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Review

Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer

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Abstract. The fraction of pyruvate dehydrogenase complex (PDC) in the active form is reduced by the activities of dedicated PD kinase isozymes (PDK1, PDK2, PDK3 and PDK4). Via binding to the inner lipoyl domain (L2) of the dihydrolipoyl acetyltransferase (E2 60mer), PDK rapidly access their E2-bound PD substrate. The E2-enhanced activity of the widely distributed PDK2 is limited by dissociation of ADP from its C-terminal catalytic domain, and this is further slowed by pyruvate binding to the N-terminal regulatory (R) domain. Via the reverse of the PDC reaction, NADH and acetyl-CoA reductively acetylate lipoyl group of L2, which binds to the R domain and stimulates PDK2 activity by speeding up ADP dissociation. Activation of PDC by synthetic PDK inhibitors binding at the pyruvate or lipoyl binding sites decreased damage during heart ischemia and lowered blood glucose in insulin-resistant animals. PDC activation also triggers apoptosis in cancer cells that selectively convert glucose to lactate.

Keywords. Pyruvate dehydrogenase, pyruvate dehydrogenase kinase, lipoyl domain, glucose, energy metabolism, starvation, diabetes, heart ischemia.

Introduction

In mammals, the conversion of pyruvate to acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex (PDC) results in net reduction of glucose or glucose precursors [1–10]. The latter includes glycogen, lactate, citric acid cycle intermediates, and amino acids that form pyruvate or citric acid cycle intermediates. When carbohydrate stores are reduced, mammalian PDC activity is down-regulated to limit the oxidative utilization of glucose in most nonneural tissues. Up-regulation of PDC allows carbohydrate to be oxidatively used in response to energy demands and is needed for the conversion of surplus dietary carbohydrate to fatty acids.

To satisfy the discrete tissue-specific roles that PDC must meet in the management of fuel consumption and storage, a set of dedicated regulatory enzymes provide highly adaptable control of the fraction of PDC in the active form (PDCa) [1–10]. Four pyruvate dehydrogenase kinase (PDK) isozymes [9–12] and two pyruvate dehydrogenase phosphatase (PDP) isoforms govern the activity state of PDC [13]. In combination these carry out a continuous phosphorylation-dephosphorylation cycle that determines the proportion of the pyruvate dehydrogenase (E1) component that is in the active, nonphosphorylated state. Increasing PDCa by inhibiting PDK activity is a drug target for diabetes [14–16], heart disease [17–22], and more recently cancer [23–25]. This review ad-

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dresses the short-term control of PDKs, stressing the regulatory mechanisms involved with emphasis on the well-characterized PDK2, the differences in regulatory properties of four PDK isoforms, the development and use of synthetic PDK inhibitors, and why inhibition of PDK activity is an important and fruitful target for treatment of the above diseases.

Short-term and long-term mechanisms act to alter the activities and levels of the PDK and PDP to thereby vary PDCa as required in managing use and storage of fuels [1–10]. To limit the loss of glucose carbons, PDC activity is restricted in many tissues when fatty acids or ketone bodies serve as primary fuel sources. Upon the initiation of exercise, the oxidative use of muscle glycogen and blood glucose is linked to increasing PDC activity. Routine heart function involves elevation of the PDC reaction following dietary intake of carbohydrate and diminishing flux through the PDC reaction as carbohydrate stores are depleted. During long-term exercise or short-term fasting, PDC activity is attenuated in skeletal muscle and stringently minimized in heart in conjunction with increased use of fatty acids and ketone bodies. Full oxidation of glucose is normally retained in neural tissues with the PDC reaction serving as a limiting step. As carbohydrate reserves are depleted, the throttling down of the PDC reaction in most tissues serves to conserve residual carbohydrate for support of neural tissue and red blood cells. In liver, depletion of carbohydrate demands that PDC activity be greatly restricted to support gluconeogenesis from three carbon sources and this is also needed to aid processing of lactate through the Cori cycle during anaerobic exercise. During starvation, elevated PDK activity emphatically down-regulates PDC in heart, skeletal muscle, liver and kidney, thereby limiting the depletion of body carbohydrate reserves (below). In cholinergic neurons, acetylcholine is made from acetyl-CoA produced by the PDC reaction [26]. In white and brown adipose tissues, liver and lactating mammary gland, an increase in PDCa follows dietary intake of carbohydrate to support fatty acid biosynthesis and then PDCa is rapidly decreased as carbohydrate becomes limiting [1–9, 27–32]. In adipose tissues, PDC activity is increased by insulin-based up-regulation of PDP activity [33–35]. Therefore, tissue-specific regulation of PDC activity constitutes a crucial means of disposing of excess carbohydrate, meeting short-term energy demands (such as stepped up muscle activity), conserving limited body carbohydrate reserves, and responding to the ongoing dynamic energy demands of neural tissues.

Components and organization of the mammalian PDC

PDK function and regulation are dependent on and influenced by the organization and catalytic function of mammalian PDC [1, 2, 9, 36–38]. Therefore, this background must be succinctly described. The irreversible PDC reaction proceeds via the sequential steps catalyzed by the E1, dihydrolipoyl acetyltransferase (E2), and dihydrolipoyl dehydrogenase (E3) components (Fig. 1). The core of the complex is an integrated structure formed by association of the multi-domain E2 and E3-binding protein (E3BP) (Fig. 2). The domain structures of E2 and E3BP were evident from their sequences [39, 40]. The C-terminal, catalytic domain of E2 forms an inner core 60mer (I domain). In this structure, 20 I-domain trimers assemble at the corners in a dodecahedron. Recent evidence supports the related C-terminal I domain of E3BP being integrated into the inner core [41]. This surprising result is based on analytical ultracentrifugation (AUC) and small angle x-ray scattering studies indicating that E2·E3BP has a smaller mass than the E2 60mer. Based on this and the capacities to bind E1 and E3, a symmetric $E2_{48}{\cdot}E3BP_{12}$ structure (Fig. 2, inner core model on the right) was proposed [41]. E2 and E3BP provide E1-binding and E3-binding domains, B and B' domains, respectively (Fig. 2) [41–44]. There are two N-terminal lipoyl domains in E2, designated L1 and L2, and one in E3BP, designated L3 in Figure 2. All these globular domains in E2 and E3BP are sequentially connected by 20–30 amino acid linker regions rich in Ala and Pro residues (Fig. 2). The somewhat stiff but mobile linker or hinge regions (labeled with H and sequentially numbered from the N terminus) support separation with a capacity for rapid and flexible movement (like a computer mouse cable) [45]. The PDK (PDK2, Fig. 2) and PDP1 also bind to E2 lipoyl domains (see below).

The five-step PDC reaction (Fig. 1) starts with the decarboxylation of pyruvate and ends with the reduction of NADH. In the three intervening reaction steps, intermediates are formed and transferred between component active sites on a lipoyl prosthetic group of the flexibly held lipoyl domains. While up to 48 E1 are bound per E2●E3BP [41], the mammalian complex generally contain 20–30 E1 tetramers ($\alpha_2\beta_2$) [9]. E1 first catalyzes the thiamine diphosphate-dependent decarboxylation of pyruvate and then the reductive acetylation of the lipoyl-lysine prosthetic groups on the lipoyl domains of E2 and E3BP. The E1-catalyzed reductive acetylation reaction requires specific structure in the lipoyl domains [45, 46] and is normally rate limiting in the mammalian complex [47]. The E1 reaction involves alternating use of the

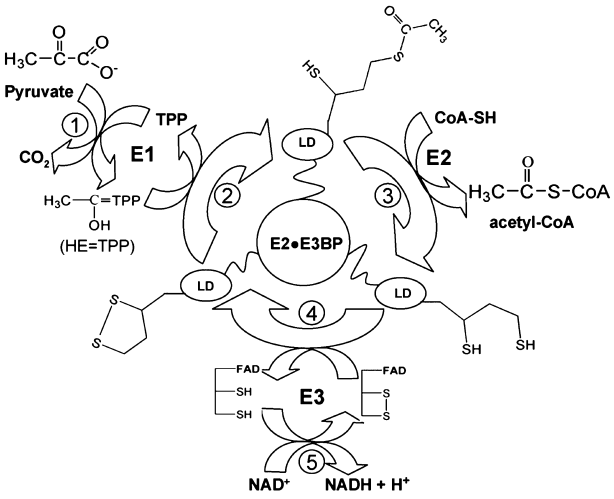

Figure 1. Overall reaction of the pyruvate dehydrogenase complex (PDC). LD is used as an abbreviation for the three lipoyl domains, L1 and L2 of E2 and L3 of E3BP (see domain structures Fig. 2). Only the changes in the reactive ends of the attached lipoyl groups are shown. The reaction sequence is described in the text.

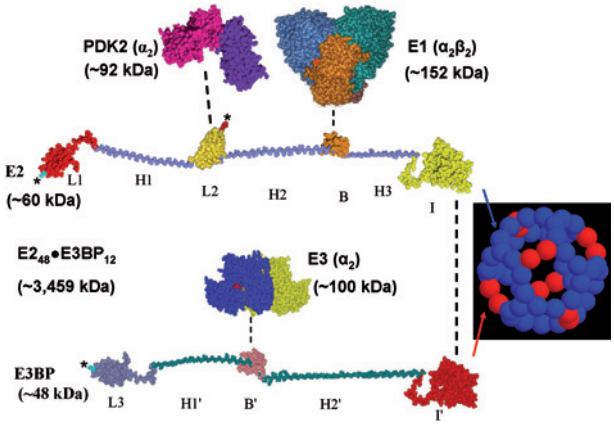

Figure 2. Domain structure and domain interactions of the E2 and E3BP components. The domains and interactions (dashed lines) are as described in the text. The black * is used to indicate position of the lipoyl groups on the lipoyl domains and red * shows the position of the active site of E1, which is at an interface between the α (upper) and β (lower) subunits of E1. All three serines of the α-subunit that undergo phosphorylation are near the E1 active site [48]. Human component subunit masses were used to calculate component masses based on established oligomer states or the proposed E2·E3BP oligomeric state that is based on the inner core model shown on the right [41]. The connecting H-labeled hinge (or linker) regions are shown in extended conformations; these are numbered consecutively from the N-terminal end in E2 and E3BP domain structures, and contain the amino acid sequence for human E2 and E3BP. Globular domains in E2 and E3BP are from various sources; the L1 and L3 domains are based on the L2 domain of human E2 [129]; the L2 domain is a different fold of L2 as found bound to PDK3 [63]; the I domains E2 and E3BP are from *B. stearothermophilus* E2-I domain [45]. Structures of human E1 [48] and human PDK2 [60] are used.

two active sites; recent structural insights contribute explanations for this linked function [48, 49]. Via trimer units of its inner core [50, 51], E2 then catalyzes the transfer of the acetyl group from dihydrolipoyl prosthetic group to CoA. Up to 12 E3 dimers per E2●E3BP are bound [41]; these accept the remaining reducing equivalents via reduction of their thiol-FAD system and then transfer them via FAD to NAD^+. Facile movement of the lipoyl domains between the E1, E2, and E3 active sites is executed by the mobile linker regions of E2 and E3BP [45]. In the E1, E2, and E3 reactions, specific binding of the lipoyl groups occurs in channels formed at the interfaces located between subunits of these components [48–50, 52–54]. The high activities of the rapid and reversible E2 and E3 reactions determine the ratio of the 108 lipoyl groups of one PDC (96/48 E2 subunits, 12/12 BP subunits) that are in the oxidized, reduced, or acetylated forms (oxidized disulfide, 6,8-dithiol, and 8-S-acetyl). Increasing the proportion of the reduced and acetylated forms directly inhibits PDC activity, but with mammalian PDC, small increases that cause little direct inhibition, elicit significant PDC inactivation by stimulating PDK activity [55, 56] (see below).

General properties of PDK and PDP isoforms

PDK isozymes together with the related branched-chain dehydrogenase kinase comprise a novel family of serine kinases, unrelated to cytoplasmic Ser/Thr/Tyr kinases [11, 12, 57–61]. Based on the order in which they were initially cloned, the four PDK isoforms, identified in mammals, are designated PDK1, PDK2, PDK3, and PDK4 [11, 12]. The ~46-kDa PDK subunits have two-domain structures (see below); the C-terminal domain is clearly related to another class of ATP-consuming enzymes [55–59] that broadly includes bacterial histidine kinases. The sequences of the same PDK isozyme in different mammals are highly conserved (>94% identity for human versus rat) [9]. The different subunits of the human isoforms share 65±4% sequence identity, with only short segments at the N terminus not being easily aligned. These sequence differences reflect substantial variation in the functional and regulatory properties of the four PDK isoforms. The PDK are normally dimers [62–64], although effector-induced changes in oligomeric state of a specific isoform have be described [65]. Insights into the structural basis for effector modulation of PDK activity are considered.

The two PDP isoforms (PDP1 and PDP2) have 52-kDa catalytic subunits that are members of the 2C class of protein phosphatases [13, 66]. Besides its catalytic subunit (PDP1c), PDP1 also contains a large (95.6-kDa) regulatory subunit (PDP1r) [67–69]. Both PDP1 and PDP2 activities require Mg^{2+} and are regulated with regard to their responsiveness to this

essential metal [13, 69, 70]. Micromolar Ca^{2+} greatly stimulates the activity of PDP1, which is found in Ca^{2+}-sensitive tissues [71–73]. Polyamines, most effectively spermine, significantly reduce the K_ms for Mg^{2+} of both PDP isoforms [13, 69, 70]. PDP2 is expressed in fat-synthesizing tissues [13] and is probably the primary target by which insulin-predicated regulation enhances PDP activity via a mechanism that, like spermine, leads to a lowering of the K_m for Mg^{2+} [74, 75]. Putative final stage mechanisms whereby insulin regulation enhances PDP activity include allosteric mediators [76, 77] and phosphorylation by PKCδ [78]. However, definitive support for such mechanisms has not been provided. There is strong evidence for multiple pathways in the insulin-induced increase in PDCa [79].

When metabolic regimens (nutritional state, hormone signaling, and tissue-specific fuel preferences) favor oxidation of fatty acids as the primary fuel, reduction of PDCa is critically important for the conservation of carbohydrate reserves [1–9]. The impact of increased use of fatty acids and ketone bodies is registered via increases in the NADH to NAD^+ and acetyl-CoA to CoA ratios, which bring about enhanced PDK-catalyzed inactivation of PDC [80–82]. A sufficient supply of carbohydrate is recognized via adequate pyruvate levels and the need for increasing PDC activity is conveyed by a reduction in phosphate potential (ATP/ADP + P_i). ADP plus pyruvate (enhanced by phosphate, P_i) act synergistically to inhibit the activity of PDK thereby fostering higher PDCa [83, 84]. Hormone-induced or workload-fostered increases in glucose transport or glycogen breakdown coupled to glycolysis elevate pyruvate; increased cellular workload elevates ADP and P_i. Dichloroacetate (DCA) binds PDK at the pyruvate-binding site [83, 85] and unlike pyruvate is not consumed in the E1 and other reactions. Studies with DCA provided both mechanistic insights [64, 83] and some of the first indications of potential medical benefits [14] from inhibiting the activity of PDK. CoA inhibition of PDK activity has also been reported [80, 84].

Control of PDK4 expression

To conserve carbohydrate reserves, the suppression of PDC activity during starvation is bolstered by enhanced expression of PDK4 particularly in heart, skeletal and other muscle tissues, kidney, and liver [7–9, 86–99]; increased expression of PDK2 is also observed in liver [30, 87] and kidney [87, 100]. As a complement to up-regulation of PDK4, PDP2 levels are decreased in heart by starvation [101]. Enhanced PDK4 expression is also fostered by a high fat (low carbohydrate) diet [8, 89, 93], diabetes [86–88], carnitine deficiency [102], extended exercise [103], and hibernation [104]. The very low tissue PDC activities that result from effector control augmented by the greatly enhanced expression of these PDKs, lead to fatty acids being used almost exclusively as an energy source in liver and kidney. This serves to direct available pyruvate (derived from amino acids or circulating lactate) through gluconeogenesis to provide glucose for the brain and other neural tissues. Elevation of insulin in conjunction with re-feeding carbohydrate prevents enhanced expression of PDK4. Under basal conditions PDK4 expression is suppressed in most tissues by maintaining the histones of the PDK4 gene in the nonacetylated state (*i.e.*, by histone deacetylase activity) [94, 96–98]. Via peroxisome proliferator-activated receptor-α (PPARα), WY-14,643 (a potent agonist for PPARα receptor) or fatty acids increase the expression of PDK4 [90, 91]. Activation of PPARδ [92, 95] also enhances PDK4 expression, and PPARγ signaling inhibits expression [95, 105]. These results suggest that increases in free fatty acid levels can directly affect the level of PDK4. Based on studies with PPARα–null mice, the control of PDK4 expression via PPARα is particularly important in liver and kidney but other means of control operate in skeletal and heart muscle [90, 106].

Glucocorticoids increase during starvation or diabetes. PDK4 expression is increased by glucocorticoids, which foster acetylation of histones associated with the PDK4 gene, and thereby enhance production of PDK4 mRNA [96]. This results from binding of retinoic acid-responsive elements to the promoter region of PDK4 gene; these block histone deacetylation and recruit a histone acetyltransferase (such as p300/CBP) [96]. Insulin inhibits PDK4 expression via the phosphatidylinositol 3-kinase pathway by activating protein kinase B, which, in turn, phosphorylates FOXO factors (Forkhead Box O family members) [96–98]. FOXO factors are needed for binding of p300/CBP, and insulin-initiated phosphorylation by protein kinase B results in the FOXO factors leaving the nucleus, thereby blocking expression [96–98, 107, 108]. In diabetic animals, due to the absence of insulin or due to insulin insensitivity, the unimpeded overexpression of PDK4 curtails glucose oxidation (see treatment of diabetes by PDK inhibitors below).

In homozygous PDK4 knockout mice ($PDK4^{-/-}$), 48-h starvation results in more than a twofold decrease in blood glucose as compared to $PDK4^{+/+}$ starved animals [99]. This is compensated in part by an increase in ketone bodies in the blood. Even with no PDK4, effector control by the other PDKs still leads to PDCa levels being greatly reduced due to starvation in heart, skeletal muscle, diaphragm, kidney, and liver.

The largest difference in the percent of PDC in the active form was observed in the diaphragm after 48 h of starvation (10.7±2.4% PDCa for PDK4$^{-/-}$ mice *versus* 1.7±0.2% for PDK4$^{+/+}$ mice) [99]. Independent of nutritional state, total PDC activity was unchanged for each tissue comparing PDK4$^{-/-}$ and PDK4$^{+/+}$ mice. Thus, PDK4 overexpression results in conservation of gluconeogenic substrates during starvation but further contributes to hyperglycemia in diabetes. The maintenance of blood glucose during starvation is substantively perturbed in animals lacking PDK4.

Focus on PDK2

Not all the PDK isoforms undergo the full set of effector responses. PDK2 is the most widely distributed among the four PDK isoforms [87, 109]. PDK2 responds in a sensitive manner to the full set of known regulatory effectors of mammalian PDK [9, 84, 109–111]. With PDK2, there is an advanced understanding of the molecular structure [60, 64], mechanisms of effector regulation [55, 65, 84, 110–113], sites of binding of effectors [64], conformational dynamics [64, 65], interactions with other components [62, 65, 84, 110, 114–116], and effector-induced changes in the oligomeric state of PDK2 [65]. Therefore, the molecular mechanisms involved in PDK2 regulation are described first, and then the comparative properties of the other PDK isoforms are succinctly covered.

In the presence of specific ions and using E2·E1 as a substrate (see below), PDK2 activity is stimulated three- to fourfold by NADH and acetyl-CoA, and is reduced by elevated pyruvate and ADP to <10% residual activity [84, 110, 111]. An important aspect of PDK function is the interaction of PDK with lipoyl domains [9, 37, 38, 62, 65, 84, 110, 114–122]. These interactions and effects on these interactions result in the largest changes in PDK function. Although there is considerable insight into PDK2-lipoyl domain interactions, there are significant and consequential differences in the lipoyl domain interactions of the other PDKs. Furthermore, detailed structural information exists only for human PDK3-lipoyl domain complex [63]. Insights from that structure will also be emphasized.

In the context of the PDK structures and understanding of the mechanism of action of known physiological effectors (and DCA), the properties of tightly binding synthetic PDK inhibitors are considered as appropriate. The the use of DCA and more recently limited use of these tightly binding inhibitors in treatment of diseases is then described along with the potential for further developments in disease treatment.

PDK structure and lipoyl domain binding by PDK

Three-dimensional structures of rat PDK2 (Fig. 2) [60], human PDK2 (Fig. 3) [64] and human PDK3 (Fig. 4) [63] have established the ligand and lipoyl domain binding sites within the PDK. PDK2 is a dimer with each subunit containing two large domains and a C-terminal intersubunit cross arm (Fig. 3a, b). In ribbon (Fig. 3a) and space-filled (Fig. 3b) presentations of the PDK2 dimer structure, the N-terminal, regulatory (R) domains (residues 6–169) are colored red in one subunit and blue in the other subunit. The ATP/ADP-binding C-terminal catalytic (Cat) domains (residues 178–364) and cross arm (residues 365–385) are colored orange or lavender in the different subunits. In the central base of the structure, the Cat domains associate via an interface primarily formed by the offset interaction of equivalent mixed β-sheet regions. Near the center of the base formed by the β-sheet interfaces, the opposed Phe331 side chains of each subunit interact with each other. From this central subunit-association base, the subunits rise in opposed directions in the shape of a large, somewhat skewed trough with the R domains forming the upper sides. An open trough PDK2 structure is exhibited in Fig. 2 (Cat domains at top and R domains below). In some crystal structures of PDK2, an additional dimer interaction is created by C-terminal cross arms spanning this trough with insertion of Trp383 (Fig. 3a) between residues of the Cat and R domain [64]. Opposite from the trough side on the outside of each subunit, the interface between the R and Cat domains forms an extended cavity with the ATP/ADP binding site located in the Cat domain at one end of the active site cavity (Fig. 3). The R domain is the site of binding of regulatory ligands (Fig. 3c) [64].

PDK2 associates with PDC by binding to the L2 domain of the E2 component (Fig. 2) via an interaction that requires both structure of the protein domain and direct binding of the lipoyl group [9, 37, 38, 62, 65, 84, 110, 113–122]. This association and movement between lipoyl domains facilitate a large increase in PDK2 activity by providing enhanced access to the E1 substrate. Indeed, PDK2 uses E1 bound to E2 with at least a 400-fold lower concentration dependence than when PDK2 uses free E1 [84]. Under typical assay conditions (0.4 mg/ml E1, physiological salts), E2 enhances PDK2 activity by 10-fold [110]; only the full outer structure of E2 (L1wL2wB) gave some enhancement of PDK2 activity, apparently by providing E1 [115]. Besides ATP, the PDK2 structures in Figure 3a and b also show the tight-binding inhibitor, Nov3r, which is also included in ribbon structure of the monomer (Fig. 3c) [64]. This inhibitor binds at the site of binding of the lipoyl group

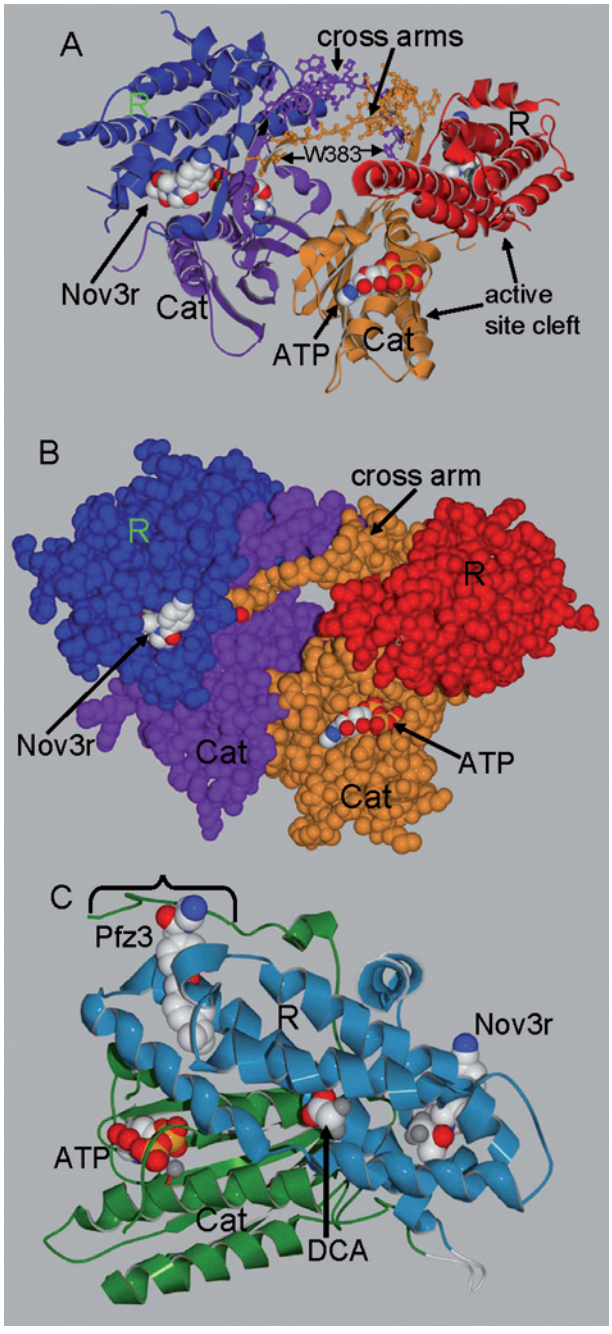

Figure 3. Cross arm in ribbon (*a*) and space-filled (*b*) PDK2 dimer structures and ligand-binding sites in PDK2 monomer (*c*). In (*a*) and (*b*), ATP and Nov3r are present as space-filled structures (CPK colored); the N-terminal R domains of PDK2 are shown in blue and red and the C-terminal Cat domains with following intersubunit cross arms are shown in orange and lavender. In (*a*), the cross arms are shown as stick structures. (*c*) ADP·Mg^{2+} bound to Cat domain (green) and Pfz3, DCA, and Nov3r binding sites [64] in the R domain (dark cyan) are shown. Most of the ribbon backbone is from the PDK2 structure with DCA/ADP bound [64]; the bracket indicates cross arm structure not observed in PDK2 crystal structure with ADP and DCA bound, but which is observed with Nov3r or Pfz3 bound. In (*c*), the interdomain loop is in white (bottom right corner).

of the L2 domain [63, 64]; Nov3r (see structure, Fig. 5, below) and related inhibitors [123–126] prevent binding of PDK2 to L2 or E2. PDK2 bind much tighter to the dimeric GST-L2 structure than the L2 monomer, supporting bifunctional binding of PDK2 to two L2 domains [62]. Reduction of the lipoyl groups of GST-L2 further enhances binding [62, 65]. With 0.1 μM E2 60mer and 30 PDK2 per 60mer, a change from oxidized, to reduced to acetylated increased the number of PDK2 bound from 7 to 12 to 16 per E2 60mer [111]. The reaction state of the lipoyl group alters PDK2 binding to L2 (below). PDK3 is activated 15-fold by E2 [110] and PDK1 by 2.5-fold [127]. In the absence of effectors, the impact of E2 on PDK4 activity is only observed at low levels of E1 because PDK4 has a much lower K_m for free E1 [128].

PDK3 binds the L2 domain very tightly and its activity is directly increased by the isolated L2 domain and more strongly by glutathione-S-transferase-L2 (GST-L2), a dimeric structure that favors bifunctional binding by both L2 domains [110]. In the absence of L2, PDK3 dimers tend to self-associate. By AUC studies, we have found that PDK3 binds to GST-L2 with a binding affinity of <5 nM. With just the L2 domain, a binding affinity of <20 nM for PDK3 binding to two lipoyl domains has been observed by AUC studies; this affinity is tighter than that estimated by other approaches [63, 114, 122]. It was proposed that PDK moves between lipoyl domains on the surface of E2 by a nondissociative, hand-over-hand mechanism [9, 37, 38, 120]. This mechanism would seem to be particularly important in the case of the tightly bound PDK3. Hand-over-hand transfer requires that a PDK dimer binds two L2 domains and forms an intermediate in which the dimer releases one lipoyl domain and then rebinds randomly to another L2 faster than it fully dissociates. Although PDK3 binds the L1 domain with a much weaker affinity than it binds the L2 domain, PDK3 binds the L1 domain with an affinity similar to that of PDK2 binding of the L2 domain [62]. PDK3 binds very tightly to the isolated dilipoyl domain of E2 (L1wL2).

The crystal structure of PDK3·L2 establishes how PDK3 binds two L2 domains (Fig. 4) [63]. The C-terminal, intersubunit cross arms play critical roles in PDK3 binding of the two L2 domains (Fig. 4). Extending beyond the intersubunit cross arm, the ongoing C-terminal tail of PDK3 loops around the lipoyl domain and up alongside the bound lipoyl group with a couple of residues contributing to the lipoyl group binding. A pocket in the trough side of the R domain contributes most of the residues binding the lipoyl prosthetic group (yellow, Fig. 4); as indicated above, this corresponds to the Nov3r binding site in PDK2 (Fig. 3). The R domain residues of this pocket

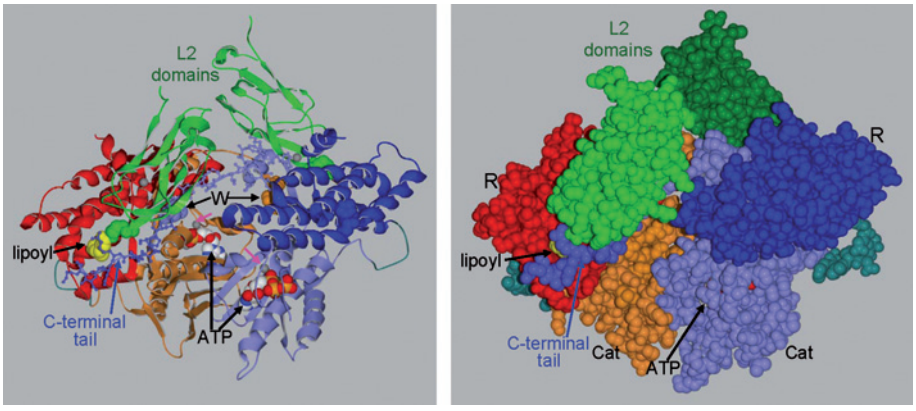

Figure 4. PDK3 dimer binding two L2 domains. Two L2 domains bound to PDK3 [63] are shown in green (with dark green used for one in right panel); the lipoyl group is yellow and shown as space-filled in left panel as is the lysine to which the lipoyl group is attached. In the left panel, the W indicates Trp389 in PDK3, which is equivalent to Trp383 in PDK2, sequence numbering is as described in aligned sequence for the four PDK isoforms in the Supplemental Materials of [64]. The C-terminal tail that follows Trp389 and wraps around L2 domain and along side lipoyl group is shown with a darker lavender color. This structure is a stick structure in the left panel, and in both panels is the left forward structure arising from the Cat domain on the right. Pink arrows point at the K^+ that is chelated in part by an oxygen of the α-phosphate of ATP [63].

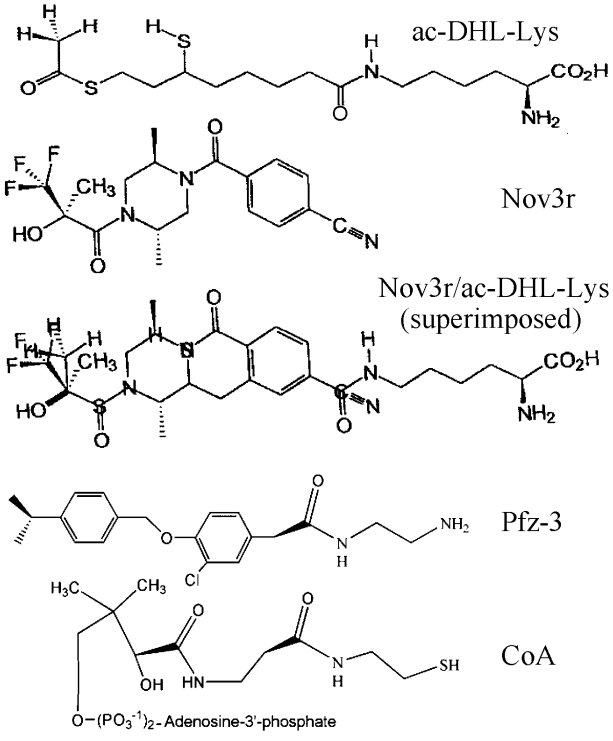

Figure 5. Comparison of Nov3r and acetyl-dihydrolipoyl-lysine structures and CoA and Pfz3 structures.

are highly conserved in the four PDK isoforms [64]. Besides the C-terminal tail and the spanning region of the incoming cross arm, the beginning of the outgoing cross arm (*i.e.*, from the subunit to which the lipoyl group is bound) and a few residues of the R domain contribute to the binding of each L2 domain. The large differences among the PDK isoforms in their affinities for binding to L2 probably reside primarily in these protein-protein interactions. As predicted based on these structures, mutations and deletions of the C-terminal regions of PDK2 or PDK3 interfere with lipoyl domain binding [116, 122].

The folding of the L2 in the PDK3·L2 crystal structure (Fig. 4) differs from that of the L2 structure determined for free L2 by NMR [129] in that the C terminus of the bound L2 is organized as part of the folded L2 domain. In Fig. 2 the structures shown for the L1 and L3 domains are, in fact, from the NMR structure of the free L2 domain. The L2 domain of E2 in Fig. 2 is from the PDK3·L2 crystal structure. Presumably, the L2 fold also associates with PDK2. A need to have the C terminus of L2 interact with the domain was previously indicated by instability of L2 mutated in the C-terminal region [46]. The folding of the C-terminal region of L2 may vary and be stabilized in more than one conformation by different protein-protein interactions.

PDK1 also preferentially binds tightly to the L2 domain and its activity is also directly increased by free lipoyl domain structures (GST-L2 = L1wL2 > L2 > L1 for binding and activation), but binding is somewhat weaker and PDK1 activity is increased less than PDK3 [127]. Although PDK4 activity is minimally affected by E2, PDK4 also binds to lipoyl domains but with a lesser preference for the L2 domain. PDK4 is the most effective among PDK isoforms in using E1 not bound to E2 as a substrate. The C terminus of PDK4 uniquely ends with a hydrophobic sequence (EVAM), and this serves as a hydrophobic signal that reduces recovery upon expression in *E. coli* [193]. Adding hydrophilic groups (GEE) interfered with binding of PDK4 to E2 [128].

Table 1. Properties of PDK2 and comparative properties of PDK1, PDK3 and PDK4 isoforms. All kinetic and binding experiments include 1.5 mM Mg^{2+}; kinetic studies were conducted with 100 mM K^+ and ~10 mM P_i [84, 110, 111, 127, 128]. Values shown for binding studies are in potassium phosphate buffer [62, 65, 84, 111] but ion requirements are from subsequent studies (Hiromasa and Roche, unpublished). Ligand binding is measured by Trp fluorescence quenching and protein binding by AUC studies.

PDK2 properties	ATP binding		ADP binding		Pyruvate binding		Pyruvate binding with ATP		Pyruvate binding with ADP		E2 activation	Stimulation by reductive acetylation	Binding to L2	Binding to GST-L2	Nov3r 50% effect
Constants	K_d	K_m	K_d	K_i	K_i	K_d	K_d	K_d			10 fold	3.5 fold	lipoyl K_d		nM
	μM				μM		μM						μM		IC50 = 6.5
	4	40	15	40	95	590	42	3.5					ox	~150 ~3	
													red	~30 ~0.4	
Effects	ADP is competitive inhibitor vs. ATP.				Uncompetitive inhibition vs ATP; pyruvate binds during catalysis to PDK2·ATP or PDK2·ADP. K_d with free PDK2.		Pyruvate binds tighter to PDK2·ADP than PDK2·ATP. With ADP, pyruvate is a noncompetitive inhibitor *vs.* ATP.				"K_m" for E1 reduced >400 fold	Increases V_{max} but in proportion increases the K_m for ATP	Lipoyl group reduction (red.) enhances binding. With E2, lipoyl acetylation further enhances binding vs. just reduced.		Blocks binding to E2; 8% activity retained with saturating Nov3r
Influence of conditions	K^+ greatly strengthens ATP and ADP binding P_i enhances ADP binding.				K^+ required; in the absence of ADP or ATP, P_i reduces pyruvate binding.		K^+ required for ATP/ADP enhanced pyruvate binding; ADP/pyruvate/P_i binding mutually favored.				E2-bound E1 by E2-bound PDK2	K^+ and anion (P_i or Cl^-) required	K^+ enhances binding to GST-L2 with reduced lipoyl group.		K^+ required
Comparison to PDK2 of:	ADP inhibition		Pyruvate/DCA inhibition		Effects ADP on pyruvate inhibition		Fold effect				Fold effect		L2 affinities	GST-L2 affinities	Effect nM
PDK1	Somewhat weaker		More sensitive to pyruvate, less sensitive to DCA		Enhanced		~2.5				~1.7		~30 fold tighter		IC50 = 30
PDK3	Stronger, P_i-enhanced		Insensitive		Minimal		~10				<1.1 ~1.8 when ADP/P_i-inhibited		1000-fold tighter		IC50 = >400 (L2 dom. of E2 strongly competes)
PDK4	Similar		Similar with DCA		Modest enhancement		>1.2 only at low E2·E1				~2.2		Weaker		AC50 = 15 (to 150%)

These residues align with the first four of the last five residues of the C-terminal tail of PDK3 (YKAKQ); this positively charged, hydrophilic segment at the very end of the C-terminal tail of PDK3 was not resolved in the PDK3·L2 structure [63].

The studies on the four PDK isoforms in our laboratory since 2000 [110] have been performed with all human components (E1, E2, E3BP, E3 and four PDK isoforms) prepared by recombinant expression in *E. coli* with any affinity tags removed from all but E3. Table 1 summarizes many of the properties of human PDK2 and comparative properties of the other three human PDK isoforms based on results from our laboratory, including the relative binding to lipoyl domains and E2 activation described above. The studies by Popov and coworkers (e.g. [59, 60, 109, 112, 114–116]) have used recombinantly expressed rat PDK with human and rat components that were recombinantly expressed.

Stimulation by reductive acetylation of lipoyl groups and related potent inhibitors

As indicated above, fatty acid catabolism increases the NADH/NAD^+ and acetyl-CoA/CoA ratios. Modest increases in these product ratios substantially enhance PDK catalyzed inactivation of PDC, most effectively by PDK2. These ratios are translated by the reversible E3 and E2 reactions interconverting the lipoyl groups between the oxidized, reduced and acetylated forms [9, 37, 38, 55, 111–113, 131–133]. Stimulation of PDK2 activity results from reduction and acetylation of the L2 domains of the E2 oligomer (see Fig. 6, below). *In vitro*, stimulation can also be achieved by E1-catalyzed reductive acetylation but this rate-limiting reaction would ordinarily not be a determining factor for the state of acetylation of lipoyl groups in respiring mitochondria. Stimulation is only observed in buffers, containing elevated K^+ and Cl^- and/or P_i [111, 131]. A mechanistic basis for the K^+ effect is suggested by recent studies (see below). With free lipoyl domains, significant stimulation by NADH and acetyl-CoA is retained with free E1 or a peptide substrate as long as E3 is available to catalyze lipoyl reduction, and the lipoyl domain-free inner core of E2 ($E2_I$) is available to catalyze the acetylation [55, 112]. Just reduction of the lipoyl group of E2 can give a 1.8-fold increase in PDK2 activity; acetylation of E2 stimulates PDK2 activity by up to 4-fold [110]. Using E2 structures with Ala substituted for the Lys residues of L1 and L2 that undergo lipoylation, nearly the same level of stimulation is retained with E2 in which only the L2 domain is lipoylated [110]. With just L1 undergoing reductive acetylation, stimulation of PDK2 is halved, and stimulation is prevented when lipoylation of L1 and L2 is prevented by Ala substitutions. Reductive acetylation of the free L2 monomer gives only a small stimulation of PDK2 activity but substantial stimulation is achieved upon acetylating L2 in the dimeric GST-L2 [110].

With E2 oligomer, maintenance of E2 with all lipoyl

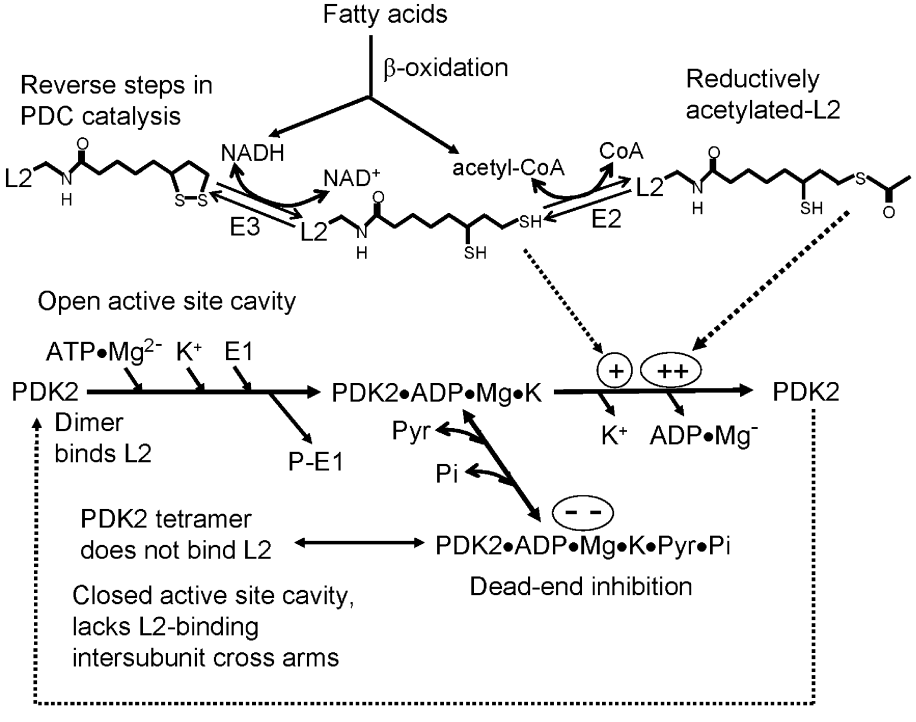

Figure 6. Summary of molecular mechanisms regulating PDK2 activity. At the top, the E3 and E2 reactions are shown acting in the reverse directions to make reduced and reductively acetylated L2 (Pyr, pyruvate). The other effects and transitions are as described in the text.

groups in the oxidized state ($E2_{ox}$) greatly reduces the capacity of E2 to enhance PDK2 activity [111]. With $E2_{ox}$, the fractional increase in activity with reduction of lipoyl groups is much larger, and then acetylation gives only modest further activation; however, the final PDK2-specific activity remains low [111, 113]. The basis for this effect and the lack of full reversibility even with extended incubation of E2 under reducing conditions are not fully understood. Treatment of PDK2 with disulfide reagents also lowers PDK2 activity but this is readily reversed by reducing conditions [111]. Apparently, the capacity to pass limited reducing equivalents among lipoyl groups (by disulfide exchange) maintains E2 lipoyl domains in a conformational state that aids kinase function.

Product stimulation increases V_{max} and the K_m for ATP, whereas with $E2_{ox}$ the reduction in PDK2 activity occurs in association with a greatly reduced K_m for ATP [111]. This trend, in which increases in k_{cat} also increase the K_m for ATP and *vice versa*, fits an ordered mechanism in which ATP binds first and the dissociation of ADP is rate limiting in the absence of stimulatory effectors [84, 111]. ADP^{3-} complexed with Mg^{2+} and K^+ at the PDK2 active site slows ADP dissociation (Fig. 6) [84]. In agreement with this mechanism, the fractional stimulation by reductive acetylation increases with the concentration of ATP until ATP is added at a near saturating level [111]. Significant PDK2 stimulation was also observed using ΔBE2 (E2 60mer lacking the E1 binding domain) [111]. Under conditions of stimulation by reductive acetylation of ΔBE2, binding studies revealed some weakening of the K_d for ATP, but a larger increase in K_d for ADP. In support of stimulation particularly enhancing ADP dissociation from PDK2, kinetic studies (fixed levels of free E1 with ATP varied) found that $K_m \gg K_d$ for ATP. Other results that fit the ordered mechanism and effector control of ADP dissociation are described below.

In the absence of inhibitors, PDK3 activity is not significantly stimulated by reductive acetylation of intact E2, but ADP-inhibited PDK3 activity is stimulated by reductive acetylation, which removes all ADP inhibition [110]. PDK3 activity is also enhanced by acetylation of free lipoyl domain structures. Reductively acetylated GST-L2 supports the highest activity (increases from 80 to 130 $nmol \cdot min^{-1} \cdot mg^{-1}$) whereas reductive acetylation of GST-L1 gives by far the highest fold-increase (from 14 to 95 $nmol \cdot min^{-1} \cdot mg^{-1}$) [110].

High-throughput screening followed by extensive synthetic refinements generated a potent class of PDK inhibitors that are amides of trifluoro-2-hydroxy-2-menthylpropionate [123–127]. Nov3r was the first high-potency inhibitor described in this class [123]. As indicated above, recent studies have established that Nov3r and related inhibitors bind tightly to PDK2 at the site of binding of the lipoyl prosthetic group of the L2 domain [64]. More specifically, based on structural alignment, these inhibitors mimic the acetyl-dihydrolipoyl group (ac-DHL-Lys, Fig. 5). While these inhibitors prevent binding of PDK2 to the L2 domain of E2, they do not fully prevent the residual PDK activity observed in the absence of E2 (*i.e.*, without E2 supporting PDK access to E1). Potent inhibition of PDK2 activity requires physiological K^+ just as stimulation by reductive acetylation of the lipoyl group. Lack of K^+ led to Mann et al. [134] to conclude that Nov3r was not a potent inhibitor of PDK2, but, in fact, PDK2 is the most potently inhibited among the PDK by this class of inhibitors [126, 135, 136].

PDK1 activity was only increased by ~2.5-fold by E2 and consequently its activity was only reduced about 60% by Nov3r [126, 135, 136]. PDK3 is less effectively inhibited by Nov3r than PDK2 because it binds the L2 domain about 100-fold tighter than PDK2 [9]. Surprisingly, PDK4 activity is stimulated by the Nov3r class of inhibitors [126, 135, 136]. At elevated E1 levels, PDK4 is not activated by binding to E2 (it has a much lower K_m for E1) [128], but PDK4 activity is stimulated by reductive acetylation of E2 [109, 113, 128]. As the only significant change among four PDKs in a residues involved in binding Nov3r in PDK2, PDK4 has a leucine replacing a phenylalanine (Phe28 of PDK2). It seems likely this change coupled to the capacity of PDK4 to effectively use of E1 in the absence of E2 explain the marked transition from potent inhibition by Nov3r to stimulation of PDK4. Nov3r probably causes changes at the active site that mimic those induced by a reductively acetylated lipoyl group binding at the same site. Table 1 compares the effects of reductive acetylation and Nov3r of the well-characterized PDK2 and the other PDK isoforms, based on results from our laboratory.

ADP and pyruvate effects on PDK2: inhibition of activity, hindering of L2 binding, and induction of tetramer formation

The potent synergistic inhibition of PDK2 activity, which is by elevated ADP and pyruvate [83, 84], is a good indicator of low energy (ADP) and available PDC substrate (pyruvate). As described in the section below, strong synergistic inhibition requires both K^+ and P_i. Early findings were that pyruvate binds directly to PDKs [137], and DCA is an effective analog to replace pyruvate [85]. By not being a substrate in the E1 reaction, DCA avoids the prob-

lems seen with pyruvate of catalytic depletion and stimulation of PDK activity by being used in the reductive acetylation of lipoyl groups [131]. Pyruvate inhibition can be studied with E1 lacking TPP [84] and pyruvate binding to PDK2 in the absence of E1 and E2 by Trp fluorescence [65].

Fig. 3c shows the location of the binding of DCA along with ADP and Mg^{2+} in the active site [64]. ADP and DCA were crystallized in one structure, whereas the positioning of Nov3r and the other ligand, designated Pfz3, are from other PDK2 crystal structures [64]. DCA is bound to the central part of the R domain. In a space-filled model, bound DCA is almost entirely enveloped in this DCA/pyruvate binding site. The carboxyl group of DCA forms a salt bridge with Arg154; DCA is sandwiched between His115 and Ile157. Replacement in PDK2 of Ile157 with phenylalanine, as found in PDK3, greatly reduces DCA/pyruvate inhibition. This is in accord with the very weak pyruvate/DCA inhibition of PDK3 [109, 110].

In E2-activated PDK2 catalysis in the presence of physiological ions (importance below), kinetic and binding studies support an ordered mechanism with ATP binding first with ATP, in the absence of effectors, dissociating with a rate constant slower than k_{cat} [84]. ADP dissociation limits PDK2 catalysis [84, 111]. The rapid E2-facilitated delivery of PDK2 to E1 is important for ADP dissociation being the rate-limiting step. The ordered mechanism with slow ADP dissociation explains the observed substantially higher K_m than K_d for ATP. Pyruvate and DCA are uncompetitive inhibitors with preferential binding to PDK2·ADP (but also to PDK2·ATP) [84]; this further slows ADP dissociation, which, in part, explains the synergistic inhibition of PDK2 by these effectors (Fig. 6).

Quenching of Trp fluorescence of PDK2 is a particularly effective means of observing binding of adenine nucleotides and pyruvate [65]. Under these nonturnover conditions, weak pyruvate binding to PDK2 is observed but ADP decreases the concentration of pyruvate, giving half-maximal quenching by 150-fold [63]; this large change is dependent on linked binding of K^+ and P_i binding (see below). All the quenching due to adenine nucleotides and most of the quenching due to pyruvate or the combination of ATP/ADP and pyruvate were caused by quenching of Trp383 (*i.e.*, quenching removed by W383F mutation) [65]. As indicated above, Trp383 anchors the intersubunit cross arms in PDK2 (an equivalent Trp anchors the cross arm in PDK3). The marked quenching by ADP plus pyruvate may occur in association with displacement of the cross arm; the cross arm is absent in the PDK2 crystal structure with ADP and DCA bound [64].

Adenine nucleotides (ATP or ADP) reduce somewhat the binding PDK2 to E2 and more specifically the L2 domain [62, 65, 115]. In the opposite direction, binding of L2 reduces the affinity of ATP at the active site [65, 115]. The combination of ADP and pyruvate cause a marked decrease in binding to GST-L2 [65]. Furthermore, these ligands cause human PDK2 to self-associate to a tetramer with a K_d of ~7.5 μM [65]. The tetramer has a higher frictional coefficient (1.61 vs 1.39), suggesting it is a looser structure. As indicated above, these conditions cause marked quenching of Trp383 and may cause release of the cross arms. Based on this and complementary hydrophobic and charged groups in the cross arms, it was suggested that the released cross arms directly participate in tetramer formation [65]. The marked reduction in binding of PDK2 to the L2 domain of E2 due to binding of ADP and pyruvate (aided by K^+ and phosphate) likely makes a major contribution (beyond slowing ADP dissociation) to the potent inhibition by these effectors.

Less information is available on the regulation of the other PDK isoforms (see Table 1). Not only is PDK3 weakly inhibited by pyruvate/DCA but ADP does not particularly enhance this inhibition [109, 110]. However, ADP is an effective inhibitor of PDK3 activity and inhibition is enhanced by P_i [110]. PDK1 and PDK4 are more sensitive than PDK3 to pyruvate/DCA inhibition but less sensitive than PDK2 [109, 127, 128].

With the intention of developing high-affinity inhibitors that bind at the DCA site, Espinal et al. [134] developed compounds with a 2,2-dichloroacetophenone (DCAP) core structure and tested these for inhibition of PDKs associated with bovine purified heart PDC. Minimal evidence was presented that these compounds indeed bind at the DCA/pyruvate site. Like DCA, one compound (*p*-methyl-DCAP, IC_{50} = 1 μM) appeared to be an uncompetitive inhibitor *versus* ATP [138]. One study comparing the effects of DCAP on the different PDKs did not include K^+ and P_i [134], which have marked effects on inhibition of PDK2 by pyruvate and DCA [84].

Fig. 3c also shows the inhibitor Pfz3 bound at a third site in the R domain of PDK2 [64]. This inhibitor was found in screening inhibition of PDK associated with porcine PDK2. It is suggested that this inhibitor may be acting as an analog of CoA (structural comparison, Fig. 5). Direct inhibition, but only to a small extent, of human PDK2 by CoA has been observed [84]. For CoA to bind where Pfz3 binds, it would have to enter from the other side of the R domain with the adenine portion of CoA near the active site cleft. A potential opening for binding of CoA may form from the pyrophosphate portion of CoA displacing Glu128 [64].

Key role of binding of K^+ and P_i in effector regulation and changes in structure and oligomeric state of PDK2

Mitochondria contain high levels of K^+ (100–130 mM). With undefined bovine PDK associated with kidney PDC (PDK2 and PDK3 indicated in later immunoblot studies), the K_m for ATP is decreased and ADP inhibition is enhanced by elevating K^+ ion levels [139]. Pyruvate inhibition is increased both by this condition and by P_i anion [83, 84], and stimulation by NADH and acetyl-CoA requires and is increased with concentration of KCl or K_xPO_4 [131]. These effects and requirements were confirmed in studies with human PDK2 [84, 111]. Indeed, no stimulation by NADH and acetyl-CoA was observed unless both K^+ and at least one anion (P_i or Cl^-) were included in assay mixtures [111].

ATP can be captured at the active site by a cold-trapping procedure in which PDK2 is transferred and diluted in cold potassium phosphate buffer and then collected on Millipore filter [84]. ATP quenching of Trp fluorescence of PDK2 gave a similar low apparent K_d of 3.5 μM ATP [65]. This high-affinity binding requires K^+ binding. Using Trp fluorescence, we have been able to demonstrate a coupling effect of ~65 for binding of K^+ and ATP (*i.e.*, change in the ratio of the equilibrium binding dissociation constants without *versus* with a saturating level of the other ligand bound [140]). In Fig. 4, left, arrows point to K^+ bound at the active site that are directly chelated by an oxygen of the α-phosphate of ATP and by other residues in the active site [63]. Similar binding was observed with ADP [63]. Ligand-induced changes in K^+ binding were proposed based on PDK2 structures [64]. Using Trp fluorescence, we have obtained direct evidence that K^+ is required for tight binding of ADP, for greatly enhancing binding by pyruvate, and for additional P_i binding that by these sequential equilibria captures ADP and pyruvate on PDK2. Interestingly, the conversion of PDK2 to a tetramer (above) upon binding of ADP and pyruvate requires K^+ and is markedly enhanced by P_i anion. Therefore, potent inhibition of PDK2 activity by pyruvate results from simultaneous binding of K^+ and P_i that stabilizes the dead-end complex (Fig. 6); formation of this dead-end complex also greatly hinders binding of PDK2 to the L2 domain and fosters PDK2 dimers associating to form a tetramer (Fig. 6).

A comparison of PDK2 crystal structures supports a hinge movement between the Cat and R domains of PDK2 that leads to opening of the active site cleft and reduction of the spacing in the trough region upon formation of the intersubunit cross arm [64]. A similar conformational change comparing the rat PDK2·ADP and PDK3·L2 structures was attributed to PDK3 binding the L2 domain [63]. L2 binding would capture and greatly stabilize the cross arm, and therefore stabilize the more open active site cavity. The induction of a more open active site is predicted to contribute to enhanced kinase activity due to favorable binding of the E1 substrate and weaker binding of ADP.

Stimulation by reductive acetylation was shown to speed up ADP dissociation and to raise the apparent K_m for ATP. Transitions in the active site region of PDK2 with Nov3r (a mimic of acetyl-dihydrolipoyl group) fit weaker binding of K^+ [64]. Direct evidence for this effect needs to be obtained. Interlinked mechanisms for inhibition and stimulation of PDK2 are shown in Fig. 6. Inhibitory conditions slow the release of ADP from the active site by favoring formation of ADP·Mg·K complex. This further favors binding of pyruvate and P_i, which is linked to the closing of the active site cleft and loss of the intersubunit cross arm and acquisition of the capacity to form a tetramer [65, 84]. With E2-favored catalysis, cross arm formation is stabilized and supports binding to and movement of PDK2 between the L2 domains of E2 to efficiently access E1. These conditions also support an open active site cavity that favors E1 binding and somewhat weakened ADP binding [64]. Stimulation by reductive acetylation of L2 induces conformational changes that weaken K^+ interactions with PDK2 and bound ATP or ADP at the active site, thereby greatly speeding up dissociation of ADP [62].

Activation of PDC by PDK inhibition for treating diabetes, heart disease, and cancer

Diabetes

As indicated above, extended starvation precipitates PDC activity, which is emphatically suppressed in most tissues by overexpressing PDK4. The same regulatory control severely confines PDC activity in diabetic animals, obstructing consumption of abundant glucose [6–9, 86–88, 93, 96]. This contributes to elevated blood glucose and protein glycation, which causes damage to the vascular system [141–144]. Glucose oxidation is also decreased in obese individuals (including man); in studies with obese rats and mice, this is linked to a low percentage of the PDC being in the active form, at least in part due to insulin resistance [1, 3, 4, 6, 32]. The potential therapeutic value of tissue-targeted control of PDK expression and the potential value of therapeutic interventions to reduce PDK4 expression in muscle in insulin resistance has been proposed [106]. However, beyond insulin, a specific intervention for reducing PDK4 expression (such as a PPARα agonist) has not been

described. Use of PDK inhibitors to elevate PDC activity and thereby promote glucose disposal in peripheral tissues of diabetic animals has been pursued as a therapeutic approach. Initial studies using DCA were encouraging [145], but this compound is a weak PDK inhibitor and a toxic metabolite [146–148]. With the objective of designing potent drugs to increase the metabolic use of glucose in individuals with type II diabetes, Glaxo [134], Novartis [123–125, 149], AstraZeneca [126, 135, 136], and Pfizer [64] have produced PDK inhibitors. Novartis and AstraZeneca developed the class of tight-binding inhibitors that are amides of trifluoro-2-hydroxy-2-methylpropionic acid [123-126, 135, 136]. As indicated above, these inhibitors, including Nov3r and AZD7545, bind at the lipoyl group binding site and effectively increase PDC activity. As a consequence of increasing PDCa, inhibitors in this class (AZD7545, compound K) were shown to reduce blood glucose in obese Zucker fa/fa rats, a model for the early stages of type II diabetes [15, 16]. Interestingly, as indicated above, this class of inhibitors actually stimulates the activity of PDK4 [126, 135, 136].

Concerns with inhibiting PDK2, which is universally distributed in body tissues, include that activation of PDC in some tissues may be deleterious or that PDK4 overexpression will override the effects of PDK2 inhibition. While PDK4 overexpression is well documented in association with type I diabetes, the situation in type II diabetes is less clear. It has been reported in obese Zucker rats that levels of expression of PDK2 and PDK4 in liver and skeletal muscle are similar to those found in lean rats [150]. A similar pattern of expression of PDK4 was also observed in hyperglycemic human patients [151]. However, there was increased PDK2 and PDK4 expression in the Otsuka Long-Evans Tokushima Fatty rat [152]. Inhibition of PDK2 not only favors glucose use by muscle tissues, including heart, but PDK2 is the primary PDK of liver [30, 87, 109]. Activation of liver PDC acts in opposition to liver gluconeogenesis, which proceeds in diabetic patients even though blood glucose is elevated. Another potential advantage of selective PDK2 activation is that retention of PDK4 activity allows it to serve as a back-up, preventing depletion of blood glucose during extended periods of diminished dietary intake of glucose.

Because the role of liver PDC is fatty acid biosynthesis and PDK2 is also the primary PDK in adipose tissue, a potential concern with PDK2 inhibition is increasing fat synthesis from glucose. In agreement with the specificity for inhibiting PDK2 [126] by AZD7545 or compound K, the increase in PDC activity in fasted rats is tempered in muscle tissues (60% PDCa) but rises to a high level in liver (90% PDCa) [16]. However, in addition to lowering blood glucose as effectively as rosiglitazone, a 4week treatment of obese (fa/fa) Zucker rats with AZD7545 had no effect on food intake or body weight gain [16]. In contrast, rosiglitazone caused weight gain in the same study [16], in agreement with previous results with Zucker rats [153] or human patients [154]. Apparently, increased glucose conversion to fatty acids upon activation of PDC is being prevented by upstream regulation, probably, in part, by retention of low phosphofructokinase activity.

Heart disease

Particularly in heart muscle, it is important to dampen PDC activity under low energy demand/nonstress conditions, and to gear up PDC activity when use of carbohydrate is needed to meet energy demands (*e.g.*, due to increased activity or medical conditions such as partial ischemia or in postischemic myocardium) [17–22, 155, 156]. Activation of PDC by inhibiting PDK activity has been a useful therapeutic target for avoiding cardiomyopathy, particularly during heart surgery or partial ischemia [17–22, 155–163]. Fatty acids are the primary mitochondrial substrate during moderately severe ischemia [1, 4, 8, 20, 164]. While glycolysis remains active, use of fatty acids favors PDC being converted to the inactive form. Ischemia, in turn, causes pyruvate to be converted to lactate, thereby increasing the acidification within the myocardium [155, 156, 164]. This disruption in cell homeostasis causes a decrease in ATP and poor Ca^{2+} uptake by the sarcoplasmic reticulum [19, 156–158]. To date, PDK inhibition by DCA has been the primary means of drug intervention to directly activate PDC. However, use of the combination of glucose-insulin-K^+ or inhibiting fatty acid oxidation (*e.g.*, trimetazidine or ranalozine) also beneficially increases PDCa [21, 161–163].

Interventions aimed at activating PDC in postischemic myocardium (primarily with rat hearts) give variable results depending on the reperfusion conditions [19, 159, 165–169]. The change in glucose oxidation depends both on the length of time of the ischemic shut down and the substrate/inhibitor conditions used in the reperfusion media. Inclusion of fatty acids along with glucose decreased responsiveness compared to use of glucose as the sole substrate. Increasing pyruvate has also been shown to improve the mechanical performance of the heart under normoxic and postischemic conditions [21, 170–175]. Preferential use of external pyruvate over glucose or lactate was enhanced by insulin or DCA [21]. A direct effect of insulin in increasing PDCa is probably due to PDP2 activation. PDP2 is found in heart but is

decreased by starvation [101]. Known regulation predicts very low PDP2 activity without insulin.

While highly beneficial for myocardial ischemia, DCA has toxic side effects [145–148]. Testing of the more potent Nov3r class of inhibitors for reducing complications due to lactate build-up during heart ischemia seems warranted. Increasing mitochondrial Ca^{2+} has been shown to be a critical event for PDC activation upon reperfusion of ischemic hearts [176–181]. Therefore, activation of PDP1 is also a critical event for lowering lactate levels.

Cancer

A longstanding observation is the development of so-called Warburg metabolism in a wide range of cancers [182, 183]. Similar to anaerobic muscle, glucose is converted through glycolysis to lactate, which is secreted. Partial hypoxia in poorly oxygenated solid tumors serves as a plausible rationale for this mode of metabolism in some cancers. However, Warburg metabolism is also found in some well-oxygenated cancer cells [183–189]. Whether under hypoxic or aerobic conditions, the reliance on glycolysis is associated with increased malignancy. Recent studies suggest that forcing cells into more aerobic metabolism suppresses cancer growth [25, 186–189]. Whereas continuously dividing cells allows fatty acids and amino acids to be directed to membrane and protein synthesis, respectively, the logic for cancer cells establishing this low efficiency metabolism is not well understood. Certainly, full oxidative use of glucose is a more efficient way of generating ATP in well-oxygenated cells. Regardless of the rationale, the transition to Warburg metabolism requires shutting down of the PDC reaction.

In the transition to Warburg metabolism, there is enhanced signaling by the hypoxia-inducing factor (HIF) in cancer cells [188–192]. Indeed, mutations that directly or indirectly instigate HIF signaling appears to be a common transition in the development of cancer [190–192]. Two recent studies [23, 24] demonstrated that HIF induces the overexpression of PDK1, which then acts to lower PDC activity. PDK1 phosphorylation can be particularly effective for maintaining inactive PDC since this isoform uniquely phosphorylates three serine residues in the alpha subunit of E1 [113, 193]. Reactivation of E1 requires the removal of all three phosphate groups. Selectively blocking HIF-induced expression of PDK1 induced apoptosis in the cancer cells [23, 24]. One study emphasized oxygen depletion due to activation of PDC as the cause of apoptosis [23]. The other study emphasized that PDC activation led to enhanced production of reactive oxygen species (ROS) and suggested that this induced apoptosis [24]. Whether oxygen depletion or ROS production, both studies supported selective PDC activation as a sufficient change to induce apoptosis. Inhibition of PDK1 and possibly other PDK isoforms would appear to be a potential target for killing cancer cells. The Nov3r-class inhibitors are high-affinity inhibitors of PDK1, but maximally cause only 60% inhibition of this isoform [126]. The reason for this is that the low (2.5-fold) activation of PDK1 by E2 is being prevented. PDK3 can also be induced by HIF [23]. Since many cancers utilize Warburg metabolism, the development of potent PDK1 and PDK3 inhibitors may provide a powerful approach for killing or, at least, greatly slowing the growth of many forms of cancer.

Conclusions

Varied control of PDC activity is needed to regulate the conversion of carbohydrate to energy or fat in mammalian tissues. The dedicated PDK/PDP system responds to metabolite and hormone signals to vary PDC activity in response to changes nutritional state. Based on advanced insights into the structure and mechanisms of short-term effector regulation, PDK2 function and control is delineated. The activity of this most widely distributed PDK is facilitated by its bifunctional binding to and movement among the L2 lipoyl domains of the E2 component; this process provides PDK2 with efficient access to its E1 substrate. At a domain level, the structural basis for PDK2 binding L2 and its lipoyl cofactor is described. Metabolic conditions favoring fatty acids or ketone bodies being the major energy source in a tissue favor reductive acetylation of L2, which leads to enhanced PDK2 activity in association with stronger binding to L2 and an increased rate of ADP dissociation. Low energy and a sufficiency of carbohydrate favor PDC activation via PDK2 inhibition that involves binding of pyruvate and P_i enforcing ADP binding. A key feature in making ADP dissociation the rate-limiting step is chelation of K^+ by bound ADP. We suggest that stimulation displaces K^+ and inhibition enforces retention of the PDK2·ADP·K^+ complex via the favored binding of additional ligands (pyruvate and phosphate). The binding of the full set of inhibitory ligands prevents binding to L2 and causes PDK2 to associate to a tetramer. Based on Trp fluorescence studies, tetramer formation is suggested to occur in association with release of an intersubunit cross arm in PDK2 that is anchored at the other subunit via Trp383. Some tight-binding inhibitors of PDK have been developed for use in preventing glucose depletion in insulin-resistant diabetes and damage due to lactic acid production in ischemic heart. Recent studies

support the potential for using PDK inhibitors to induce apoptosis in cancer cells operating with Warburg metabolism.

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