) FASEB J. 4, 3224–3233
2. Roche, T. E., Baker, J., Yan, X., Hiromasa, Y., Gong, X., Peng, T., Dong, J., Turkan, A., and Kasten, S. A. (2001) J. Biol. Chem. 292, 6921–6933
3. Hiromasa, Y., Fujisawa, T, Aso, Y., and Roche, T. E. (2004) Biochemistry 43, 14976–14985
4. Knoechel, T. R., Tucker, A. D., Robinson, C. M., Phillips, C., Taylor, W., Bungay, P. J., Kasten, S. A., Roche, T. E., Rice, R. A., and Hamilton, J. A. (2001) J. Biol. Chem. 276, 37443–37450
5. Knoechel, T. R., Tucker, A. D., Robinson, C. M., Phillips, C., Taylor, W., Bungay, P. J., Kasten, S. A., Roche, T. E., Rice, R. A., and Hamilton, J. A. (2001) J. Biol. Chem. 276, 37443–37450
6. Aicher, T. D., Anderson, R. C., Beberntz, G. R., Coppola, G. M., Jewell, C. F., Knorr, D. C., Liu, C., Sperbeck, D. M., Brand, L. J., Strohschein, R. J., Gao, J., Vinulch, C. C., Shetty, S. S., Dragland, C., Kaplan, E. L., DeGrande, D., Islam, A., Liu, X., Lottiez, R. J., Maniara, W. M., Walter, B. E., and Mann, W. R. (1999) J. Med. Chem. 42, 2741–2746
7. Kato, M., Chuang, J. L., Tse, S.-C., Wynn, R. M., and Chuang, D. T. (2005) EMBO J. 24, 1763–1774
8. Liu, S., Baker, J. C., Andrews, P. C., and Roche, T. E. (1995) Arch. Biochem. Biophys. 326, 916–940
9. Liu, T.-C., Korotchkina, L. G., Hyatt, S. L., Vettakkorumakankav, N. N., and Patel, M. S. (1995) J. Biol. Chem. 270, 15545–15550
10. Eftink, M. R. (1991) Methods Enzymol. 208, 125–127
11. Lalovic, R. (2000) Principles of Fluorescence Spectroscopy, Kluwer Academic Publishers, New York
12. Chen, Y., and Barkley, M. D. (1998) Biochemistry 37, 9976–9982
13. Chiou, F., Randall, D. D., Roche, T. E., Burgett, M. W., Wilkerson, J. W., and Reed, L. J. (1972) Arch. Biochem. Biophys. 151, 328–340
14. Philo, J. S. (2000) Anal. Biochem. 289, 151–163
15. Staffard, W. F. (2000) Modern Spectroscopic Methods: Acquisition and Interpretation of Data for Biological and Synthetic Polymer systems (Schuster, T. M., and Laue, T. M., eds) pp 119–137, Birkhauser, Boston
16. Correia, J. J. (2000) Vol. Methods Enzymol. 321, 81–100
17. Staffard, W. F. (2000) Methods Enzymol. 323, 303–325
18. Correia, J. J., Shacko, B. M., Lam, S. S., and Lin, K. (2001) Biochemistry 40, 1473–1482
19. Schuck, P. (1998) Biochemistry 7, 7503–7512
20. Schuck, P. (2003) Anal. Biochem. 320, 104–124
21. Vivian, J. T., and Schuck, P. R. (2000) Biochemistry 8, 2093–2109
22. Callis, P. R., and Vivian, J. T. (2003) Chem. Phys. Lett. 39, 409–414
23. Callis, P. R., and Liu, T. (2004) J. Phys. Chem. B 108, 4248–4259
24. Roche, T. E., Hiromasa, Y., Turkan, A., Gong, X., Peng, T., Yan, X., Kasten, S. A., Bao, H., and Dong, J. (2003) Eur. J. Biochem. 270, 1050–1056
25. Klyuyeva, A., Tuganova, A., and Popov, K. M. (2005) Biochemistry 44, 13573–13582
26. Loewenthal, R. S., and Fersht, A. R. (1991) Biochemistry 30, 6775–6779