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REGULATION OF THE PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX

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CONTENTS

INTRODUCTION

To maximize catalytic efficiency in metabolic pathways of both prokaryotic and eukaryotic cells, enzymatic components are occasionally clustered or complexed physically. Organization of multiple catalytic functions into a single enzyme complex can be accomplished in two ways. First, multifunctional enzymes utilize a single polypeptide chain to catalyze more than one enzymatic function; an example of a multifunctional enzyme is the mammalian fatty acid synthase. A more common means for clustering enzymatic functions is the assembly of multiple catalytic components into complexes held together by noncovalent bonds. The most prominent examples of multienzyme complexes are the α-keto acid dehydrogenase multienzyme complexes that catalyze the oxidative decarboxylation of pyruvate, α-keto-

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glutarate, and branched chain α-keto acids (see Ref. 105 for several recent reviews). In this review article we discuss briefly several catalytic and molecular features of the pyruvate dehydrogenase multienzyme complex. We then describe selectively some of the characteristics of the regulatory mechanisms that modulate the activity of this enzyme complex in its normal metabolic function in the energy-generating pathways of aerobic cells/tissues.

PYRUVATE DEHYDROGENASE CATALYTIC AND MOLECULAR PROPERTIES

In the pyruvate dehydrogenase reaction, pyruvate is oxidatively decarboxylated to acetyl-CoA, CO_2, and NADH by a series of sequential reactions catalyzed by the three component enzymes of the pyruvate dehydrogenase complex: pyruvate dehydrogenase (E1), EC 1.2.4.1; dihydrolipoamide transacetylase (E2), EC 2.3.1.12; and dihydrolipoamide dehydrogenase (E3), EC 1.8.1.4. E1 catalyzes the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoyl moiety of E2. E2, in turn, catalyzes the transfer of the acetyl group to free CoA. The reduced lipoyl moiety of E2 is reoxidized by E3, with NAD^+ as the final electron acceptor (Figure 1).

Pyruvate dehydrogenase complex has been purified from numerous plant, microbial, and mammalian sources. Pyruvate dehydrogenase complexes from prokaryotes and eukaryotes have molecular weights in the millions; the subunit composition of bovine kidney PDC is shown in Table 1. The complex is organized around a central core composed of multiple copies of the E2 subunit; in *Escherichia coli* and other gram-negative bacteria, the core consists of 24

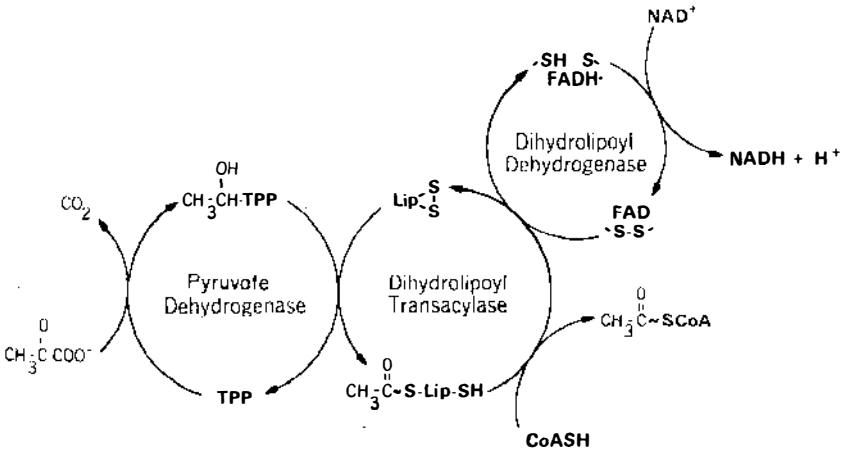

Figure 1 Catalytic mechanism for the pyruvate dehydrogenase reaction.

Table 1 Subunit composition of mammalian pyruvate dehydrogenase complex

Subunit	M_r	Subunits		Subunits per molecule of complex
		No.	M_r	
Native complex	8,500,000			
El tetramer	154,000			30
E1α		2	41,000	
E1β		2	36,000	
E2 core	3,100,000		52,000	60
E3 dimer	110,000			6
E3		2	55,000	
X			50,000	6
Kinase	~100,000	1	48,000	
		1	45,000	
Phosphatase	~150,000	1	97,000	
		1	50,000	

copies of E2, arranged as a hollow cube of 8 trimers (75); in eukaryotes, such as yeast and mammalian systems, the core consists of 60 copies of E2 arranged in an icosahedral dodecahedron with 532 symmetry (99).

E1, pyruvate dehydrogenase, differs in structure between prokaryotic and eukaryotic systems: In *E. coli*, E1 exists as a single polypeptide of $M_r \approx$ 100,000 (39), whereas in yeast and mammalian systems, functional E1 consists of an $\alpha_2\beta_2$ tetramer composed of two copies each of E1α and E1β (1). In the yeast and mammalian pyruvate dehydrogenase complexes, 30 E1 tetramers are arrayed about the surface of the E2 core (98).

E3, dihydrolipoamide dehydrogenase, is representative of a class of ubiquitous flavin-containing dehydrogenases (9). In PDC (pyruvate dehydrogenase complex), E3 exists as a dimer of M_r 110,000, six of which are bound to the surface of the E2 core (98).

Recently, a fourth polypeptide, known as protein X, has been found in yeast and mammalian complexes (21, 132). Approximately six copies of protein X are associated tightly with the E2 core, and evidence indicates that protein X is involved in binding of E3 to the complex (35, 66).

Two other proteins are associated with the mammalian forms of pyruvate dehydrogenase. Pyruvate dehydrogenase kinase catalyzes the phosphorylation of three serine residues on the E1α subunit, causing inactivation of the complex (144). The kinase is a heterodimer consisting of an α subunit, of M_r 48,000, which is thought to possess the catalytic activity, and a β subunit, of M_r 45,000 (124). The kinase is present in very small quantities and is bound tightly to the complex (61). Pyruvate dehydrogenase phosphatase

catalyzes the dephosphorylation of E1α, with the concomitant activation of the complex (61). The phosphatase is bound rather loosely to the complex and is a heterodimer of M_r 140,000–150,000; the subunits have M_r of 97,000 and 50,000 (129).

Human pyruvate dehydrogenase complex has been the subject of intense scrutiny because of clinical manifestations of several genetic deficiencies. The genes encoding both pyruvate dehydrogenase subunits (E1α and E1β), dihydrolipoamide transacetylase (E2), and dihydrolipoamide dehydrogenase (E3) have been cloned and sequenced; some of these genes have been isolated as distinct tissue- or growth stage-dependent isotypes.

cDNAs encoding human E1α have been cloned and sequenced by several different laboratories (20, 23, 48, 60). Differential expression of testis-specific versus somatic isoforms of the E1α gene has been reported (127); the testis-specific form has been localized to chromosome 4, while the X chromosome contains the somatic E1α gene (5, 19, 126). Evidence has been presented for the existence of a 5′-upstream enhancer region common to several enzymes involved in mitochondrial energy production, including E1α (74, 131). The gene encoding human E1β has been isolated and sequenced (13, 47, 51, 60) and has been localized to chromosome 3 (13).

Pyruvate dehydrogenase (E1) catalyzes the oxidative decarboxylation of pyruvate, and the reaction is the rate-limiting step in the overall reaction catalyzed by the pyruvate dehydrogenase complex. The reaction is completely dependent upon the cofactor, thiamin pyrophosphate; titrations with the transition state analog thiamine thiazoline pyrophosphate, which irreversibly inhibits pyruvate dehydrogenase, indicate that there are two thiamin pyrophosphate binding sites per tetramer (40). The enzymatic reaction also requires Mg^{2+}, which presumably complexes with the pyrophosphate linkage of thiamin pyrophosphate to facilitate binding (133).

Thiamin pyrophosphate contains an acidic proton that dissociates and results in the carbanion of thiamin pyrophosphate. This species then attacks the carbonyl carbon of pyruvate and forms CO_2 and enzyme-bound 2-α-hydroxyethylthiamin pyrophosphate, which then reacts with oxidized lipoic acid, in the presence of the other cofactors, to generate S-acetylhydrolipoate and free thiamin pyrophosphate.

The gene encoding dihydrolipoamide transacetylase (E2) has been cloned and sequenced from several sources, including human liver (130), human heart (79), and human placenta (30). Some differences exist among the deduced amino acid sequences; tissue-specific isoforms of E2 may occur (79).

E2 functions both as a structural and as a catalytic element; in addition to forming the core about which the other component elements of PDC are arranged, E2 catalyzes the transfer of acetyl groups from S-acetylhydrolipoate to CoA (8). Gene sequencing and genetic engineering have elucidated the

multidomain structure of E2 (67, 130). The N-terminal portion of the protein consists of two identical copies of a lipoyl-bearing region (107). Each copy is capable of being lipoylated and acetylated (87), and each is adjacent to a conformationally flexible amino acid segment that is required for proper function of the complex (78); this "tether" links the lipoyl domains to the next domain, the putative E1 binding site. The C-terminal portion of E2 comprises the catalytic region. All dihydrolipoamide transacetylases contain the sequence His-Xaa-Xaa-Xaa-Asp-Gly near the C-terminus; this highly conserved sequence is present in chloramphenicol acetyltransferase (CAT) and is thought to be involved in the catalytic mechanism (38). Asp-431 to Asn, Ala, or Glu substitutions in the yeast E2 lead to substantial decreases in kcat; however, His-427 to Asn or Ala substitutions have no significant effect on E2 activity (80). Replacement of a conserved serine residue in *E. coli* E2, analogous to a conserved serine in CAT, supports the active-site hypothesis (109); an Arg-416 to Asp mutation in *Azotobacter vinelandii* E2, again a residue thought to be involved in the active site by analogy to CAT, had no effect on the catalytic activity of E2 (114).

Dihydrolipoamide dehydrogenase (E3) has been cloned and sequenced from human liver (95) and human small cell carcinoma (H378) (85) and has been localized to chromosome 7 (86). E3 catalyzes the oxidation of dihydrolipoic acid to lipoic acid, with the subsequent reduction of NAD^+ to NADH. Each molecule of the E3 homodimer contains single FAD and NAD^+ binding sites, as well as a highly conserved active-site residue (His-452) in the C-terminal interface region (9).

REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

The location of the various α-keto acid multienzyme complexes in the energy-generating pathways of most aerobic tissues/cells is illustrated in Figure 2. The mammalian pyruvate dehydrogenase complex is associated with the mitochondrial inner membrane and is a key enzyme in cellular metabolism; it irreversibly commits three-carbon intermediates derived from the catabolism of carbohydrate (i.e. glucose) or certain amino acids to conversion to acetyl-CoA. Subsequently, acetyl-CoA is oxidized in the tricarboxylic acid cycle or incorporated into long-chain fatty acids. Because of its location at a significant branch-point in the metabolic pathways channeling intermediates into the tricarboxylic acid cycle, the pyruvate dehydrogenase complex is an ideal candidate as a regulatory enzyme. For example, in liver the activity of pyruvate dehydrogenase must be suppressed under gluconeogenic conditions when pyruvate is needed to synthesize oxaloacetate for the pyruvate carboxylase reaction. During lipogenesis, increased pyruvate dehydrogenase

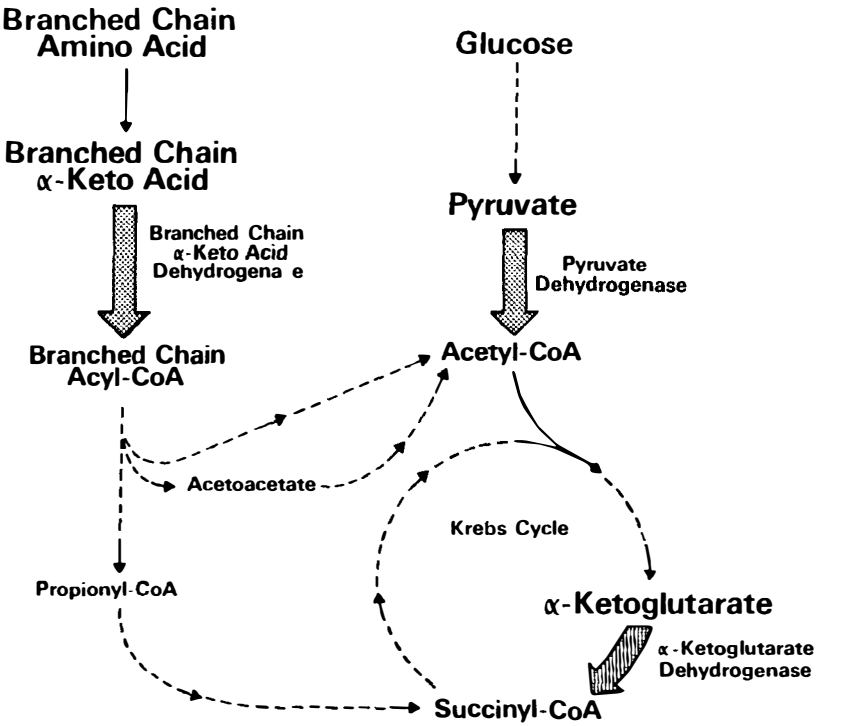

Figure 2 Location of α-keto acid dehydrogenase multienzyme complexes in cellular energy metabolism.

activity is required to supply acetyl-CoA for fatty acid or sterol biosynthesis. Also, pyruvate dehydrogenase in cardiac tissue needs to be able to react rapidly to increased work load by increasing its activity. In the presence of alternative substrates such as fatty acids or ketone bodies, suppression of pyruvate dehydrogenase activity is necessary to allow "sparing" of carbohydrate for other tissues (such as the brain) that are dependent upon glucose for essential energy metabolism. Thus, the regulatory systems for pyruvate dehydrogenase need to be sensitive both to the bioenergetic and biosynthetic requirements of various tissues of diverse function.

Two separate types of regulatory mechanisms have been characterized in order to accommodate the complexities of tissue- specific metabolic requirements in which pyruvate dehydrogenase is involved in higher organisms. The first mechanism, which is also the simplest, is merely end-product inhibition of the catalytically active pyruvate dehydrogenase complex. The second mechanism involves covalent modification of the complex by a phosphoryla-

tion/dephosphorylation mechanism, mediated by a specific protein kinase that is tightly bound to the enzyme complex and by a specific phosphoprotein phosphatase that is much less tightly associated with the pyruvate dehydrogenase complex.

For many years, investigators have observed experimentally that, in the presence of an alternative substrate such as fatty acid or ketone bodies, carbohydrate utilization by tissues such as heart is inhibited (31, 120, 143). A severe inhibition of pyruvate oxidation is responsible for this "carbohydrate-sparing" effect, and early attempts to elucidate the mechanism responsible for this type of inhibitory action on pyruvate dehydrogenase centered on end-product inhibition of the enzyme complex by NADH and acetyl-CoA. Studies using a partially purified preparation of pig heart pyruvate dehydrogenase demonstrated that the enzyme complex is inhibited by the end products of the pyruvate dehydrogenase reaction, NADH and acetyl-CoA (32, 55). Similar feedback inhibition of pyruvate dehydrogenase has been demonstrated using pyruvate dehydrogenase from a wide variety of cell and tissues. Values for the inhibition constant (K_i) from several mammalian systems for NADH (approximately 50 μM) and acetyl-CoA (5–10 μM) are similar to the K_m values for NAD^+ (approximately 60 μM) and CoASH (approximately 5 μM). Thus, under metabolic conditions causing elevated $NADH^+/NAD^+$ or acetyl-CoA/CoASH, the pyruvate dehydrogenase reaction should be inhibited.

The regulation of pyruvate dehydrogenase by a phosphorylation/dephosphorylation mechanism was first demonstrated in 1969 in the laboratory of Reed (70), using purified bovine kidney pyruvate dehyrogenase complex. The kinase and phosphatase reactions have since been demonstrated in a wide range of eukaryotic tissues (53, 69, 122, 128, 136). Pyruvate dehydrogenase is phosphorylated and inactivated by its specific, intrinsic, cAMP-independent protein kinase, using MgATP as substrate (14, 49). Reactivation of the enzyme complex is accomplished by a Mg^{2+}- and Ca^{2+}-dependent phosphoprotein phosphatase, which removes the phosphoryl groups from the phosphorylated enzyme.

The pyruvate dehydrogenase kinase/phosphatase system is regulated by a number of effectors, which are summarized in the cartoon depicted in Figure 3. Pyruvate dehydrogenase kinase is inhibited competitively with respect to ATP by ADP (69, 122, 138), and noncompetitively by pyruvate (14). ADP inhibition of the pyruvate dehydrogenase kinase is increased by the presence of K^+ or NH_4^+ because of lowering of the apparent K_i for ADP (106). On the other hand Robertson et al (102) demonstrated that monovalent cations stimulate pyruvate dehydrogenase kinase from bovine heart. The pyruvate dehydrogenase kinase is inhibited by NAD^+ and CoASH, and stimulated by NADH and acetyl-CoA (2, 3, 15, 93). Calcium also inhibits phosphorylation; half-maximal inhibition occurs at 0.5 mM (137). The coenzyme thiamin

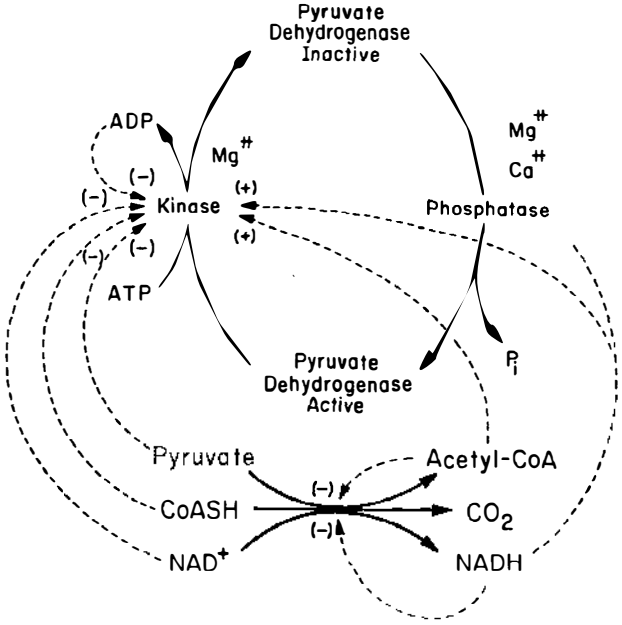

Figure 3 Regulatory mechanisms for pyruvate dehydrogenase by feedback inhibition and covalent modification via the protein kinase/phosphoprotein phosphatase mechanism.

pyrophosphate inhibits phosphorylation, apparently by binding at the catalytic site of E1 to promote a conformational change, which in turn causes one of the serine hydroxyl groups on the α subunit of the pyruvate dehydrogenase to become less accessible to the pyruvate dehydrogenase kinase (7).

Regulation of the pyruvate dehydrogenase phosphatase appears to be less complicated than the regulation of the kinase. The phosphatase reaction requires Mg^{2+}, exhibiting a K_m of about 2 mM (121). Ca^{2+} stimulates the phosphatase reaction indirectly by facilitating binding of the phosphatase to the transacetylase core, thus enhancing dephosphorylation (94). NADH inhibits phosphatase activity and the inhibition is reversed by NAD^+ (93).

Another mechanism that has been proposed for the regulation of pyruvate dehydrogenase involves acetylation and/or acylation of the enzyme complex. Stimulation of the pyruvate dehydrogenase kinase activity by acetyl-CoA and NADH is thought to be due to acetylation and reduction, respectively, of the transacetylase lipoyl moieties (56, 104). Acetylation of dihydrolipoyl moieties of the pyruvate dehydrogenase complex has been demonstrated using [3-^{14}C]pyruvate and [1-^{14}C]acetyl-S-CoA (10, 11); the acetylated enzyme displays enhanced kinase activity, which is stable to gel filtration (10), and

the extent of acetylation correlates with the degree of protein kinase activation (11). Reed's laboratory, using purified protein kinase and tryptic peptide substrates from the E1 dehydrogenase subunit, provided evidence, however, that NADH and acetyl-CoA have direct stimulatory effects on the pyruvate dehydrogenase kinase and that pyruvate has a direct inhibitory effect on the kinase (100).

Jackson & Singer (52) demonstrated that pyruvate dehydrogenase could be inactivated by incubation with α-ketoisovalerate and suggested that the formation of isobutyrylated lipoate residues was responsible. Further, they suggested that inactivation of pyruvate dehydrogenase by α-keto-isovalerate may contribute to the toxicity of elevated branched chain α-keto acids, as seen in maple syrup urine disease. Studies from this laboratory using rat liver mitochondria and the bovine heart complex (101) demonstrated directly the acylation of the E2 transacetylase by α-ketoisovalerate. Acylation of pyruvate dehydrogenase led to enhanced inactivation of the enzyme by kinase-mediated phosphorylation of the E1 component.

Pettit et al (92) demonstrated that pyruvate dehydrogenase kinase activity could be regulated by exchange of protein thiols and disulfides. These results suggested that thiol groups were responsible for maintaining the protein kinase in an active conformation; inhibition of the kinase by disulfides was specific and was reversed by thiols. Pettit et al suggested that naturally occurring disulfides may play an important role in the regulation of this protein kinase.

Regulation of Pyruvate Dehydrogenase in Specific Tissues

The discovery that a specific kinase and phosphatase interconvert purified pyruvate dehydrogenase complex between an active and inactive form immediately indicated that the enzyme must be phosphorylated to a greater or lesser extent in vivo and that the phosphorylation state in vivo must control the relative activity of the enzyme. Incubations of beef heart mitochondria in the presence of [γ-^{32}P]ATP and SDS gel analysis of mitochondrial extracts demonstrated that the γ-phosphate of labelled ATP could be detected in the α subunit of pyruvate dehydrogenase, and thereby provided direct support in an intact metabolic system for pyruvate dehydrogenase inactivation by phosphorylation (118).

Estimates of the relative pyruvate dehydrogenase activity state in vivo using measurements of enzymatic activity in extracts of freeze-clamped tissue both before and after incubation with purified phosphatase have been made (140). In this method, the first assay measures the amount of "active" enzyme, whereas the second assay measures the amount of "total" enzyme, or the amount expressed only after complete activation by dephosphorylation. The active/total ratio reflects the percent activity in vivo. Measurement of the

percent activity has been used extensively by many investigators to demonstrate pyruvate dehydrogenase regulation in vivo under a variety of conditions.

Heart and kidney tissue in fed rats normally contain about 70% active pyruvate dehydrogenase (140). However, fasting or induction of experimental diabetes decreases active enzyme to about 15% (140). In human skeletal muscle after a 12-hr fast, the enzyme is only about 20% active (123). Similar effects occur in heart muscle of normal and obese hyperglycemic mice (71). These effects can be reversed by insulin administration or refeeding. Brain tissue also contains about 70% active enzyme, but starvation has no effect on the activation state of the enzyme in brain (122). In contrast, liver and adipose tissue normally contain only about 20% active enzyme, and starvation or diabetes has no effect on the activation state in these tissues (137). Also, age appears to affect both the activation state and regulation of the enzyme. For example, the fetal rat liver enzyme exists in the fully activated state, in contrast to the normal liver (135), and the adipose tissue enzyme decreases as the rat matures (54). The metabolic factors responsible for the observed in vivo percent active enzyme are complex. Under some selected experimental conditions, such as in isolated mitochondria in the presence of various uncouplers and inhibitors, separate effects on the kinase and phosphatase can be estimated, whereas under steady-state conditions, such as in isolated mitochondria or in perfused organs, only the overall regulatory effects of certain parameters can be measured. In the case of the heart enzyme, originally it was suggested that pyruvate dehydrogenase exists naturally in its inactive form, and that activation occurs as Mg^{2+} becomes available to initiate dephosphorylation by the phosphatase (117, 139). Studies in rabbit heart mitochondria subsequently indicated that this may not be the case and that the enzyme most likely exists in the active form under most metabolic conditions, even in the presence of high ATP concentrations, in agreement with the observed percent active enzyme found in heart tissue from normally fed rats (116). In contrast to the idea that Mg^{2+} stimulates the phosphatase in vivo, decreased pyruvate oxidation in intact heart mitochondria, and hence pyruvate dehydrogenase inactivation, depends on increased Mg^{2+} and occurs at various ATP concentrations (115, 116). In the presence of ATP, metabolic conditions resulting in the release of Mg^{2+}, for example, uncoupler plus ADP (62), lead to an inhibition of pyruvate oxidation, presumably through the kinase/phosphatase interconversion system (116). Only during prolonged incubation in the absence of substrate or ATP does Mg^{2+} cause an activation of pyruvate dehydrogenase by phosphatase stimulation, and hence these conditions usually suffice for determining "total" enzyme only as an experimental tool.

Studies of the steady-state activity of pyruvate dehydrogenase in rat heart mitochondria, where both kinase and phosphatase are active, and under

conditions where the effects of each effector couple can be segregated from the others, indicate that the CoA/acetyl-CoA, NAD^+/NADH, and ADP/ATP ratios all contribute to the steady-state activity (3, 42). At a fixed ratio of two effector couples, for example, shifting the third couple up or down increases or decreases the steady-state activity of pyruvate dehydrogenase. At a fixed ADP/ATP ratio of 3.5, which might plausibly represent the ratio during state 3 respiration, shifting either the CoA/acetyl-CoA or NAD^+/NADH ratio results in a 2-fold change in steady-state activity, but shifting both ratios at the same time produces an additive effect of more than a 4-fold change in steady-state activity. At a lower ADP/ATP ratio, shifting the CoA/acetyl-CoA ratio only affects the steady-state activity under highly oxidized conditions, such as $NAD^+/NADH = 11$.

In the perfused rat heart, the percent active enzyme complex depends on the perfusate pyruvate concentration (25). At low pyruvate concentrations the enzyme is almost 100% active. As the pyruvate concentration increases to 1 mM, the percent active enzyme steadily decreases to about 50% active. Then, as pyruvate continues to increase from 1 mM to 10 mM, the percent of active enzyme returns to almost 100% active. The decrease in the percent of active enzyme is consistent with decreases in the CoA/acetyl-CoA and NAD^+/NADH ratios measured in the same hearts, but the return to 100% active enzyme at high pyruvate concentrations cannot be explained by changes in these ratios. The reactivation is more consistent with kinase inhibition by pyruvate (25). Moreover, pyruvate concentrations have a major role in determining the activation state during flow-induced ischemia in the heart (90).

Regulation of the activation state by changing the concentration of infused pyruvate also involves regulation of pyruvate entry into the mitochondria via the monocarboxylate transporter. In the perfused rat heart, transport inhibition by α-cyanocinnamate and α-cyano-4-hydroxycinnamate suggests that pyruvate enters the mitochondria at low concentrations primarily via the transport system, whereas at high pyruvate concentrations a degree of passive diffusion occurs that cannot be blocked by either transport inhibitor (134). At low to intermediate pyruvate concentrations (0.2–1 mM), addition of transport inhibitor to the perfused heart decreases the flux of pyruvate through the enzyme complex and simultaneously converts the enzyme to 100% active form (134). This data further supports the idea that lowering the pyruvate concentration regulates interconversion, and suggests the possibility that factors influencing exchange rates on the translocator, for example, changes in concentrations of counter ions leaving the mitochondria, might make pyruvate entry rate limiting and thus link pyruvate dehydrogenase activity to metabolic processes producing counter ions (134).

Increases in fatty acid concentrations provide heart tissue with its preferred

fuel source and cause major metabolic changes in the tissue. Studies conducted during the 1960s showed that the availability of fatty acids, ketones, acetate, or long-chain acylcarnitine derivatives substantially decreases pyruvate oxidation in rat heart (28, 97). Subsequently, many similar experiments have confirmed these effects and characterized them in terms of feedback inhibition and interconversion between the active and inactive forms of pyruvate dehydrogenase. In rat heart mitochondria, for example, palmitoylcarnitine oxidation in the presence of 50 μM pyruvate inhibits pyruvate dehydrogenase activity 61%, whereas there is little or no inhibition as the pyruvate concentration increases to 500 μM (43). Although the CoA/acetyl-CoA ratio clearly plays a major regulatory role under these conditions, the relative importance of feedback inhibition of the overall enzyme complex versus direct effects on the kinase is difficult to measure (3, 44). Moreover, not only may the CoA/acetyl-CoA ratio be important but so too may be the absolute concentration of acetyl-CoA (84).

In the perfused rat heart, substrates such as acetoacetate, β-hydroxybutyrate, and octanoate rapidly inhibit as much as 90% of the flux of [$^{14}CO_2$] production from labelled pyruvate (83). However, the percent of active enzyme decreases only by about 50%, which suggests that more than one regulatory effect governs overall flux through the enzyme under these conditions. Direct feedback inhibition by measured increases in acetyl-CoA concentrations in these hearts more than likely accounts for the additional inhibition of flux beyond the decrease in the percent active enzyme. Similarly, in the presence of dichloroacetate to inhibit kinase activity, acetate inhibits the flux of pyruvate through the enzyme complex. Even at 10 mM dichloroacetate, when the enzyme is 100% active, acetate causes a 35% decrease in flux through the enzyme complex. Moreover, in the presence of 10 mM pyruvate, when the enzyme again is 100% active, acetate and octanoate respectively inhibit 27% and 64% of flux through the enzyme complex but change only 2% and 6% of the percent of active enzyme. These experiments demonstrated that both regulatory systems, feedback inhibition and interconversion, operate in the perfused heart.

The regulatory effects of fatty acids and ketone bodies on the pyruvate dehydrogenase complex in the isolated perfused rat liver have been studied extensively in our laboratory. We observed that infusion of fatty acids into livers at a low, physiological concentration (0.05 mM) of pyruvate actually stimulated the decarboxylation of [1-^{14}C]pyruvate. However, at higher pyruvate concentrations, the often observed inhibition of the pyruvate dehydrogenase reaction occurred (24, 89, 113, 145). On the basis of these experimental results, we proposed that fatty acids or any other precursor of acetoacetate (i.e. α-ketoisocaproate, acetate, or β-hydroxybutyrate) stimulate pyruvate dehydrogenase in the liver by an exchange transport mechanism via

the monocarboxylate translocator. This suggestion is illustrated schematically in Figure 4, which indicates that when ketogenesis (intramitochondrial acetoacetate synthesis) is stimulated at low cytosolic pyruvate levels, pyruvate-acetoacetate exchange on the translocator is stimulated. The increase in mitochondrial pyruvate inhibits the pyruvate dehydrogenase kinase, thereby activating the pyruvate dehydrogenase complex (88).

Under normal physiological conditions, glycolysis and the pyruvate dehydrogenase complex play a very important role in brain energy metabolism (63). In contrast to the enzyme in other tissues, pyruvate dehydrogenase activity in brain is not reduced by starvation (122), and remains about 70% active (4, 122). The insensitivity of brain pyruvate dehydrogenase to starvation is thought to be due to the ability of pyruvate to protect the enzyme from inactivation (4); the ability of brain mitochondria to accumulate a high concentration of pyruvate (64) might then maintain the enzyme in an active form and ensure a continuous supply of acetyl-CoA to the citric acid cycle.

Schaffer & Olson (112) demonstrated a rapid increase in [1-^{14}C] pyruvate decarboxylation and in the active proportion of pyruvate dehydrogenase following synaptosomal membrane depolarization. Alternative substrates such as octanoate, acetate, and 3-hydroxybutyrate had no effect on the activation state of the enzyme, in contrast to other tissues (112). Studies using hippocampus slices have demonstrated that pyruvate dehydrogenase becomes phosphorylated during high frequency synaptic stimulation (6). Further studies have suggested a link between activity of brain pyruvate dehydrogenase and calcium uptake by brain mitochondria. Conditions favoring dephosphorylation of brain pyruvate dehydrogenase also increase pyruvate-supported calcium uptake (6).

Regulation of Pyruvate Dehydrogenase by Hormones

Several hormones influence regulatory mechanisms that control the activity of the pyruvate dehydrogenase complex in several tissues. For example, insulin stimulates pyruvate dehydrogenase activity several-fold in adipose tissue. While this response has been studied extensively, one cannot account for pyruvate dehydrogenase activation simply on the basis of changes in Ca^{2+} or changes in other known regulatory molecules within the mitochondria (16, 53, 135). In liver insulin appears to have only a very minor effect on enzyme activity (91). An indirect effect of insulin is to lower plasma free fatty acids in the whole rat and thereby cause enzyme activation by changing the substrates and metabolites in the liver. In the perfused rat heart, conflicting results have been reported. In diabetic rats, nicotinic acid alone lowers the concentration of free fatty acids but does not increase the percent active enzyme, whereas nicotinic acid plus insulin increases the percent active enzyme threefold in these hearts (81). Nevertheless, pyruvate dehydrogenase

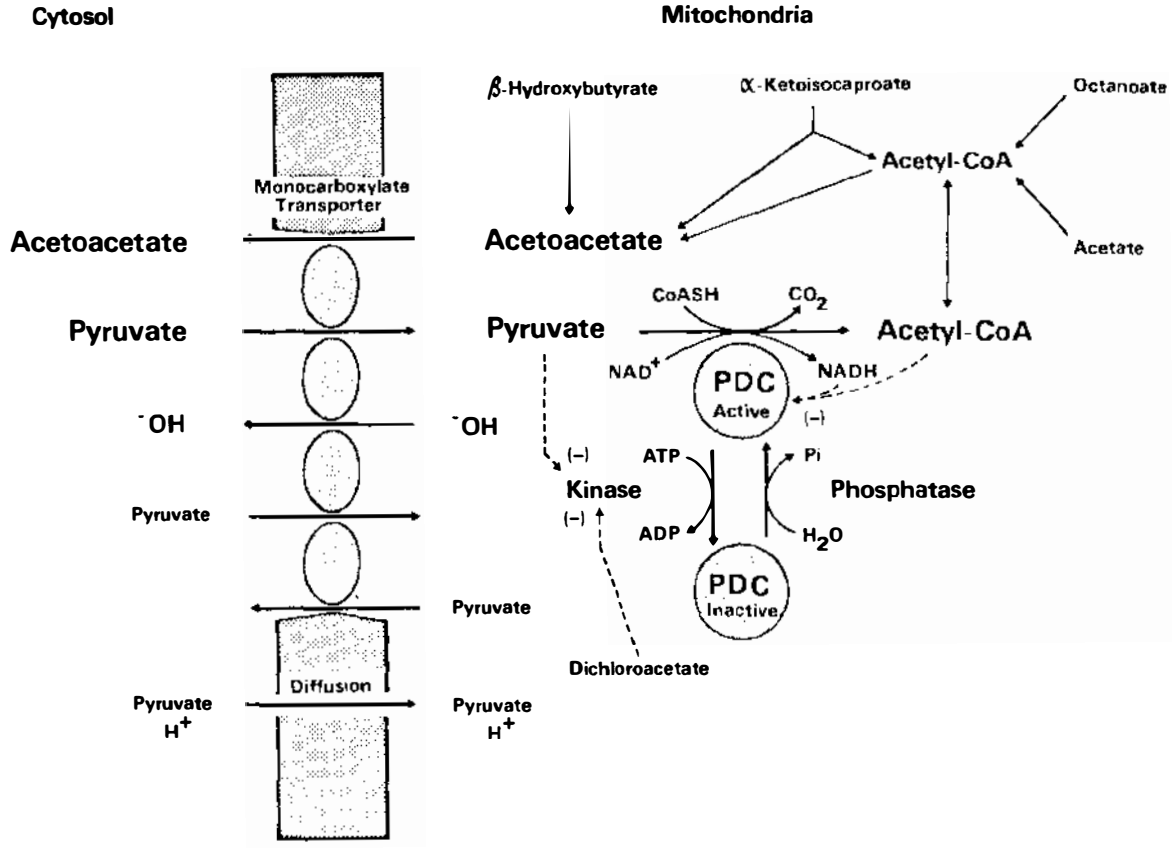

Figure 4 Regulation of the pyruvate dehydrogenase complex by pyruvate/acetoacetate exchange transport on the mitochondrial monocarboxylate transporter.

activation by insulin in diabetic hearts has not been confirmed by others (56). A separate report indicates that in beating mouse heart cells, in culture, insulin stimulates pyruvate oxidation, thereby suggesting enzyme activation in this system (119). Insulin has an activating effect on pyruvate dehydrogenase in lymphocytes (17). Cell-free preparations, from lymphocytes previously treated with insulin, are capable of stimulating pyruvate dehydrogenase activity (17, 18).

The precise signaling mechanism, from the insulin receptor on the cell surface to the pyruvate dehydrogenase complex located on the mitochondrial inner membrane, remains unresolved. Several mechanisms for the pyruvate dehydrogenase response to insulin have been proposed. Alteration of the phosphorylation state of intracellular proteins is a classical mode of insulin signal transduction. However, cell lines harboring mutant insulin receptors that are defective in tyrosine kinase activity are capable of insulin-stimulated pyruvate dehydrogenase activation (36). Several second messengers, activated by insulin binding to its receptor on the cell surface, have been proposed, including H_2O_2, Ca^{2+}, G proteins, and phosphatidylinositol metabolites (142).

One proposal for insulin signal transduction involving pyruvate dehydrogenase phosphatase activation depends upon the production of a small mediator molecule on the outside of the plasma membrane, which then interacts with yet another receptor on the cell surface. Several putative mediators of insulin responses have been characterized (37, 57, 65, 72, 111). One such mediator is derived from the glycophospholipid anchor of a membrane protein (65). Two extracellular enzymes, a *p*- aminobenzamidine-sensitive protease and phospholipase C, catalyze the release of the mediator into the extracellular space (65), where it could be available for both autocrine and paracrine regulation of pyruvate dehydrogenase. This mediator has a molecular weight of ~1000–1500, as judged by gel filtration; it contains D-chiro-inositol, galactosamine, and mannose; evidence suggests that the mediator contains ethanolamine, organic phosphorus, and possibly amino acids (65). Purified mediator can stimulate pyruvate dehydrogenase phosphatase in solution (65) by decreasing the divalent cation requirement of the phosphatase (68). A recently discovered, nonmetal ion-dependent, pyruvate dehydrogenase phosphatase from bovine heart mitochondria is also stimulated roughly threefold by this mediator (68).

A second type of small molecule mediator has been characterized (72, 110, 111); this substance is derived from treatment of an insulin-sensitive glycophospholipid with a phosphatidylinositol-specific phospholipase C (PI-PLC) (37). The polar head group resulting from this treatment was purified by gel filtration chromatography, and its activating effect in whole-cell assays was comparable to that of insulin, although the effect of the mediator declined after 10 min, as opposed to the longer-lasting effect of insulin (37). This

mediator contains inositol, phosphate, glucosamine, and other monosaccharides (110).

Anti-inositol-glycan antibodies can selectively block a portion of the insulin-derived stimulation of pyruvate dehydrogenase (108); PI-PLC or a commercial nonspecific PLC can mimic the activating effect of insulin, but this activation is significantly less than that observed in insulin stimulation (73). Either or both of these small mediators may be responsible, in part, for insulin-triggered activation of pyruvate dehydrogenase.

Mediation of pyruvate dehydrogenase phosphatase by low-molecular-weight species that are generated at the cell surface would require the presence of transport mechanism(s) across the plasma membrane and the inner mitochondrial membrane. The long-term potentiation of pyruvate dehydrogenase phosphatase by insulin cannot be accounted for by these small mediators, especially in light of the observation that toluene-permeabilized mitochondria, isolated after insulin stimulation, maintain the active phosphatase under conditions in which the mediator could freely diffuse out of the mitochondria (26). Perhaps the existence, in the inner mitochondrial membrane, of a large molecular weight regulator that could be activated by the small mediator and then, in turn, cause activation of the pyruvate dehydrogenase phosphatase could account for the insulin effect.

In contrast to insulin, β-adenergic agonists and glucagon stimulate pyruvate oxidation to only a small extent in the perfused rat heart at intermediate to low pyruvate concentrations (46, 76). However, the effect can be demonstrated more clearly in the presence of fatty acids. For example, at 0.5 mM pyruvate, β-hydroxybutyrate decreases pyruvate dehydrogenase activity from about 50% to 28% and simultaneously decreases pyruvate oxidation. Under these inhibitory conditions, addition of isoproterenol increases the level of enzyme activity to about 72% and increases pyruvate oxidation approximately 3-fold (46). Addition of the β-receptor blocker propranolol prevents the β-agonist effect. Morever, the fact that both verapamil and A23187 prevent the β-agonist effect strongly suggests that Ca^{2+} movements are involved in the activation effect of β agonists in the perfused heart (46). Experiments with ruthenium red in the perfused rat heart also demonstrated a similar dependence on Ca^{2+} for enzyme activation (77).

Pyruvate Dehydrogenase Deficiency States

The pyruvate dehydrogenase complex is involved in several pathological conditions. Defects in one or more of the component enzymes of the complex are a major cause of primary lactic acidosis in infants and young children. Brain tissue, with its absolute requirement for aerobic glucose oxidation, appears to be a primary site for these metabolic lesions, with concomitant neurological symptoms. E1α is the major site of disruption, and mutations in

the coding region of the gene account for the majority of the lesions reported (12, 22, 27, 41); one of these mutations involves a 21-base pair insertion of a tandem repeat which alters a serine phosphorylation site (22) that may be related to defective dephosphorylation of this subunit (58). The location of the E1α gene on the X chromosome may expose it to greater chance of damage (82). A mutant form of E1β, containing three frame shifts and an unusual 3′ noncoding region, has been sequenced (51). Rapid proteolysis of uncomplexed E1α or E1β seems to indicate that a defect in one of the genes, which causes either low levels of expression of the subunit or production of a defective subunit, may lead to deficiencies of both pyruvate dehydrogenase subunits (50, 59). Although not as prevalent as E1 deficiencies, abnormalities in E2 and protein X (103) and E3 (45) have been reported.

Primary biliary cirrhosis is an autoimmune disease characterized by the production of antibodies against components of the pyruvate dehydrogenase complex (for recent review, see 33). The major autoantigen appears to be the E2 subunit, and the primary epitope is the inner lipoic acid binding domain. This site is conserved in protein X, thus making it another target of the autoimmune response (125). A third target is E1α; autoantibodies appear to be targeted against one of the functional sites of the protein (29).

Defects in TPP-containing enzymes, including pyruvate dehydrogenase, have been reported in brain and peripheral tissues from patients suffering from Alzheimer's disease (34).

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