Marked Differences between Two Isoforms of Human Pyruvate Dehydrogenase Kinase*

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Jason C. Baker, Xiaohua Yan, Tao Peng, Shane Kasten, and Thomas E. Roche‡

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

Pyruvate dehydrogenase kinase (PDK) isoforms 2 and 3 were produced via co-expression with the chaperonins GroEL and GroES and purified with high specific activities in affinity tag-free forms. By using human components, we have evaluated how binding to the lipoyl domains of the dihydrolipoyl acetyltransferase (E2) produces the predominant changes in the rates of phosphorylation of the pyruvate dehydrogenase (E1) component by PDK2 and PDK3. E2 assembles as a 60-mer via its C-terminal domain and has mobile connections to an E1-binding domain and then two lipoyl domains, L2 and L1 at the N terminus. PDK3 was activated 17-fold by E2; the majority of this activation was facilitated by the free L2 domain (half-maximal activation at 3.3 μM L2). The direct activation of PDK3 by the L2 domain resulted in a 12.8-fold increase in k_cat along with about a 2-fold decrease in the K_m of PDK3 for E1. PDK3 was poorly inhibited by pyruvate or dichloroacetate (DCA). PDK3 activity was stimulated upon reductive acetylation of L1 and L2 when full activation of PDK3 by E2 was avoided (e.g., using free lipoyl domains or ADP-inhibited E2-activated PDK3). In marked contrast, PDK2 was not responsive to free lipoyl domains, but the E2–60-mer enhanced PDK2 activity by 10-fold. E2 activation of PDK2 resulted in a greatly enhanced sensitivity to inhibition by pyruvate or DCA; pyruvate was effective at significantly lower levels than DCA. E2-activated PDK2 activity was stimulated at least 3-fold by reductive acetylation of E2; stimulated PDK2 retained high sensitivity to inhibition by ADP and DCA. Thus, PDK3 is directly activated by the L2 domain, and fully activated PDK3 is relatively insensitive to feed-forward (pyruvate) and feed-back (acetylating) effectors. PDK2 was activated only by assembled E2, and this activated state beget high responsiveness to those effectors.

The pyruvate dehydrogenase complex (PDC)1 catalyzes the irreversible conversion of pyruvate to acetyl-CoA and NADH with the departure of CO₂. The inactivation of PDC by phosphorylation (1) limits the commitment of glucose-connected fuels to undergoing complete oxidation or to being transformed to fatty acids (2). The fractional PDC activity is set by the competing steady state activities of two classes of dedicated enzymes, the pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP). These highly regulated enzymes catalyze the phosphorylation and dephosphorylation, respectively, of the pyruvate dehydrogenase (E1) component of PDC (1–5).

PDC is regulated by at least four related PDK isoforms (4–7) and two PDP isoforms, with related catalytic subunits (8). The PDK isozymes and the related branched chain dehydrogenase kinase (9) form a unique family of serine kinases. They are distantly related to bacterial histidine kinases, sharing five conserved motifs (4–6). The bacterial kinases form a stable histidine-phosphate intermediate and generally transfer this phosphate to an aspartic acid side chain (10, 11). The mitochondrial kinases phosphorylate serine substrates without forming a stable histidine phosphate; indeed, slow (relative to kinase turnover) autophosphorylation of a serine and no autophosphorylation of the conserved histidine was found with the branched chain kinase (12).

The availability of the individual PDK isoforms allows determination of their unique functional and regulatory properties, a step toward understanding how the required tissue-specific regulation of PDC activity is achieved. Kinase-catalyzed inactivation of PDC plays a key role in limiting glucose oxidation when more abundant fatty acids are used to provide oxidative energy (2). This routinely occurs in many tissues but is particularly important during starvation (2, 13–18), when limited glucose must be conserved for glucose-utilizing tissues such as brain. PDC is similarly down-regulated due to high PDK activity in the diabetic state (2, 15–22). In both cases, this occurs with PDK overexpression (1, 14–22). When fatty acid oxidation is not used by a tissue or when glucose is being converted to fatty acids, the activity of PDC must be regulated very differently. Consequently, PDC limits the nearly exclusive use of glucose as an oxidative energy source in neural tissues and facilitates the conversion of glucose to fatty acids in adipose tissue when there is surplus glucose. Thus, it seems likely that different PDK (and PDP) isoforms have developed to meet the distinct tissue-specific and metabolic state-specific requirements for proper tuning of PDC activity. Variation in the distribution of specific mRNAs for the PDK isoforms supports this conclusion (22–24).

The organization of PDC plays an important role in supporting PDC activity and in the regulation of PDC by PDKs and PDPs. The core structure of PDC is formed by association of 60 dihydrolipoyl acetyltransferase (E2) subunits. E2 is a segmented protein with four domains connected by mobile linker
regions (25, 26). Twenty trimers of the C-terminal inner domain of E2 assemble at the vertices of dodecahedral; these trimers catalyze the transacylation reaction (27, 28). Via linker regions, this inner core domain is first connected to an E1-binding domain and then two lipote-bearing domains, an inner domain (L2) and an N-terminal domain (L1). The dihydrolipoyl dehydrogenase-binding proteins (E3BP) has a similar segmented structure (29, 30); about 12 E3BP associates via its C-terminal domain with the inner core of E2 (29–32). E3BP then contains an E3-binding domain and a lipoyl domain (designated L3) set off by linker regions. The assembled E2-E3BP are estimated to bind 6–12 E3 and 20–30 E1 αββ tetramers (3). Bovine PDK and PDP activities have also been shown to be markedly enhanced via binding to this central E2-E3BP core structure via the lipoyl domain regions of E2 (30, 34, 35). By using recombinant constructs of the L1 and L2 domains of E2, unspecified isoforms of bovine PDK were shown to bind preferentially to the L2 domain via an interaction that requires the lipoyl prosthetic group (36, 37). Here we begin the characterization of purified human PDKs and evaluate the functional activation and the changes in regulatory properties of two kinase isoforms, PDK2 and PDK3, as a result of their specific interactions with the lipoyl domains of human E2 and human E3BP. Our ability to produce individual lipoyl domains and E2–60-mer structures with or without E3BP allow us to sort the direct effects of individual lipoyl domains and unique contributions of the assembled complexes to activated PDK function and regulation.

Acetyl-CoA and NADH, common products of the PDC reaction and the catabolism of fatty acids, stimulate bovine PDK activity resulting in the feed-back throttling down of PDC activity (2, 38, 39). Increases in the NADH/NAD+ and acetyl-CoA/CoA ratio stimulate PDK activities by increasing the proportion of reduced and acetylated lipoyl groups on the lipoyl domain of E2 (39–43). Pyruvate and ADP, serving as signals indicating abundant substrate and low energy, act synergistically to inhibit PDK activity by direct binding to PDKs (44, 45). Evidence has been presented that sensitivity to these effectors varies with the particular PDK isoform (23). By using an all human system, we establish that marked changes in catalytic efficiency and effector responsiveness of human PDK2 and PDK3 occur as a consequence of their association with E2.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human E2, K46AE2, and K173AE2 were prepared as described previously (37, 46). Recombinant human E2–E3BP was prepared as described elsewhere (37, 46). Recombinant human E2–K46AE2 was produced as described previously (47). The acetyltransferase-catalyzing outer dodecahedral core of E2, E2α, was prepared by tryptic removal of the exterior E1 binding and lipoyl domain region from bovine kidney E2, followed by pelleting the E2 through sucrose layers (42). Homogeneous lipoyl domain constructs were prepared in a fully lipoylated state either as free domains or fused to glutathione S-transferase (GST) as described elsewhere (49) by log phase expression in Escherichia coli BL21 that prevented the modifications of the lipoyl domains introduced by E. coli JM109 (33). The lipoyl domain constructs of E2 used include the following: outer lipoyl domain, L1; inner lipoyl domain, L2; bilipoyl structure, L1-L2; and the lipoyl domain of E3BP, designated L3. The specific design for constructing, expressing, and preparing purified GST-L3 and, from this, preparing free L3 (residues 1–98) will be described elsewhere.2 Porcine heart E2 and thrombin were purchased from Sigma, [3-3H]ATP from NEN Life Science Products, and TALON affinity purification columns from CLONTECH. From E3 expression system was provided by M. Patel, and human E3 was prepared as described previously (50). PreScission protease is a product of American sham Pharmacia Biotech; BL21(DE3) E. coli and pET28a vector were obtained from Novagen. GroEL/ESL plasmid was kindly provided by Dr. Anthony Gatenby at DuPont (51). Synthetic DNA was obtained from Integrated DNA Technologies, Inc., Coralville, IA.

Expression—Vector Construction—The domains harboring the human PDK2 and PDK3 cDNA inserts were a kind gift from Kirill M. Popov and Robert A. Harris (6). For PDK2, PCR was performed with Pfu DNA polymerase (Stratagene) using primers that produced a 1.2-kilobase pair product encoding mature PDK2 with a 5′ NehI site and a 3′ HindIII site introduced. The product was ligated into pET28a vector at the NehI and HindIII sites using T4 DNA ligase (New England Biolabs; standard procedure) (52). The resulting plasmid was co-transformed along with a plasmid encoding GroEL and GroES into E. coli BL21(DE3) made competent by the method of Inoue et al. (53). Selection for the presence of both plasmids was done on LB plates containing 50 μg/ml kanamycin and 50 μg/ml chloramphenicol. The pET28a vector provides a start codon and encodes an N-terminal polyhistidine sequence followed by a sequence encoding a thrombin cleavage site. Along with this construct, which provided high quality PDK2, a second expression vector was constructed in which the thrombin cut site coding region was replaced by a region coding for the PreScission protease cleavage site. This was made by removing DNA from the plasmid between NeoI and Ndel sites and replacing it with a synthesized DNA segment encoding to a cut site specific for human rhinovirus 3C protease (54).

For construction of PDK3 expression plasmid, a 270-base pair PCR fragment was generated using primers that matched the 5′ SacI site at the beginning of the mature sequence and a 3′ BamHI site matching the unique internal BamHI site in PDK3 cDNA. This PCR-amplified region of PDK3 was then recovered and used as a template for a second round of PCR that changed the 5′ restriction site to Nhel and maintained the 3′ BamHI site. After restriction treatment, this was ligated with the remaining PDK3 coding region produced by digestion with BamHI and XhoI and pET28a plasmid opened from Nhel to XhoI sites. This was followed by transformation and subsequent re-engineering, as described for PDK2, to produce two expression vectors, one encoding a thrombin cut site and the other a PreScission protease site, with each expressing N-terminal polyhistidine tags. All constructs were confirmed by DNA sequencing performed by the Automated Sequencing Facility at Kansas State University.

Expression and Purification of Recombinant PDK2 and PDK3—PDK plasmid-containing bacteria were grown at 37 °C to mid-log phase (A600 =0.6) in LB media containing 50 μg/ml kanamycin and 50 μg/ml chloramphenicol. Then expression was induced with 0.5 mg/ml isopropyl-β-D-thiogalactoside at 24 °C for 16 h. Bacteria were harvested by centrifugation at 4,000 × g for 20 min at 4 °C and frozen at −80 °C. Following thawing, bacterial pellets were resuspended to 10% (w/v) in 0.1% Pluronic-F68 plus 25 mM imidazole. PDK was then eluted with buffer containing 100 mM NaCl, 1 mM ethylene glycol. Ice water-cooled suspensions were sonicated by six repetitions of 50% pulseing at 250 watts for 30 s followed by at least 1 min of cooling. Supernatants were cleared by centrifugation at 10,000 × g for 20 min at 4 °C and 0.1% Pluronic-F68 was added to the supernatant to a level of 0.1% (w/v). 1 ml of equilibrated TALON resin was added per 50 ml of supernatant, and this suspension was gently mixed for 60 min at 4 °C. The mixture was transferred to a column, and the gel resin was washed first with 4 column volumes of H buffer + 0.1% Pluronic-F68 + 20 mM imidazole and then with 3 column volumes of H buffer containing 0.1% Pluronic-F68 plus 25 mM imidazole. PDK was then eluted with buffer containing 100 mM imidazole. PDK containing fractions were pooled, and 1 ml dithiothreitol, 1 ml EDTA, and either 50 or 50 μg/ml chloramphenicol was added for a 1- or a 3-h incubation on ice, respectively. Following thrombin digestion, EGTA was added to 2 mM and glycerol was then added to 20%. Following PreScission protease digestion, protease was removed by passing the mixture through a column with 0.25 ml of G-50 Sephadose equilibrated with HG buffer (16 mM Hepes-Na, pH 8.0, 0.5 mM EGTA, 0.1% Pluronic-F68, 1% ethylene glycol, 20% glycerol). PDK preparations were desalted on a Sephadex G-25 column equilibrated with HG buffer for PDK2 and HG buffer containing 0.15% NaCl for PDK3. PDK preparations were stored unfrozen at −20 °C. Final PDK recovery was typically 5–10 mg per liter of bacterial growth media for PDK2 and 1–4 mg per liter of media with 25% culture supernatant. Water purity was obtained using the Co2+-containing Talon system with nickel-affinity columns. PDKs were stored for over 9 months in an unfrozen state at −20 °C with <30% loss of activity. However, aged preparations of PDK3 needed to be preincubated with E2 at 4 °C for 60 min to exhibit maximal activity. PDK2 could be concentrated to >10 mg/ml, whereas PDK3 had to be maintained at <0.3 mg/ml to avoid
development of insoluble aggregates.

Kinase Activity Assays—PDK activity was measured in duplicate as the initial rate of incorporation of [32P]phosphate into E1 (42, 46) using 0.1 mM [γ-32P]ATP (150–500 cpm/μmol) at 30 °C, unless otherwise stated. For comparative purposes with assays of prior kinase preparations, kinase activities were evaluated in 60 mM Tris-Hepes, pH 7.3, with no inorganic ions and in the three buffer formulations used with purified PDKs as follows: MOPS-K+ buffer, 50 mM MOPS-K+, pH 7.3, 20 mM KPO4, pH 7.3, 60 mM KCl, 0.4 mM EDTA, 2 mM MgCl2 (42, 46); phosphate buffer, 20 mM KPO4, pH 7.0, 2 mM MgCl2, 0.2 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 0.1% Pluronic F-68 (55); Tris buffer, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 50 mM β-mercaptoethanol (23). Subsequently, the MOPS-K+ buffer system was used as described previously (42, 46). With the standard levels of kinase and E1, elevated (E2-activated and stimulated) kinase initial rates were nearly always within 20% of control values from repetitions of the same experiment.

For studies of pyruvate inhibition of PDK activities in the presence of E2, human E1 was prepared completely free of thiamine pyrophosphate by reconstitution assays using high levels of E1 and excess levels of E2-detagging followed by removal of the GST-fused human rhino- virus 3C protease.

Activity of PDK Isoforms—In Tris-Hepes buffer, which lack inorganic ions, PDK2 and PDK3 had specific activities of 690 ± 7 and 437 ± 4 nmol-min⁻¹·mg⁻¹, respectively, at 30 °C using 0.1 mM ATP, 0.23 mM human E2–60-mer, and 28 E1 tetramers per E2–60-mer. The specific activity of PDK2 preparation in the presence of E2 in Tris at 37 °C was 4-fold higher than reported for rat PDK2 (23). The standard kinase assay buffers (23, 36, 55) differ greatly in their ion content ("Experimental Procedures"). The patterns for variation kinase activities with the assay buffer including variations in the extent of E2 activation were notably different for PDK2 and PDK3 (Table I). In all cases, E2 markedly increased PDK2 activity. PDK2 had the highest activity at 30 °C in the phosphate buffer system both in the absence of E2 (44 nmol-min⁻¹·mg⁻¹) and in the presence of E2 (184 nmol-min⁻¹·mg⁻¹). However, in the phosphate buffer system, E2 facilitated only a 2-fold increase in PDK2 activity, whereas a 10-fold increase in activity was observed in both the MOPS-K+ and Tris buffers (Table I). When PDK2 activity is measured with a fresh E2 that contains reduced lipoyl groups, specific activities in MOPS-K+ can rise to 130–150 nmol-min⁻¹·mg⁻¹ at 30 °C (e.g. control values Figs. 4A and 5A). The substantial decrease in PDK2 specific activity from the Tris-Hepes buffer (690 nmol-min⁻¹·mg⁻¹) versus the MOPS-K+ buffer (140 nmol-min⁻¹·mg⁻¹) for the same preparation) is primarily due to inhibition by anions. The marked influences of ions in altering PDK2 effector responses will be described elsewhere.

PDK3 activity at 30 °C was also highest in the absence of E2 in the phosphate buffer system at 40 nmol-min⁻¹·mg⁻¹ PDK3, yet in the presence of E2 the highest activity, 308 nmol-min⁻¹·mg⁻¹, was obtained in the Tris buffer (Table I). As with PDK2, E2 caused only a 4-fold increase in PDK3 activity in the phosphate buffer yet facilitated 17- and an 11-fold increases in the MOPS-K+ and Tris buffers, respectively. Phosphate lowers PDK3 activity observed in the phosphate buffer and contributes to the even lower activity in the MOPS buffer. Phosphate reduced PDK3 activity both by direct inhibition and by elevating the Km value of PDK3 for ATP (below). In Tris-Hepes, 5, 10, and 15 mM phosphate anion (Tris as counter ion) lowered PDK3 activity by 10, 16, and 29% and by 15, 33, and 49% in the presence of 0.1 mM ADP (ADP, alone, reduced PDK3 activity by 39%). Thus, ADP enhanced the inhibition of PDK3 by P1.

Table I reports maximal specific activities and apparent Km values for PDK2 and PDK3 obtained at 30 °C in the presence of 6.4 μM E2 and 24 μg of E1 with ATP varied from 0.05 to 0.3 mM. With the MOPS-K+ buffer system, the apparent Km value of

[3] The use of commercially available thiamin preparations to remove thiamin from the PDKs caused problems in subsequent studies analyzing the GST-lipoyl domains. (J. C. Baker, X. Yan, and T. E. Roche, unpublished observations.). Even the presence of thiamin inhibitors (a mixture of EGTA, aprotonin, leupeptin, benzamidine, and 4-(2-aminoethyl)-benzenesulfonyl fluoride), lipoyl domains were released from GST. Apparently, a contaminating protease in the thimbin preparations utilized the thimbin site between GST and L2.

[4] X. Yan and T. E. Roche, manuscript in preparation.
TABLE II

| Buffer system | PDK2a | PDK2b |
|---------------|-------|-------|
| K<sub>a</sub>(app) | V<sub>max</sub>(app) | K<sub>a</sub>(app) | V<sub>max</sub>(app) |
| Phosphate     | 40    | 243   | 29    | 232   |
| Tris          | 23    | 152   | 6.3   | 505   |
| MOPS-K<sup>-</sup> | 5.7   | 93    | 12.5  | 179   |

<sup>a</sup> With different PDK2 preparations tested in the MOPS-K<sup>-</sup> buffer system, specific activities have ranged from 85 to 156 nmol/min·mg. Higher activities were in part due to reduced lipoyl groups in the E2 source used for activation (e.g. Fig. 4A and Fig. 5A, control in the absence of E3).

<sup>b</sup> As PDK3 preparations were stored, it was increasingly important to preincubate PDK3 with E2 for at least 60 min at 4 °C prior to assays to maintain the specific activity (~85% retained after several months). The range of specific activities of different PDK3 preparations measured in the MOPS buffer system was from 161 to 191 nmol/min·mg⁻¹.

PDK2 for ATP is substantially lower, 5.7 μM, than with the other buffer systems, and the apparent V<sub>max</sub> of 93.1 nmol/min/mg is also reduced. However, the apparent K<sub>a</sub> values of PDK3 for ATP in Tris buffer system, 6.3 μM, and this buffer also gave the highest apparent V<sub>max</sub> of 505 nmol/min/mg of PDK3. With more than double the maximum velocity and a lower K<sub>a</sub>, the catalytic efficiency of PDK3 is estimated to be at least 5.5-fold higher in Tris buffer than in the other buffer systems. At 37 °C, the maximum velocity of PDK3 in this buffer exceeded 800 nmol/min/mg. The apparent K<sub>a</sub> values of PDK2 and PDK3 for ATP were elevated in the phosphate buffer system to 40 and 29 μM, respectively. Although providing a lower specific activity for each PDK, the MOPS-K<sup>-</sup> buffer system was used in further characterizing these kinases based on the trend with bovine PDKS in which elevated K<sub>a</sub> and phosphate levels enhanced the regulatory responses with known effectors (38, 40, 45, 56).

**L2 Activation**—Bovine kidney PDKS have been shown to bind primarily to the L2 domain of E2 (36, 37). Thus, we first tested the effect of variation of the level of free L2 on PDK2 and PDK3 activity. PDK2 activity was not activated by L2 and was inhibited <30% by high levels (30–60 μM) L2. Dimeric GST-L2 modestly activated PDK2 activity with a maximum increase obtained with 16–19 μM GST-L2 subunit (8–9.5 μM GST dimer) followed by a decrease in PDK2 activity at higher GST-L2 levels until inhibition rather than activation was observed with >75 μM GST-L2 subunit. In marked contrast, PDK3 was profoundly activated by L2. Fig. 1 shows the effects of variation in the level of the L2 domain on PDK3 activity. PDK3 was half-maximally activated by 3.8 μM L2 and approached maximal activation (8.9-fold) with 15 μM L2. There was a sharp transition to maximal PDK3 activity (109 nmol/min·mg⁻¹) at the higher L2 levels.

**Comparison of PDK Activations by Different Lipoyl Domain Sources**—Based on these trends, the effects of various free lipoyl domain constructs were evaluated at 15 μM lipoyl domain, and their effects were compared with the activation of PDK2 and PDK3 by E2 and E2-E3BP that were added at 6.4 μM E2 subunit level (~0.1 μM E2–60-mer). Lipoyl domain structures included the outer (L1) and inner (L2) lipoyl domains of E2, the bilipoyl construct (L1-L2), lipoyl domain of E3BP, L3,
E2-bound PDK2 may enhance the sensitivity to DCA inhibition, although an alternative explanation is considered under “Discussion.”

In marked contrast to PDK2, DCA inhibition of PDK3 was not enhanced by E2 with 1 mM DCA repeatedly giving 13% inhibition but greater inhibition by higher levels of DCA. At 0.2 mM, ADP alone gave 50–60% inhibition of PDK3 in the presence (Fig. 4B) or absence of E2 and the combination of ADP and DCA reduced PDK3 activity by ~70%. Because of the lack of sensitivity to DCA (and pyruvate, below), the response of PDK3 to alternative effectors was tested. Carnitine, acetylcarnitine, malate, spermine, and calcium had no effect on PDK3 activity in the presence of E2 (data not shown). Binding of a saturating level of TPP (0.2 mM) to the E1 substrate reduced free PDK3 activity by 30%, but the activities of L2- or E2-activated PDK3 were reduced 10% by addition of 0.2 mM TPP to E1 lacking TPP (below).

Pyruvate Inhibition—DCA is normally used in regulatory studies since, unlike pyruvate, it cannot serve as a substrate in E1-catalyzed reductive acetylation of lipoyl groups that stimulate kinase activity (40). To evaluate pyruvate inhibition in the presence of E2, human E1 was prepared completely free of TPP (see “Experimental Procedures”). Fig. 4A shows that, as with DCA, PDK3 was not significantly inhibited by pyruvate in the presence of E2 with or without ADP. Pyruvate inhibition was also very weak in the absence of E2 (data not shown). In contrast, PDK2 was markedly inhibited by pyruvate in the presence of E2; indeed, 0.1 or 0.3 mM pyruvate was as effective, respectively, as 0.3 or 1.0 mM DCA (Fig. 4A).

Pyruvate was a somewhat more effective inhibitor than DCA in the absence of E2, but E2 also greatly enhanced pyruvate inhibition. Unlike E2, GST-L2 at 15 μM did not enhance pyruvate or DCA inhibition of PDK2.

Stimulation of PDK2 and PDK3 by NADH and Acetyl-CoA—As indicated in the Introduction, stimulation by NADH and acetyl-CoA are mediated by reduction and acetylation of lipoyl prosthetic groups. No stimulation of PDK2 was observed in the absence of TPP (Fig. 4A). Fig. 5 shows the stimulation of PDK2 and PDK3 activities after being preincubated with E2 and E3 and also presents results when PDK2 was preincubated with E2 without E3. In the presence of E3, NADH stimulated PDK2 activity by 1.6-fold and the combi-
tion of NADH and acetyl-CoA stimulated PDK2 by more than 3.5-fold. Inclusion of ADP greatly inhibited PDK2 activity but did not change the fold stimulation by the combination of products as was found with bovine PDC kinases (57) (see “Discussion”). E3 caused a decrease in E2-activated PDK2 activity; simultaneously, there was a time-dependent (probably oxygen aided) decrease in reduced lipoyl groups on the E2–60-mer. This was observed as a decrease in the capacity to acetylate sites with [1-14C]acetyl-CoA in the absence of added NADH; this decreased from nearly 30% of lipoyl domains of E2 after preincubation with E1 for 120 min to <10% of the lipoyl domains for E2–60-mer incubated with E1 and E3. Thiol exposure leads to the reduced lipoyl groups on E2. (Recombinant E2 was purified until the last step in the presence of 10 mM β-mercaptoethanol; the preincubation of concentrated E1 and E2 for 120 min was in the presence of 0.8 mM DTT.) The addition of acetyl-CoA in the absence of E3 or NADH (added in all cases at a ratio of 3:1 with NADH) gave appreciable stimulation of PDK2 by acetylation these reduced lipoyl groups. In the absence of E3, acetyl-CoA stimulation was not further enhanced by inclusion of NADH (Fig. 5, left panel). Our studies (below) with free lipoyl domains definitively support the requirement that NADH reduce lipoyl groups via the E3 reaction to stimulate kinase activity (42, 43); however, the lack of elevation of PDK2 activity by NADH (Fig. 5) in the presence of E3 beyond that achieved with E2 in the absence of E3 indicates that reduction of 30–35% of the lipoyl groups of E2 is enough to maximize the increase in PDK2 activity due to lipoate reduction.

In marked contrast, PDK3 did not significantly respond to the addition of NADH and acetyl-CoA in the presence of E2 (Fig. 5, right panel). Therefore, the high PDK3 specific activity in the presence of E2 was not further increased by reduction and acetylation of lipoyl groups. This result was somewhat unexpected from previous work on resolved bovine PDC but agrees with a previous study on PDK3 in the Tris buffer system (23). Interestingly, the addition of acetyl-CoA alone, provided a small enhancement of PDK3 activity to yield the highest activity. This occurred in conjunction with acetylation of the low proportion of available reduced lipoyl groups of E2 (not estimated in this separate experiment from that with PDK2).

**Product Stimulation of PDK2 and PDK3 Facilitated by Free Lipoyl Domain Sources**—In agreement with previous studies (38–43), in the absence of a lipoyl-bearing domain source, PDK2 and PDK3 activities were not enhanced even in the presence of E3 and E2 (acetyltransferase-catalyzing inner core of E2, lacking the bilipoyl domain and E1 binding domain). Similarly, in the absence of E3, NADH failed to stimulate with all free lipoyl domain sources, and without inclusion of E2, acetyl-CoA had no effect on PDK2 and PDK3 activities (data not shown). Table IV shows the capacities of individual lipoyl domains to mediate NADH and acetyl-CoA stimulation of PDK2 and PDK3 activities. With free lipoyl domain sources, only the dimeric GST-L2 mediated a significant (2.4-fold) stimulation of PDK2. Free L2 supported a 34% increase, and L1 was ineffective; however, dimeric GST-L1 facilitated a small (but reproducible) stimulation of PDK2 (24%). These results indicate that interaction of PDK2 with a lipoyl domain source with the potential for bifunctional (GST-held) or multivalent (E2) binding aids stimulation of PDK2 by reductive acetylation (cf. “Discussion”).

Based on results with E2, which failed to support PDK3 stimulation, the responses of PDK3 with various free lipoyl domain constructs were unexpected (Table IV). L1 facilitated a substantial stimulation by NADH and acetyl-CoA, and the dimeric GST-L1 supported a 6.9-fold stimulation of PDK3 upon being reductively acetylated by reaction of these products. With L2-containing structures, higher absolute PDK3 activities were obtained, but the fractional stimulation of PDK3 was lower than that obtained with L1-containing structures. Results with the bilipoyl domain L1-L2 structure indicate that PDK3 preferentially binds L2. However, L1 gains in its capacity to enhance PDK3 activity upon being reductively acetylated. Overall, our results suggest that PDK3 activity reaches a maximum in the presence of E2 such that reduction and acetylation of lipoyl groups cannot further increase the limiting catalytic step rate (see “Discussion”). Below, we evaluate the related prospect that inhibition of E2-activated PDK3 allows product stimulation of PDK3 activity to be observed, and we further evaluate the roles of L1 and L2 in E2–60-mers when only one lipoyl domain can undergo reductive acetylation.

**Effects of Mutant E2 Structures on PDK Regulation**—Since lipoylation is required for PDK binding to E2, we evaluated PDK function and regulation with E2 mutant structures that have amino acid substitutions for the Lys that undergoes lipoylation, Lys-46 in L1 and Lys-173 in L2 (37). With K46A-E2 and K173A-E2, the capacity of lipoylated L2 or L1, respectively, to support activated function and product stimulation was determined in the presence and absence of inhibitors (ADP + DCA). As shown in Fig. 6, left panel, when only the L2 was lipoylated (K46A-E2) or with native E2, PDK2 underwent ~4-fold stimulation by NADH + acetyl-CoA. Strong inhibition was retained by DCA/ADP (~80%) in the absence or presence

![Table IV](image)

**Table IV**

| Lipoyl domain construct | PDK2 | PDK3 |
|-------------------------|------|------|
| No effector             | NADH + acetyl-CoA | No effector | NADH + acetyl-CoA |
| L1                      | 5.2 (96) | 6.3 | 7.0 (111) |
| L1-L2                   | 5.2 (100) | 6.2 | 16.2 (261) |
| GST-L1                  | 7.9 (124) | 13.9 | 95 (683) |
| GST-L2                  | 7.7 (134) | 68 | 101 (148) |
| L1-L2                   | 15.0 (236) | 80 | 107 (134) |
| L1-L2                   | 6.3 (131) | 71 | 94 (132) |

* Numbers in parentheses are percent of the control activity.
We have characterized two PDKs using an all human component system in which the PDKs, the E1, and all E2 constructs were purified with affinity tags removed, thereby leaving only six or fewer non-native amino acids on their N termini. Our preparations have high specific activities compared with prior preparations of purified PDKs (23, 55). PDK2 and PDK3 specific activities varied with standard buffer conditions as did the magnitudes by which E2 enhanced the kinase activities. We have characterized effector regulation of the PDK activities in a MOPS-K⁺ buffer that contains ions that lower activity, but these ions were required for full expression of the regulatory responses of bovine PDK (38, 40, 45, 56). Under the same conditions, our human PDK2 had a specific activity at least 4-fold higher than rat PDK2 (23). Their use of kinase-depleted porcine heart PDC as a source of E2 and E1 supported very high human PDK3 activity (23).

Our focus has been on how E2 via its lipoyl domains facilitates the largest changes in kinase activity. We have dissected the roles of individual lipoyl domains and gained insights into the importance of having the lipoyl domains of E2 housed in an E2–60-mer structure. Besides employing free lipoyl domains as monomers, we tested dimer structures in which the flexibly held domains are fused to opposing corners on one side of the GST dimer; that space should allow the two lipoyl domains to interact with a PDK oligomer. PDK2 and PDK3 behaved quite differently in virtually every response evaluated. In common, E2 greatly enhanced the rates at which PDK2 and PDK3 phosphorylate E1. However, the enhancements appear to result from different mechanisms.

A major contribution to elevation of PDK3 activity is the capacity for the lipoyl domains of E2, most effectively the L2 domain, to facilitate directly a very large increase in PDK3 activity, primarily through increasing the $k_{cat}$ of PDK3. This would seem to require that the L2 domain induces critical structural changes in PDK3 that speeds up or transforms the rate-limiting step(s) in PDK3 catalysis. Although most of the 17-fold activation of PDK3 by E2 might be attributed to this direct activation by binding to the L2 domain, binding of PDK3 to E2 is not the equivalent to binding to L2 in E2-bound PDK3 must have rapid access to many E2-bound E1. In marked contrast, free monomeric lipoyl domains had no direct effect on the catalytic efficiency of PDK3. Dimeric GST-L2 gave a small increase in activity suggesting the importance of bifunctional binding a PDK2 oligomer by L2 domains. In comparison to the free domain, L2 domains in the E2–60-mer may be in a conformation that, in binding PDK2, induces a critical conformational change in PDK2 to thereby enhance kinase activity. However, E2 activation of PDK2 may entirely result from increased productive encounters produced by the restricted orientations of the E2-bound E1 and PDK2 with access being sustained as a dissociative or direct transfer mechanism. Previous studies on bovine PDK(s), which are tightly bound to E2 (58), led to the suggestion that required encounters of a kinase with many tightly bound E1 (59, 60) are facilitated by “hand over hand” transfer on the surface of E2 by either E1 tetramers (61) or by the kinase oligomer (36).

Contrary to the trend found in past studies on bovine kidney and heart PDKs (e.g., Refs. 43 and 62), E2-activated PDK2 was inhibited more potently by pyruvate than by DCA. When even a small portion of E1 retains a bound TPP, pyruvate is used by E1 to acylate the lipoyl groups of E2 and, thereby, stimulate PDK activity (40). This prevents detection of the full potency of pyruvate inhibition through direct binding to a PDK (44). By using TPP-free E1, pyruvate inhibited PDK2 activity to the same extent as 3-fold higher levels of DCA. E2 transformed PDK2 from being poorly to potently inhibited by these inhibitors. Free lipoyl domains failed to enhance the inhibition and, therefore, to support a mechanism in which this gain in sensitivity was elicited by the conformation of PDK2 upon binding to the lipoyl domains of E2 (data not shown). Within the crowded environment at the surface of E2, the PDK2-lipoyl domain interaction may be distinct and support a conformational change in PDK2 that enhances pyruvate binding to PDK2. An alternative mechanism seems likely. PDK2 probably shares the property of bovine PDKs wherein DCA or pyruvate inhibits by binding to the PDK-ADP reaction intermediate and not to

**Human Pyruvate Dehydrogenase Kinase Isoforms 2 and 3**

**DISCUSSION**

We have characterized effector regulation of the PDK activities in a MOPS-K⁺ buffer that contains ions that lower activity, but these ions were required for full expression of the regulatory responses of bovine PDK (38, 40, 45, 56). Under the same conditions, our human PDK2 had a specific activity at least 4-fold higher than rat PDK2 (23). Their use of kinase-depleted porcine heart PDC as a source of E2 and E1 supported very high human PDK3 activity (23).

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free PDK (45). Then, a capacity of an E2–60-mer to remove E1 availability as a limitation for the rate of PDK catalysis could increase the steady state level of PDK-ADP. Such an E2-facilitated transition would then increase pyruvate binding and inhibition. Systematic kinetic and binding studies should allow that mechanism to be evaluated.

In contrast, although binding to L2 had an enormous impact in enhancing catalysis by PDK3, it failed to alter the weak inhibition of PDK3 by DCA or pyruvate. To date, we have found no condition that elevates the inhibition by these ligands including addition of ADP or ions that enhance pyruvate and ADP inhibition of bovine PDKs. The very high activity of PDK3 and lack of pyruvate inhibition suggest a need for alternative inhibitors. E2-activated PDK3 activity was not significantly inhibited by loading saturating TPP on the E1 substrate, and PDK3 was insensitive to known activators of the opposing phosphatase (Ca<sup>2+</sup> or spermere). ADP and phosphate were inhibitors of PDK3 suggesting this kinase would respond to the intramitochondrial phosphate potential (ATP/(ADP + P<sub>i</sub>); however, healthy tissues generally maintain an elevated phosphate potential. There would appear to be a need for an additional means of reducing PDK3 activity.

The extensive studies on bovine kidney PDKs need to be re-evaluated based on properties of specific PDK isoforms. The sequences associated with bovine kidney E2 closely match those for PDK2 (upper band) and PDK3 (lower band). Pyruvate and DCA were found to only partially inhibit the PDKs associated with bovine kidney PDC (45). This partial inhibition is apparently a consequence of differences in sensitivity of two PDKs, one (PDK2) very sensitive to inhibition and the other (PDK3) poorly inhibited by DCA or pyruvate.

As with rat PDK2 (23), PDK2 was effectively stimulated by NADH and acetyl-CoA. We have demonstrated that GST-L2 can support a much stronger stimulation of PDK2 activity (2.4-fold, Table IV) than the L2 monomer (34%). Because GST-L2 is a dimer, this indicates that the multimeric nature of lipoyl domains around the surface of the assembled E2 core contributes to elevating the E2 activity due to reductive acetylation. Stimulation by free lipoyl domains required E3 to use NADH in reducing lipoyl groups and the assembled inner domain of E2 to catalyze the transacetylase reaction as was found in earlier studies (42, 43). Mutation to prevent acetylation of the L1 domain in E2 allowed high stimulation of PDK2; unexpectedly, when only L1 could undergo reductive acetylation (K173A-E2), substantial stimulation of PDK2 was observed in marked contrast to the results with free L1 structures. It seems likely that acetylated L1 is more effective because it is so concentrated at the surface of E2 (>1.0 mg/ml), although alternative explanations (altered L1 structure or a role of nonlipoylated L2) are possible. Within experimental error, the inhibitory effects of DCA plus ADP and the stimulatory effects of products operated independently in affecting PDK2 activity (Fig. 6). Given the high responsiveness of PDK2 to activation by feed-back control and to inhibition by pyruvate and ADP, it is significant that, among the PDK isoforms, PDK2 was found to be the most broadly distributed in animal tissues (23).

With just E2 present, PDK3 was not stimulated by products. However, PDK3 was shown to be effectively stimulated when conditions were used that prevented the full, direct E2 activation of PDK3 by using a free lipoyl domain source, inhibiting E2-activated PDK3 with ADP or preventing lipoylation of the L2 domain of E2 by mutation. With E2–60-mers, independent of these changes, reductive acetylation raised PDK3 activity to nearly the same level. In combination these results indicate that fully E2-activated PDK3 is operating with reaction rate “ceiling” that cannot be elevated by reductive acetylation of the lipoyl group E2s. When activity is reduced, stimulation can occur, but the enhanced rate cannot rise above the ceiling rate. Apparently, E2-activated PDK3 catalysis becomes limited by a step that is not influenced by feed-back stimulation.

With bovine kidney PDC, ADP-inhibited kinase activity underwent a greater fractional stimulation by PDC products (57). Again this can be explained only by invoking the response of the combination of both PDKs. Upon being inhibited by ADP (not shown) or ADP plus DCA (Fig. 6), the fractional stimulation of PDK2 by NADH plus acetyl-CoA is unchanged, but PDK3 undergoes a transition from not being stimulated in the absence of ADP to undergoing stimulation of its ADP inhibited activity. Therefore, the enhanced fractional stimulation of bovine kidney PDC kinase by NADH plus acetyl-CoA may reflect the portion of kinase activity supplied by PDK3 with product stimulation reversing PDK3 inhibition by ADP.

In general, our results support the past trend of studies with the E1-associated bovine PDK in which the L2 domain was found to be the most effective in supporting PDK activity (36, 37, 40). We have detected a greater capacity for the L1 domain to support enhanced function of human PDK2 and PDK3. There may be conditions that enhance kinase-L1 interactions. Inclusion of the third lipoyl domain (L3) by using the assembled E2-E3BP subcomplex only modestly reduced PDK2 and PDK3 activities. GST-L3 inhibited PDK2 and modestly activated PDK3 supporting some interaction with this domain. Further studies will be needed to evaluate the consequences of an ongoing binding competition between the lipoyl domains toward the modulation of kinase activities. Chen et al. (63) presented evidence with nematode PDKs from *Ascaris suum* and *Caenorhabditis elegans* that C-terminal regions (final 84–87 amino acids, respectively) of these PDKs are required for binding to E2, and activity is retained following removal of this region. However, mutation of a highly conserved glycine residue at position 319 of rat PDK2 within this C-terminal region caused more than a 90-fold increase in the K<sub>m</sub> value for ATP (48). Further studies are needed to establish the roles of this region and the basis for binding to the lipoyl domains of E2.

By using an all human system, we have found tremendous differences between PDK2 and PDK3. For the most part, these ensue from marked differences in how binding to the lipoyl domains of E2 alters the catalytic efficiency and the responsiveness to known effector control. The combination of PDK2 and PDK3 activities appears to explain many of the regulatory responses observed with bovine kidney PDC that contains the related isoforms.

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