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Minireview

Short-term regulation of the mammalian pyruvate dehydrogenase complex

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In this minireview the main mechanism of control of mammalian pyruvate dehydrogenase complex (PDHC) activity by phosphorylation–dephosphorylation is presented in the first place. The information recently obtained in several laboratories includes new data about isoforms of the PDH converting enzymes (kinase and phosphatase) and their action in view of short-term regulation of PDHC. Moreover, interesting influence of exogenous thiamine diphosphate (TDP) and some divalent cations, especially Mn^{2+}, on the kinetic parameters of PDHC saturated with endogenous tightly bound TDP, is discussed. This influence causes a shortening of the lag-phase of the catalyzed reaction and a strong decrease of the K_m value of PDHC mainly for pyruvate. There are weighty arguments that the effects have an allosteric nature. Thus, besides reversible phosphorylation, also direct manifold increase of mammalian PDHC affinity for the substrate by cofactors seems an important aspect of its regulation.

Keywords: pyruvate dehydrogenase complex, regulation, phosphorylation, dephosphorylation, allosteric effect, thiamine diphosphate

The multienzyme pyruvate dehydrogenase complex (PDHC) catalyzes the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2 (Reed, 2001). The main product, a CoA-activated two-carbon unit, can be condensed with oxalacetate in the first reaction of the citric acid cycle, or used for fatty acid and cholesterol synthesis. So, the several-step process of oxidative decarboxylation of pyruvate occupies a central position in cellular metabolism, because it links glycolysis with the citric acid cycle and lipid biosynthesis. In eukaryotic cells, the PDHC is located in the mitochondria matrix and may be regulated in numerous ways (Wałajtys *et al*., 1974; Wieland, 1983).

PDHC STRUCTURE

The mammalian PDHC is of great complexity. Its structural core is composed of 60 lipoate-containing subunits of dihydrolipoamide acetyltransferase (EC 2.3.2.12; abbreviated E2) (Yeman, 1989; Reed, 2001). Each E2 subunit consists of an inner domain (ID), a binding domain (BD), and two lipoyl domains (LD_1, LD_2) connected by flexible linkers (Fig. 1). The inner domains are arranged in a pentagonal dodecahedron and the others form an extended "swinging arm" including the lipoate residues (Perham, 2000). Thirty heterotetrameric ($\alpha_2\beta_2$) molecules of TDP-containing pyruvate dehydrogenase (EC 1.2.4.1; abbreviated E1) are joined to the binding domains of E2 (Reed, 2001; Fries *et al*., 2003). Twelve homodimeric molecules of FAD-containing dihydrolipoamide dehydrogenase (EC 1.8.1.4; abbreviated E3) are connected with the binding domains of the so called "E3-binding protein" which differs from typical E2 subunit because it contains only one lipoyl domain (LD_3) and is not catalytically active (Harris *et al*., 2002; Hiromasa *et al*., 2004). Detailed information about the PDHC structure can be found in recently published reviews (Perham, 2000; Reed, 2001; Patel & Korotchkina, 2003).

Studies of the structural organization of E1 may be especially useful for understanding its catalytic and regulatory mechanisms. For example, the crystal structure of the holo-form of human pyruvate dehydrogenase at 1.95 Å resolution, recently determined by Ciszak *et al.* (2003), gives evidence

Abbreviations: BD, binding domain; E1, TDP-containing pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, FAD-containing dihydrolipoamide dehydrogenase; ID, inner domain; LD_1, LD_2 and LD_3, lipoyl domains; PDHC, pyruvate dehydrogenase complex; PDHK, pyruvate dehydrogenase kinase; PDHP, pyruvate dehydrogenase phosphatase; TDP, thiamine diphosphate.

that a shuttle-like motion of E1 heterodimers ($\alpha\beta$) is possible. This is in accordance with a flip-flop mechanism of the catalytic action of animal E1 (Khailova *et al*., 1990). Site-directed mutagenesis, kinetic analysis and X-ray crystallography of human E1 revealed that Pro-188, Met-181 and Arg-349 in the α-subunit, and Trp-135 in the β-subunit play important structural roles. Moreover, the αPro-188 and αMet-181 residues were found to be essential for binding of TDP (Korotchkina *et al*., 2004).

REGULATION BY REVERSIBLE PHOSPHORYLATION

Phosphorylation–dephosphorylation is undoubtedly the main mechanism of control of mammalian PDHC activity. It was discovered over 30 years ago (Linn *et al*., 1969; Wieland & Jagow-Westermann, 1969) and has been intensively investigated ever since (Wałajtys *et al*., 1974; Strumiło *et al*., 1981; Wieland, 1983; Denton *et al*., 1996; Kolobova *et al*., 2001; Holness & Sugden, 2003; Roche *et al*., 2003; Bao *et al*., 2004a). For realization of the mechanism, PDHC uses two specific enzymes: pyruvate dehydrogenase kinase (EC 2.7.1.99; PDHK) and pyruvate dehydrogenase phosphatase (no EC number; PDHP). Phosphorylation and concomitant inactivation of E1 is catalyzed by PDHK, whereas dephosphorylation and reactivation of E1 by PDHP (Linn *et al*., 1969; Wieland, 1983). The regulatory enzymes are both integrated with PDHC; they bind to PDHC mainly *via* the E2-lipoyl domains (Harris *et al*., 2002) (Fig. 1).

CHARACTERIZATION OF PDH KINASES

PDHK is a specific kinase that phosphorylates three serine residues in three separate sites of the E1-component of the PDHC, using ATP as a donor of phosphate group (Wieland, 1983; Reed, 2001; Kolobova *et al*., 2001; Bao *et al*., 2004a). Mammalian PDHK has been shown to exist in four isoforms (Gudi *et al*., 1995; Bowker-Kinley *et al*., 1998; Harris *et al*., 2002). It should be noted that genes which encode $PDHK_1$, $PDHK_2$ and $PDHK_4$ are more strongly expressed in the muscle tissues and liver. On the other hand, genes encoding $PDHK_3$ and $PDHK_2$ are expressed in the kidney, brain, and testis (Harris & Huang, 2001; Patel & Korotchkina, 2003). This determines the respective differences in tissue distribution of mammalian PDHK isoforms and their mRNAs. Recently the three-dimensional structure of rat PDHK (isoform 2) has been established (Steussy *et al*., 2001). Interestingly, the mammalian PDHKs exhibit low homology with other eukaryotic serine protein kinases, but are similar to bacterial histidine protein kinases (Harris *et al*., 2002). However, their catalytic mechanisms are different, because PDHKs phosphorylate a serine residue directly, without phosphorylation of a histidine intermediate (Steussy *et al*., 2001). Each PDHK isoform is a homodimer composed of subunits of molecular masses between 39 and 48 kDa (Patel & Korotchkina, 2003).

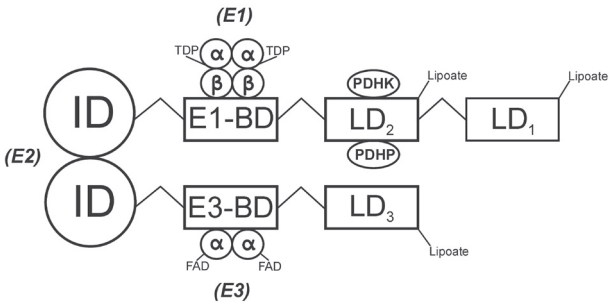

Figure 1. Scheme of a fragment of the mammalian pyruvate dehydrogenase complex structure (from Harris *et al*., 2002, modified).
Only two subunits of E2 with E1 and E3 components are shown. In complete PDHC 60 inner domains are arranged in a pentagonal dodecahedron. Abbreviations: ID, inner domain; BD, binding domain; LD, lipoyl domain.

REGULATORY PROPERTIES OF PDH KINASES

Mammalian PDHC contains only 1–3 molecules of PDHK (Yeaman, 1989) which bind mainly to the mobile lipoyl domains of E2-subunits (Liu *et al*., 1995). Only the $PDHK_4$ isoform prefers the lipoyl domain of the "E3-binding protein" (Roche *et al*., 2003). Such localization of PDHKs on the "swinging arms" and their transfer from one lipoyl domain to another gives the possibility of reaching almost all phosphorylation sites in the 30 molecules of E1 (Korotchkina & Patel, 2001). PDHKs are activated *via* conformational changes when bound to the lipoyl domains (Roche *et al*., 2003; Bao *et al*., 2004b). Moreover, the activity of PDHK is stimulated by the reduction and acetylation of the lipoyl residues occurring during PDC action (Harris *et al*., 2002). Numerous studies with PDHC isolated from various mammalian tissues indicate that PDHK is stimulated by NADH and acetyl-CoA (Wieland, 1983; Yeaman, 1989; Reed, 2001). A high $NADH/NAD^+$ ratio causes reduction of the lipoyl groups through the reversal of the reaction catalyzed by dihydrolipoamide dehydrogenase (E3), and a high acetyl-CoA/CoA ratio promotes acetylation of the reduced lipoyl groups by E2 (Patel & Korotchkina, 2003). High NADH and acetyl-CoA concentrations are found in mitochondria when fatty acid oxidation is intensive, for example during starvation, in high fat diet and diabetes (Wu *et al*., 2000; Harris *et al*., 2002). Phosphorylation and concomitant inactivation of PDHC by stimulated PDHK is very typical for such metabolic states. On the oth-

er hand, high NAD^+ and CoA concentrations lead to PDHK inhibition, and the PDHC remains active (Wieland, 1983; Holness & Sugden, 2003). Pyruvate also exerts an inhibitory influence on PDHK (Bao *et al*. 2004a). Isoform $PDHK_2$ is more sensitive to inhibition by pyruvate and stimulation by NADH and acetyl-CoA than the other ones (Harris *et al*., 2002; Bao *et al*., 2004a). Competitive inhibition of PDHK by ADP *versus* ATP was also considered to be essential in the regulation of the PDHC (Strumiło *et al*., 1981; Wieland, 1983). Studies of $PDHK_2$ showed that ADP dissociation from active sites is a limiting step in E2-activated catalysis by isoform 2 (Bao *et al*., 2004b). Conversion of all lipoyl groups in the E2-oligomer to the oxidized form greatly reduced k_{cat} and the K_m of $PDHK_2$ for ATP. On the other hand, reduction and, to a greater extent, reductive acetylation of the lipoyl groups increased $PDHK_2$ binding to E2 and stimulated it (Bao *et al*., 2004a). Thus, fluctuations of pyruvate concentration and the NADH/NAD^+, acetyl-CoA/CoA and ATP/ADP ratios in varied hormonal and nutritional states significantly change PDHK activity and, in an opposite manner, the activity of the PDHC. It should also be added that a lipoprotein which strongly inhibits the PDHC has been isolated from bovine adrenal mitochondria (Strumiło *et al*., 1984). The degree of inhibition was higher at lower ATP concentrations. It was proposed that the mitochondrial lipoprotein could specifically shield the active site of PDHK or the phosphorylation sites of E1 during catalytic action.

As presented above, the E1-component of mammalian PDHC has three phosphorylation sites. Isoforms of PDHK show different activity during phosphorylation of the sites. Their relative activities toward site 1 are: $PDHK_2 > PDHK_4 > PDHK_1 > PDHK_3$; toward site 2: $PDHK_3 > PDHK_4 > PDHK_2 > PDHK_1$ (Patel & Korotchkina, 2003). In the case of site 3, the differences in the activity of the PDHK isoforms are not so clear. According to Kolobova *et al*. (2001), $PDHK_1$ can phosphorylate all three sites, whereas $PDHK_2$, $PDHK_3$ and $PDHK_4$ each phosphorylates only sites 1 and 3. Earlier, it was reported that only phosphorylation at sites 1 and 2 results in PDHC inactivation, yet additional phosphorylation of site 3 inhibits reactivation of E1 by PDH phosphatase (Wieland, 1983). However, according to new data, phosphorylation of either site leads to PDHC inactivation, but in a different way (Korotchkina & Patel, 2001; Kolobova *et al*., 2001). For example, phosphorylation of site 1 of human pyruvate dehydrogenase prevents its interaction with the substrate, pyruvate, and a lipoyl domain, whereas modification of site 3 hinders E1 interaction with TDP (Patel & Korotchkina, 2003). Conversely, exogenous TDP added to the reaction medium results in inhibition of PDHK activity (Walsh *et al*., 1976; Czygier & Strumiło, 1995a). TDP especially decreases the amount of phosphate that $PDHK_1$ incorporates in sites 2 and 3, and $PDHK_2$ in site 2 (Kolobova *et al*., 2001). Interesting data were obtained in kinetic investigation of aurochs heart PDHC: completely dephoshporylated PDHC showed a positive cooperativity of pyruvate binding sites at low substrate concentrations, yet partial phosphorylation of the complex (to 35% reduction of its activity) abolished the cooperativity (Czygier & Strumiło, 1995b).

Besides the short-term regulation by the metabolites, a long-term control of PDHK amount at the transcriptional level is very important. This aspect is presented thoroughly in the recently published reviews (Harris *et al*., 2002; Patel & Korotchkina, 2003). In this minireview, only several examples of long-term regulation will be quoted. Of the four PDHK isoforms greater changes in the amount of enzyme protein were observed for $PDHK_4$ and $PDHK_2$. Starvation and diabetes increase the level of $PDHK_4$ expression in most tissues (Harris *et al*., 2001). Insulin treatment of diabetic rats reverses the changes (Harris *et al*., 2002). Starvation also increases $PDHK_2$ expression in the liver and kidney (Wu *et al*., 2000).

CHARACTERIZATION OF PDH PHOSPHATASES

PDHP is a specific phosphatase that removes P_i from phosphorylated sites of E1 and reactivates the component and the whole PDHC. Mammalian PDHP consists of two nonidentical subunits of 52 and 96 kDa (Chen *et al*., 1996). The smaller subunit performs the catalytic function, whereas the larger one is a flavoprotein performing a regulatory function (Lawson *et al*., 1997). There are two isoforms of PDHP encoded by different genes for the catalytic subunits (Huang *et al*., 2003). The isoforms $PDHP_1$ and $PDHP_2$ show some differences in activity, regulation, and tissue distribution (Karpova *et al*., 2003). It is known that binding of $PDHP_1$ to the mammalian PDHC occurs thorough the lipoyl domain LD_2 and requires the presence of Ca^{2+} which plays a bridging role (Roche *et al*., 2003). As with the binding of $PDHP_2$, the existing information is insufficient for unequivocal conclusions.

REGULATORY PROPERTIES OF PDHP

The reaction catalyzed by both isoforms of PDHP requires Mg^{2+} concentrations in the millimolar range (Linn *et al*., 1969; Reed, 2001). Magnesium ions may be replaced by Mn^{2+}. The K_m values of pig heart PDHP for $MgCl_2$ (2.5 mM) and $MnCl_2$ (1.8 mM) are similar (Wieland, 1983), but in the case of bovine adrenal PDHP the constants (1.20 and 0.35 mM, respectively) differ over 3-fold, and a higher PDHP

activity was observed in the presence of Mn^{2+} compared to Mg^{2+} (Strumiło, 1983). In addition to Mg^{2+}, the isoform $PDHP_1$ requires Ca^{2+} at micromolar concentrations. Ca^{2+}, besides its participation in the interaction of $PDHP_1$ with the LD_2 domain, strongly stimulates $PDHP_1$ activity (Huang *et al*., 1998; Roche *et al*., 2003). Moreover, a specific regulatory protein for $PDHP_1$ has been isolated and partially characterized (Lawson *et al*., 1997). According to the recently obtained data, $PDHP_1$ is more sensitive to Mg^{2+} than is $PDHP_2$ (Harris *et al*., 2002). Polyamines, especially spermine, markedly decrease the K_m values for Mg^{2+} of both PDHP isoenzymes (Roche *et al*., 2003). In general, the short-term regulation of PDHP activity is not so varied as is the regulation of PDHK. PDHP activity is influenced mainly by divalent cations. For example, muscle contraction causes an increase in mitochondrial Ca^{2+} which can then stimulate $PDHP_1$ activity and the whole PDHC (Harris *et al*., 2002; Patel & Korotchkina, 2003). Insulin can stimulate PDHPs *via* their phosphorylation by protein kinase Cδ, which is activated and translocated to mitochondria of muscle and liver cells by the hormone (Caruso *et al*., 2001). In additional to the short-term regulation by divalent ions, polyamines and, probably, insulin, a long-term regulation of PDHP *via* changes in its expression at the genetic level is a confirmed fact. Starvation and diabetes decrease $PDHP_1$ expression, especially in rat heart and kidney (Huang *et al*., 2003), whereas insulin increases it (Harris *et al*., 2002; Holness & Sugden, 2003).

ISOSTERIC INHIBITION BY END-PRODUCTS

In numerous kinetic studies of PDHC isolated from varied mammalian tissues, its competitive inhibition by acetyl-CoA and NADH *versus* CoA and NADH has been shown (Wieland, 1983; Yeaman, 1989; Czygier & Strumiło, 1994). The K_i values for NADH were in the same range as the K_m values for NAD^+. It has been suggested that NADH inhibition of PDHC at the isosteric level (at the active sites of the E3 component) is important for the control of mammalian PDHC activity (Wieland, 1983). The inhibition of E2 caused by acetyl-CoA may be a little less significant for PDHC regulation since the K_i values for acetyl-CoA were usually higher than the K_m values for CoA (Wieland, 1983). For example, in the case of PDHC from the human heart the difference was nearly 3-fold (Kiselevsky *et al*., 1990). Yet in such states as starvation or diabetes when the mitochondrial ratios of acetyl-CoA/CoA and NADH/NAD^+ are very high in some tissues, acetylation and reduction of lipoyl groups in backward reactions catalyzed by E2 and E3 can lead to an inhibition of pyruvate decarboxylation catalyzed by E1 (Wieland, 1983; Patel & Korotchkina, 2003). The end-product inhibition at the active centers of the E2 and E3 components seems to be an essential addition to the allosteric regulation of PDH kinase by acetyl-CoA and NADH, as discussed above. Both mechanisms concern the same, universal and clear principle of regulation — "negative feedback inhibition".

ALLOSTERIC REGULATION BY SOME COFACTORS

Many investigators have considered that TDP plays only a catalytic role in the active sites of E1. For a long time it has been suggested that mammalian PDHC loses TDP almost completely during isolation, because the measured PDHC activity was usually very low in the absence of added TDP (Walsh *et al*., 1976; Sumegi & Alkonyi, 1983). The measurements were based on the initial rate of the reaction catalyzed by PDHC. However, a far higher PDC activity was observed when the reaction rate was measured for a longer period, because in the absence of added TDP the maximum rate was achieved only after a lag-phase of several minutes (Strumiło, 1988). It was established that the duration of the lag-phase and subsequent reaction rate depends strongly on the concentration and type of divalent ions present (Mg^{2+}, Ca^{2+}, Mn^{2+}). The shortest lag-phase and the highest activity of PDHC with endogenous TDP is observed in the presence of Mn^{2+} (Czerniecki & Czygier, 2001). Kinetics studies of bovine, bison, and pig heart PDHC saturated with endogenous TDP in the active sites of E1 showed that the addition of exogenous TDP leads to a disappearance of the lag-phase and manifold reduction of the K_m value for pyruvate, but a more moderate decrease of the K_m for CoA and NAD^+ with no considerable change in the maximum reaction rate (Strumiło *et al*., 1996; 1999; Strumiło & Czygier, 1998). The apparent K_m value for TDP is about 0.2 μM which means that the cofactor is very effective. Generally, an increase of an enzyme's affinity for substrates without alteration in the V_{max} are typical signs of positive allosteric modulators in view of the model of Monod *et al*. (1965). TDP acts exactly in such a manner. For example, the K_m values of pig heart PDHC for pyruvate in the absence and presence of added TDP are 76.7 ± 6.6 and 19.0 ± 2.7 μM, respectively (Strumiło *et al*., 1999). The increase of PDHC affinity for the substrate is 4-fold. It has also been established that TDP added to PDHC changes both the UV and circular dichroism spectra, and lowers the fluorescence emission of the complex containing bound molecules of endogenous TDP in its active centers (Strumiło *et al*., 2002). The spectral data indicate conformational changes of the PDHC. Thus, TDP as an allosteric modulator can quickly induce conformational and functional changes of the multienzyme complex. The divalent ions

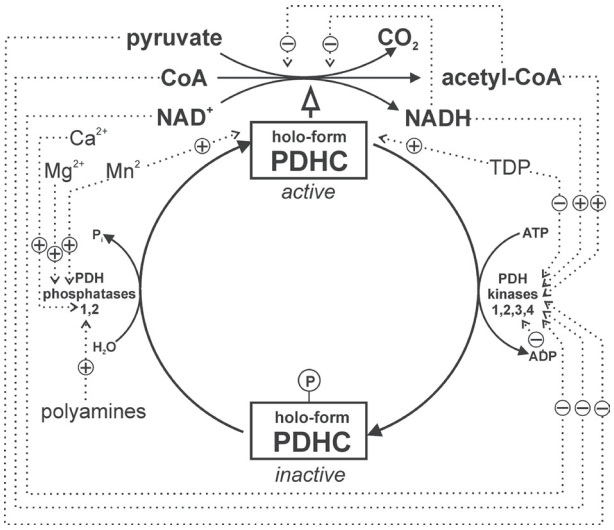

Figure 2. Short-term regulation of the mammalian pyruvate dehydrogenase complex by main effectors.

Mg^{2+}, Ca^{2+}, and especially Mn^{2+} increase the effect of TDP (Czerniecki & Czygier, 2001). Participation of the main effectors in the control of PDHC activity is summarized in Fig. 2.

The data obtained suggest that TDP plays, in addition to its catalytic function, the important role of a positive regulatory effector of mammalian PDHC. Researchers who have the possibility of structural investigations by modern methods ought to notice the allosteric regulation of PDHC. Until now, the structural studies of pyruvate dehydrogenase have been limited to the processes in the active sites of the enzyme and the interactions among its subunits (Ciszak *et al*., 2003; Korotchkina *et al*., 2004).

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