A Unifying Mechanism for Stimulation of Mammalian Pyruvate Dehydrogenase Kinase by Reduced Nicotinamide Adenine Dinucleotide, Dihydrolipoamide, Acetyl Coenzyme A, or Pyruvate*

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Marked increases in the rates of phosphorylation and inactivation of the pyruvate dehydrogenase complex by pyruvate dehydrogenase, (PDH) kinase can be induced by NADH, acetyl-coenzyme A or, under certain conditions, pyruvate. Our results support the hypothesis that reduction (by NADH) or acetylation (by acetyl-CoA or pyruvate) of the lipoic moiety of the dihydrolipoyl transacetylase component of the complex is required for stimulation of the PDH kinase activity by these effectors.

NADH and free dihydrolipoamide give half-maximal stimulation of PDH kinase activity at about 4 and 7 μM, respectively. Consistent with a common mechanism for stimulation by these effectors, a combination of 50 μM NADH and 50 μM dihydrolipoamide gives no additional stimulation beyond either added individually at the same concentration. At a concentration of 50 μM, NADH or dihydrolipoamide facilitates acetyl-CoA stimulation, which does not occur in the absence of a reducing compound. At the same thiol concentration, dithiothreitol, dithioerythritol, or β-mercaptoethanol fail either to stimulate PDH kinase activity or to facilitate acetyl-CoA stimulation. Dihydrolipoamide (50 μM) stimulates PDH kinase activity when added in addition to a 40-fold higher concentration of one of these other thiol compounds. Thus, the effects of dihydrolipoamide on PDH kinase activity are very specific and closely parallel NADH stimulation and NADH-facilitated acetyl-CoA stimulation of PDH kinase activity.

Depending on conditions, pyruvate either stimulates or inhibits PDH kinase activity. Acetyl-CoA and low pyruvate concentrations stimulate PDH kinase activity to about the same level and the combination of pyruvate and acetyl-CoA gives no additional stimulation. Acetyl-CoA and pyruvate have parallel salt dependence with greater than 20 mM KCl required to detect stimulation by either. These results are consistent with a common mechanism for stimulation of PDH kinase activity by acetyl-CoA and pyruvate. Phosphate anion, which itself lowers PDH kinase activity, enhances inhibition of PDH kinase activity by high concentrations of pyruvate. However, phosphate anion does not abolish pyruvate stimulation of PDH kinase activity, which is still observed with low pyruvate concentrations and parallels acetyl-CoA stimulation.

Pyruvate dehydrogenase complex, containing acetylated dihydrolipoyl moieties, was prepared by treatment with [3-14C]pyruvate followed by gel filtration. Stimulation of PDH kinase persists with gel filtered enzyme and addition of pyruvate gives no additional stimulation. Furthermore, addition of acetyl-CoA, NADH or a combination of acetyl-CoA and NADH gives little or no additional stimulation of PDH kinase activity. These results further support the proposed involvement of the lipoic moieties since acetylation of lipoic moieties is associated with enhanced PDH kinase activity and elimination of stimulation by pyruvate, acetyl-CoA, and NADH.

The transition state analog, thiamin thiazolone pyrophosphate, blocks acetylation of the lipoic moieties by [3-14C]pyruvate and prevents pyruvate stimulation of PDH kinase activity. However, the analog, although an inhibitor of PDH kinase activity, does not prevent stimulation of PDH kinase activity by NADH or acetyl-CoA.

Other results are presented which indicate that pyruvate stimulation does not result from removal of inhibition of PDH kinase activity by thiamin pyrophosphate.

Mammalian pyruvate dehydrogenase complex(es) are regulated by an interconversion cycle involving phosphorylation with concomitant inactivation which is catalyzed by an endogenous pyruvate dehydrogenase, (PDH) kinase and dephosphorylation, with concomitant reactivation, which is catalyzed by pyruvate dehydrogenase, (PDH) phosphatase (1, 2). The MgATP-dependent activity of the PDH kinase is modulated by a large number of metabolites. NADH and acetyl-CoA

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1 The abbreviations used are: PDH, active, nonphosphorylated form of pyruvate dehydrogenase; PDH, inactive, phosphorylated form; Mops, 3-(N-morpholino)propanesulfonic acid; TPP, thiamin pyrophosphate; TTPP, thiamin thiazolone pyrophosphate; HETPP, α-hydroxyethyl thiamin pyrophosphate.
stimulate PDH, kinase activity (3); ADP, NADH, CoA, and TPP inhibit PDH, kinase activity (3-5). Pyruvate may stimulate or inhibit PDH, kinase activity depending on the conditions (4, 6). In addition, the response of PDH, kinase to the above effectors may be influenced by fluctuations in the levels of free Mg²⁺, K⁺, and phosphate anion (3, 7-9). Thus the activity of PDH, kinase is probably modulated by changes in the intramitochondrial ATP:ADP ratio, NADH:NAD⁺ ratio, acetyl-CoA:CoA ratio, the pyruvate pool, and other conditions that alter the interactions of these metabolites with PDH, kinase. Studies have been reported in which, through the manipulation of these various pools by use of inhibitors, uncouplers, and other conditions, regulation by these different parameters can be demonstrated in isolated mitochondria (e.g. Refs. 10 to 13).

Metabolites, which are also substrates or products for the overall reaction catalyzed by the pyruvate dehydrogenase complex, are conspicuous among the large array of modulators of PDH, kinase activity. This observation raises the question as to whether these metabolites regulate PDH, kinase activity by allosteric interactions following binding at catalytic sites or at regulatory sites. Recently we presented evidence supporting a unifying mechanism which proposes that several of the regulatory effects are mediated through changes in the distribution of the lipoyl moiety of the dihydrolipoyl transacetylase component of the complex between the oxidized, reduced, and acetylated forms (14).

Specifically, we have proposed that NADH stimulates PDH, kinase activity through the reduction of the lipoyl moiety bound to the dihydrolipoyl transacetylase component and subsequent interaction of the dihydrolipate at a site that activates PDH, kinase activity. Acetyl-CoA stimulation of PDH, kinase activity was proposed to require acetylation of the reduced lipoyl moiety with the resultant activity enhanced beyond that due to stimulation by the dihydrolipoyl moiety. We observed: (a) direct stimulation of PDH, kinase by low concentrations of free dihydrolipoamide; (b) a requirement for a reductant (NADH, diithiothreitol, or free dihydrolipoamide) for acetyl-CoA stimulation; (c) prevention of both NADH and acetyl-CoA stimulations by arsenite (which reacts preferentially with reduced diithiol compounds); and (d) thiamin pyrophosphate-dependent pyruvate stimulation of PDH, kinase activity which was prevented by NADH plus arsenite, but not by arsenite alone.

Pyruvate stimulation of PDH, kinase probably is not a major regulatory feature for the regulation of the pyruvate dehydrogenase complex in vivo. It appears that, following decarboxylation of pyruvate, the reductive acetylation of the lipoyl moiety is slower than the subsequent steps catalyzed by the pyruvate dehydrogenase complex. Thus, the fraction of the lipoyl moieties in the acetylated form would tend to reflect the acetyl-CoA:CoA ratio and NADH:H⁺ ratio. Nevertheless, stimulation by pyruvate affords an important approach for analyzing the mechanisms of stimulation of PDH, kinase activity.

An alternative to our proposed mechanism for pyruvate stimulation of PDH, kinase activity is that it results from removal of TPP inhibition by formation of α-hydroxyethyl thiamin pyrophosphate. We have conducted experiments to distinguish these possibilities. We have also investigated the following predictions of our proposed mechanism: (a) that there should be a close parallel between pyruvate and acetyl-CoA stimulation and between stimulation by NADH and dihydrolipoamide; (b) that there should not be any additivity for stimulation by saturating levels of the above pairs; (c) that stimulation by dihydrolipoamide should be very specific and easily distinguished from effects of other thiol and diithiol compounds; and (d) that pyruvate stimulation should persist following acetylation and removal of pyruvate.

**EXPERIMENTAL PROCEDURES**

**Materials** — Imidazole (grade III), 3-(N-morpholino)propanesulfonic acid, NAD (grade I), NADH (grade III), potassium pyruvate, thiamin thiazolone pyrophosphate, ATP, GTP, dithiothreitol, thiamin pyrophosphate, and NAD (grade III), thiamin pyrophosphate, dithiothreitol, and dihdrothreitol, and lipoamide, were purchased from Sigma. CoA, acetyl-CoA, and adenine nucleotides were obtained from P-L Biochemicals, Inc. (γ-32P)ATP and (3-14C)pyruvate came from New England Nuclear. Thiamin thiazolone pyrophosphate was a gift from Dr. G. E. Lienhard, Dartmouth Medical School.

Dihydrolipoamide was prepared by reduction of lipoamide with NaBH₄ (15). To prevent reoxidation and chelation by metal ions, all buffers in which dihydrolipoamide was dissolved were degassed and made 0.1 mm in EDTA.

The pyruvate dehydrogenase complex was isolated from bovine kidney and porcine liver mitochondria as described previously (16). The enzyme was stored at -70°C in the presence of 0.5 mm dithiothreitol.

**Assays** — PDH, kinase activity was determined by measuring the initial rate of 32P incorporation from (γ-32P)ATP (4). The incubation mixtures contained 40 mM Mops (adjusted to pH 7.2 with imidazole), 0.5 mM EDTA, 1.5 mM MgCl₂, and 0.10 mg of kidney pyruvate dehydrogenase complex. The concentration of KCl was 60 mM in all assays except where varied or replaced by potassium phosphate (pH 7.9). Acetyl-CoA, CoA, and pyruvate were added 10 s prior to the addition of ATP. The concentrations of these and other allosteric effectors are described in figure and table legends. All samples were incubated for 60 s at 30°C and PDH, kinase activity was initiated by addition of a mixture of ADP and (γ-32P)ATP (50,000 to 75,000 cpm/nmol). The (γ-32P)ATP concentration was 0.15 mm and the ADP concentration was 0.75 mm (ADP/ATP = 5). The final volume of the reaction mixtures was 0.1 ml. The reaction was terminated after 20, 30, 60, or 90 s, and a 0.05-ml aliquot was assayed for protein-bound radioactivity (4).

For effects of pyruvate, acetyl-CoA, and NADH on the Michaelis constant for ATP and the inhibition constant for ADP, the incubation mixture was the same as above, except that (γ-32P)ATP concentration was varied and ADP, when added, was at a concentration of 0.2 mm.

For determination of protein-bound acetyl groups, the incubation mixture was the same as above, except for the addition of 0.3 mm (3-14C)pyruvate (20,000 cpm/nmol). The (3-14C)pyruvate was added 10 s before a mixture of unlabeled ATP and ADP was added at the concentrations described above. The reaction time was matched with the time of the particular kinase assay in which pyruvate stimulation was observed. A 0.05-ml aliquot was assayed for protein-bound radioactivity (17).

**Preparation of Acetylated Pyruvate Dehydrogenase Complex** — Kidney pyruvate dehydrogenase complex, 3.5 mg in 40 mM Mops/imidazole (pH 7.2), 0.1 mM EDTA, and 3 mm MgCl₂ were incubated with 75 nmol of (3-14C)pyruvate in a final volume of 0.3 ml for 30 s at 30°C. The reaction mixture was transferred to ice, and loaded on a Sephadex G-20 gel permeation column (0.9 x 15 cm). The column was equilibrated at 4°C with 40 mM Mops/imidazole, containing 0.1 mm EDTA and 3 mm MgCl₂. The pyruvate dehydrogenase complex eluted following the void volume, and was eluted in a total volume of 1.2 ml. Protein concentration was assayed by the biuret method.

2 α-Hydroxyethyl thiamin pyrophosphate is used to designate the "active aldehydic" intermediate produced following decarboxylation of pyruvate in addition to the protonated form of this metastable intermediate.
PDH, kinase activity. The indicated concentration of NADH was terminated after 40 s. Other conditions for assays were as described under "Experimental Procedures."

Inactivation of Pyruvate Dehydrogenase Complex with Thiamin Thiazolone Pyrophosphate—Kidney pyruvate dehydrogenase complex, 2.1 mg in 40 mM Mops/imidazole (pH 7.2), 0.1 mM EDTA, and 3 mM MgCl₂ were incubated with 60 nmol of TTPP (thiamin thiazolone pyrophosphate) in a final volume of 1.05 ml for 5 min at 30°. Inactivation of the pyruvate dehydrogenase complex to less than 1% activity was verified by a NAD-reduction assay (4).

RESULTS

Comparison of Dihydrolipoamide and Other Thiol Compounds to NADH in Both Directly Stimulating and Facilitating Acetyl-CoA Stimulation of PDH₆ Kinase Activity—Previously we reported that a low concentration of NADH (0.02 mM) or dihydrolipoamide (0.025 mM) stimulated PDH₆ kinase activity (14). The concentration dependence of these effects is shown in Figs. 1 and 2. Half-maximal stimulation of PDH₆ kinase by NADH or by dihydrolipoamide was observed at about 4 μM NADH or 7 μM dihydrolipoamide.

We have proposed that NADH stimulation of PDH₆ results from the reduction of the lipoyl moieties bound to the dihydrolipoyl transacetylase and subsequent interaction of the dihydrolipoate at a site that activates PDH₆ kinase activity (14). Whether indirectly through the reduction of the bound lipoyl moieties or through direct interaction, we attribute stimulation of PDH₆ kinase activity by free dihydrolipoamide to interaction of a reduced lipoate at the same site.

This proposal predicts that a combination of NADH and dihydrolipoamide, at concentrations saturating for the stimulating effect, would not give any additivity. The results in Table I support this hypothesis. Stimulation by 50 μM NADH or 50 μM dihydrolipoamide was 147 or 148% and the combination similarly yielded 144%.

In contrast to dihydrolipoamide, which gave maximal stimulation of PDH₆ kinase activity at 50 μM, dithiothreitol or dithioerythritol at 50 μM or β-mercaptoethanol at 100 μM not only failed to stimulate, but slightly inhibited, PDH₆ kinase activity (Table I). As previously observed (14), 2 mM dithiothreitol gave slight stimulation of PDH₆ kinase activity. However, addition of 50 μM dihydrolipoamide on top of 2 mM dithiothreitol gave much higher levels of stimulation. Similar pronounced increases were observed with 50 μM dihydrolipoamide on top of 2 mM dithioerythritol and 5 mM β-mercaptoethanol (Table I). These data clearly support the conclusion that stimulation by dihydrolipoamide results from a highly specific interaction rather than a general thiol effect.

Acetyl-CoA failed to stimulate PDH₆ kinase in the absence of an added thiol or other reductant such as NADH. Low concentrations of either NADH or dihydrolipoamide (50 μM) facilitated a high level of acetyl-CoA stimulation beyond that due to dihydrolipoamide (or NADH) alone. However, 50 μM dithiothreitol or dithioerythritol or 100 μM β-mercaptoethanol did not allow significant stimulation of PDH₆ kinase by acetyl-CoA. At a concentration of 2 mM, dithiothreitol and, to a lesser extent, dithioerythritol were effective in facilitating acetyl-CoA stimulation (Table I). However, β-mercaptoethanol, at a concentration of 5 mM, did not allow acetyl-CoA stimulation. These results further support the high specificity of dihydrolipoamide and its similarity to NADH. The high level of acetyl-CoA stimulation observed in the presence of dithiothreitol is consistent with our previous observation that, at high concentrations, dithiothreitol can serve as a source of reducing power for the catalytic reduction of NAD⁺ by the pyruvate dehydrogenase complex (14).

TABLE I

Comparison of dihydrolipoamide and other thiol compounds to NADH in both directly stimulating and facilitating acetyl-CoA stimulation of PDH₆ kinase activity

| Reductant added | Concentration | PDH₆ kinase activity (% of control) with addition of: |
|-----------------|--------------|-----------------------------------------------|
|                 | None         | Dihydrolipoamide (0.05 mM) | Acetyl-CoA |
| None            | 100          | 147                              | 99         |
| NADH            | 0.05         | 148                              | 144        | 216 |
| NADH            | 0.50         | 140                              | 132        | 217 |
| Dihydrolipoamide| 0.05         | 147                              | 230        |
| Dithiothreitol  | 0.05         | 93                               | 143        | 102 |
| Dithiothreitol  | 2.00         | 115                              | 150        | 204 |
| Dithioerythritol| 0.05         | 88                               | 145        | 110 |
| Dithioerythritol| 2.00         | 110                              | 149        | 175 |
| β-Mercaptoethanol | 0.10         | 86                               | 139        | 93  |
| β-Mercaptoethanol | 5.00         | 87                               | 142        | 91  |

Fig. 1 (left). Concentration range for NADH in stimulation of PDH₆ kinase activity. The indicated concentration of NADH was added at the beginning of the incubation. PDH₆ kinase activity was terminated after 40 s. Other conditions for assays were as described under "Experimental Procedures."

Fig. 2 (right). Concentration range for dihydrolipoamide in stimulation of PDH₆ kinase activity. The indicated concentration of dihydrolipoamide was added at the beginning of the incubation. PDH₆ kinase activity was terminated after 40 s. Other conditions for assays were as described under "Experimental Procedures."
Effects of Pyruvate, Acetyl-CoA, and NADH Stimulations on ADP Inhibition of PDH Kinase—Pyruvate, NADH, and acetyl-CoA did not stimulate PDH kinase activity at low KCl concentrations (cf. Fig. 3 discussed below). Since ADP is a more effective inhibitor of PDH kinase at higher levels of KCl (7), the stimulations observed at high KCl concentrations might be at least partially due to reduction of ADP inhibition. The degree of stimulation of PDH kinase activity was increased with ADP inhibited enzyme. However, at 60 mM KCl, pyruvate, NADH, and acetyl-CoA did not change either the Michaelis constant for ATP or the competitive inhibition constant for ADP with respect to ATP.

Most of the assays of PDH kinase activity in the present paper were conducted with an ADP:ATP ratio of 5:1. Our pyruvate dehydrogenase complex preparations have usually contained high levels of endogenous PDH kinase activity. ADP inhibition of PDH kinase improves our ability to make initial velocity measurements with low depletion (i.e., phosphorylation) of the protein substrate. For the above kinetic studies, a particular preparation with lower than normal PDH kinase activity was employed.

Comparison of Acetyl-CoA and Pyruvate Stimulation of PDH Kinase Activity—We have proposed that both pyruvate and acetyl-CoA stimulations of PDH kinase are mediated through the acetylation of the lipoyl moiety which subsequently activates PDH kinase (14). This predicts that there would be no additivity in stimulation of PDH kinase activity by a combination of pyruvate and acetyl-CoA. Since, under some conditions, pyruvate stimulates PDH kinase and, under other conditions, pyruvate inhibits PDH kinase, we selected conditions for this comparison which minimize pyruvate inhibition based on the results in Figs. 3 and 4, described below. The important parameters were a relatively low pyruvate concentration (0.1 mM), the presence of 60 mM KCl, and the absence of phosphate anion. Since stimulation of PDH kinase activity by acetyl-CoA requires a reductant, 2 mM dithiothreitol was included in assay mixtures. Pyruvate or acetyl-CoA, alone, stimulated PDH kinase activity to approximately the same level (Table II). The combination of pyruvate and acetyl-CoA did not yield any additional stimulation. The absence of any additivity clearly supports the premise that acetyl-CoA and pyruvate stimulate PDH kinase activity by the same mechanism. The results described below also support this conclusion.

Effects of Low or High Concentrations of Pyruvate and Acetyl-CoA on PDH Kinase Activity at Various Concentrations of KCl or Potassium Phosphate—Previous studies indicated that pyruvate inhibition of PDH kinase involves a direct interaction of pyruvate with PDH kinase (4). Pyruvate inhibition had been observed to be somewhat variable. We have observed variation with the nature and levels of the salts that were present. Pettit et al. (3) have reported a KCl requirement for acetyl-CoA and NADH stimulation of PDH.

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Effects of low and high concentrations of pyruvate (Pyr) and acetyl-CoA (Ac-CoA) on PDH kinase activity at various concentrations of KCl. Assay conditions were as described under "Experimental Procedures," with the addition of the following conditions. Dithiothreitol (2 mM) was included in the incubation mixture for assays in which acetyl-CoA was added. Acetyl-CoA, at a concentration of 0.25 mM, and pyruvate at the concentrations indicated, were added 10 s prior to addition of the mixture of ATP and ADP. PDH kinase activity was terminated after 40 s.

| Compounds added | PDH kinase activity % |
|-----------------|------------------------|
| None            | 100                    |
| DTT             | 119                    |
| DTT + pyruvate  | 228                    |
| DTT + acetyl-CoA| 204                    |
| DTT + pyruvate + acetyl-CoA | 217             |

**TABLE II**

Comparison of acetyl-CoA and pyruvate stimulation of PDH kinase activity

Assay conditions were as described in Table I, except as indicated below. Dithiothreitol (DTT), was added at a concentration of 2 mM at the beginning of the incubation. Pyruvate, at a concentration of 50 μm, and acetyl-CoA, at a concentration of 0.25 mM, were added 20 and 10 s, respectively, prior to addition of the mixture of ATP and ADP. In the absence of effectors other than ADP, 2.9 nmol of 32P were incorporated/min/mg of kidney pyruvate dehydrogenase complex.
kinase activity. We have conducted studies at 0 to 90 mM KCl or 0 to 60 mM potassium phosphate (pH 7.2) to select conditions that enhanced pyruvate inhibition or pyruvate stimulation of PDH kinase activity and to compare the salt dependence of pyruvate stimulation and acetyl-CoA stimulation. In Fig. 3, the control values decrease with increasing KCl primarily due to increased ADP inhibition (7) but also due to a slight decrease in kinase activity at higher KCl concentrations (i.e. in the absence of ADP). The results in Fig. 3 reveal a close parallel between pyruvate and acetyl-CoA stimulation; with either effector, KCl concentrations greater than 20 mM were required before significant stimulation was observed. Both effectors were inhibitory in the absence of KCl. The lower level of stimulation achieved with 2 mM pyruvate than with 0.1 mM pyruvate presumably reflects some pyruvate inhibition of PDH kinase activity at the higher concentration.

The results, shown in Fig. 4, demonstrate that phosphate anion enhances pyruvate inhibition of PDH kinase. In contrast to the results obtained with KCl, higher concentrations of pyruvate inhibited PDH kinase activity at all potassium phosphate concentrations. However, at high potassium phosphate concentrations, low concentrations of pyruvate stimulated PDH kinase activity in a manner parallel to acetyl-CoA. Thus, although phosphate enhanced pyruvate inhibition, it did not prevent pyruvate stimulation of PDH kinase activity.

The data shown in Figs. 3 and 4 are for bovine kidney pyruvate dehydrogenase complex. Similar salt dependence was observed with porcine liver pyruvate dehydrogenase complex.

It should also be noted that phosphate anion does inhibit PDH kinase activity. For instance, at 30 to 60 mM K+ with phosphate as the counterion, PDH kinase activity was about 50% of the level with Cl− as the counterion

**PDH Kinase Activity in Acetylated and Nonacetylated Pyruvate Dehydrogenase Complex Prepared Free of Pyruvate** — If pyruvate stimulation occurs through the acetylation of the lipoyl moiety, then with highly acetylated complex, stimulation should persist following removal of pyruvate. Acetylated pyruvate dehydrogenase complex was prepared by treatment with 0.25 mM [3-14C]pyruvate followed by gel filtration in the presence of a buffer containing 10 μM TPP. Removal of virtually all of the pyruvate by gel filtration was confirmed by a parallel experiment in which 11-[3-14C]pyruvate was used. Control pyruvate dehydrogenase complex also underwent gel filtration and the protein concentrations of the gel-filtered enzymes were measured. There was no change in the level of acetylation of the [3-14C]pyruvate-treated enzyme from the beginning to the end of the PDH kinase assays. An important condition for achieving this result was conducting the studies in the absence of dithiothreitol.

The results, shown in Fig. 5, show that stimulation due to pyruvate treatment persisted after removal of pyruvate. Addition of 0.1 mM pyruvate to the gel-filtered, acetylated pyruvate dehydrogenase complex not only failed to cause further stimulation but slightly inhibited PDH kinase activity. These results support the hypothesis that pyruvate stimulation results from formation of acetylated lipoyl moieties.

It is also possible that pyruvate stimulation could result from removal of TPP inhibition by formation of α-hydroxyethyl thiamin pyrophosphate (HETPP). However, since gel filtration was conducted in the presence of 10 μM TPP, a significant rate of exchange of TPP with HETPP should have removed the stimulatory effect as explained by this postulate.
Acetylated pyruvate dehydrogenase complex was prepared as described under "Experimental Procedures" except that TPP and dithiothreitol at concentrations of 12 μM and 2 mM, respectively, were included in the reaction mixture. Acetylated pyruvate dehydrogenase complex was collected in a volume of 1.2 ml, protein quantitated, and diluted to 2 mg/ml. Control pyruvate dehydrogenase complex (not gel filtered) was diluted to 2 mg/ml in 40 mM Mops/imidazole (pH 7.2) containing 0.1 mM EDTA, 3 mM MgCl₂, and 2 μM TPP. Acetylated pyruvate dehydrogenase complex contained 5.0 nmol of bound acetyl groups/mg of pyruvate dehydrogenase complex. Further incubation with [3-¹⁴C]pyruvate increased this value to 7.1.

Assay conditions for PDH₂ kinase activity were as described under "Experimental Procedures," with allosteric effectors added at the following concentrations: 0.1 mM pyruvate; 10 μM TTP; 2 mM dithiothreitol (DTT); 0.25 mM acetyl-CoA; 0.5 mM NADH; and 0.25 mM CoA. PDH₂ kinase activity was terminated after 30 s.

**Table III**

| Additions                  | PDH₂ kinase activity associated with: | Acetylated pyruvate dehydrogenase complex |
|---------------------------|--------------------------------------|------------------------------------------|
|                           | Control pyruvate dehydrogenase complex | Acetylated pyruvate dehydrogenase complex |
|                           | % control                            | % control                                |
| None                      | 4.5                                  | 100                                      |
| Pyruvate                  | 7.3                                  | 162                                      |
| TPP                       | 2.5                                  | 56                                       |
| Pyruvate + TPP            | 5.7                                  | 127                                      |
| DTT                       | 5.2                                  | 116                                      |
| NADH                      | 6.4                                  | 142                                      |
| DTT + Acetyl-CoA          | 7.2                                  | 160                                      |
| NADH + Acetyl-CoA         | 7.1                                  | 158                                      |
| CoA                       | 3.6                                  | 80                                       |

Stimulation of Mammalian Pyruvate Dehydrogenase₂ Kinase

**Table IV**

| Additions | Nanomoles bound acetyl groups/mg protein | PDH₂ kinase activity |
|-----------|-----------------------------------------|----------------------|
|           | nmol ³²P incorporated/mg protein/min     |                      |
| Control   | 4.4                                     | 6.7                  |
| Pyruvate  | 8.6                                     | 4.8                  |
| TPP       | 0.1                                     | 0.7                  |
| TTPPP     |                                         |  0.8                 |

It has structural similarities to the metastable enamine intermediate produced following decarboxylation of pyruvate. Kinetic studies have revealed that thiamin pyrophosphate has a much lower Michaelis constant than binding constant, suggesting formation of a high affinity intermediate. Thiamin thiazolone pyrophosphate (TTPP) also binds very tightly to Escherichia coli (19) and kidney pyruvate dehydrogenase complexes (20).

Further studies with thiamin thiazolone pyrophosphate aided in showing that acetylation of the lipoyl moieties was required for pyruvate stimulation of PDH₂ kinase activity. In Table IV, the activity of PDH₂ kinase and the level of acetylation of lipoyl moieties were compared for a preparation of kidney pyruvate dehydrogenase complex that was dependent on added TPP (5% activity in the absence of added TPP) and this enzyme to which TPP or TTPPP had been added. The latter enzyme was incubated with TTPPP until it was virtually completely inactive in the overall reaction catalyzed by the pyruvate dehydrogenase complex.

Both TPP and TTPPP inhibited PDH₂ kinase activity (Table IV). Assaying TTPPP binds at the same site as HETPP, this result and the results described in the previous section are consistent with HETPP causing inhibition of PDH₂ kinase activity. With the TTPPP-treated enzyme, there was an increase in both the level of protein-bound acetyl groups and pyruvate stimulation of PDH₂ kinase activity. In contrast, TTPPP prevented acetylation of lipoyl moieties and eliminated pyruvate stimulation. These findings further support the proposal that pyruvate stimulation of PDH₂ kinase activity requires acetylation of bound lipoyl moiety.

In other studies with TTPPP-treated pyruvate dehydrogenase complex (not shown), acetyl-CoA and NADH stimulations of PDH₂ kinase activity were observed. Also phosphate-enhanced pyruvate inhibition of PDH₂ kinase activity was observed.

**DISCUSSION**

Our results demonstrate a close parallel between NADH and dihydrodipicolinate and between acetyl-CoA and pyruvate in stimulating PDH₂ kinase activity. These comparisons reveal similar levels of stimulation and a complete lack of additivity. Significantly higher levels of PDH₂ kinase activity are achieved with acetyl-CoA or pyruvate than with NADH or dihydrodipicolinate. This result suggests that these pairs stimulate PDH₂ kinase activity by different mechanisms. A common mechanism, such as stimulation of PDH₂ kinase...
Activity resulting from release of inhibition by the oxidized form of the lipoyl moiety (21), seems unlikely. According to our proposed mechanism (14), illustrated in Fig. 6, the acetylated form of the lipoyl moiety would be more effective than the reduced form in stimulating PDH kinase activity.

Our results clearly indicate that the stimulation of PDH kinase activity by free dihydrolipoamide does not result from a general thiol effect but is highly specific. At the same concentrations, other thiol compounds could not replace dihydrolipoamide in stimulating PDH kinase or facilitating acetyl-CoA stimulation. In addition, dihydrolipoamide could stimulate PDH kinase activity in the presence of much higher concentrations of other thiol compounds.

At the concentration that gives half-maximal stimulation, dihydrolipoamide is present at a slightly lower concentration than the subunits of dihydrolipoyl transacetylase and, therefore, lower than the stoichiometry of protein-bound lipoyl moieties (17). Thus it seems most likely that the free dihydrolipoamide directly stimulates PDH kinase activity. However, the catalytic reduction of the covalently bound lipoyl moiety might occur following the reduction first at the active site of the dihydrolipoyl dehydrogenase component by the free dihydrolipoamide. Even lower concentrations of NADH (and, therefore, stoichiometries with respect to bound lipoyl moieties) stimulate PDH kinase activity.

As indicated in the introductory statement, pyruvate stimulation of PDH kinase activity probably is not an important factor in the modulation of this regulatory enzyme in vivo. However, among the evidence supporting an indirect mechanism for stimulation of PDH kinase activity, studies with pyruvate are of central importance. Our studies show that higher PDH kinase activity persists following treatment with pyruvate and gel filtration to remove free pyruvate. An important observation is that NADH and acetyl-CoA can no longer stimulate PDH kinase activity associated with acetylated pyruvate dehydrogenase complex. This is consistent with the concept that NADH and acetyl-CoA cannot stimulate PDH kinase activity because stimulation occurs by the reduction and acetylation of lipoyl moieties and this effect has been saturated by acetylation of the lipoyl moieties by pyruvate.

TPP inhibits PDH kinase activity (5). A possible explanation of pyruvate stimulation of PDH kinase is that formation of the active aldehyde intermediate following decarboxylation of pyruvate might release this inhibition. This proposal is not consistent with the observation that with acetylated pyruvate dehydrogenase complex, deficient in TPP, addition of TPP inhibits PDH kinase activity and addition of pyruvate does not reverse this inhibition.

Since under some conditions pyruvate inhibits PDH kinase activity, it was important to select conditions for studying pyruvate stimulation that minimize pyruvate inhibition. Our studies involving variation in the levels of KCl and potassium phosphate revealed not only a close parallel in the salt dependence for stimulation of PDH kinase activity by pyruvate and acetyl-CoA but also enhancement by phosphate anion of pyruvate inhibition of PDH kinase activity. Dichloroacetate also inhibits PDH kinase activity (22). In Mops/sodium buffer, inhibition by dichloroacetate is also increased by increasing potassium phosphate in the range of 2 to 20 mM. This suggests that pyruvate and dichloroacetate may inhibit PDH kinase activity by binding at the same site. In previous studies (4) of pyruvate inhibition of PDH kinase activity, the inhibition constant for pyruvate was observed to be somewhat variable. This may be a consequence of a mixture of stimulation and inhibition of PDH kinase activity resulting from variation in the apparent inhibition constant.

PDH kinase has a low molecular activity, ~15 mol of $^{32}$P incorporated/min/mol of PDH kinase. For the overall reaction catalyzed by the pyruvate dehydrogenase complex, there is a molecular activity of about 175 mol/min/mol of subunit of the dihydrolipoyl transacetylase. Thus for each turnover of the PDH kinase, the lipoyl moieties of the transacetylase undergo several transformations between the oxidized, reduced, and acetylated forms. Since PDH kinase activity is much slower than interconversion of the lipoyl moieties, there is an increased probability of association of a stimulatory form between catalytic turnovers by PDH kinase. It is also possible that unique and specific lipoyl moieties may be associated with PDH kinase that can undergo reduction and acetylation and that these have a particularly high probability of interacting with PDH kinase. Thus our proposed mechanism does not require the interaction of the reduced and acetylated forms of the lipoyl moieties at a site causing stimulation of PDH kinase activity to be directly proportional to the affinity of substrates and products. Recently we observed that very low NADH/NAD + ratios and acetyl-CoA/CoA ratios effectively reduce the activity of liver or kidney pyruvate dehydrogenase complex by enhancing PDH kinase activity while much higher ratios are required for product inhibition of the overall reaction catalyzed by the complex (16).

In the process of resolving the pyruvate dehydrogenase complex into its component enzymes, the PDH kinase tends to remain tightly associated with the core dihydrolipoyl transacetylase component (23). Based on binding studies with $[^{14}C]$ATP, there appear to be about 2 to 4 PDH kinase molecules associated with our preparations of kidney pyruvate

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**Fig. 6.** Model for lipoic acid mediated stimulation of PDH kinase activity. $F_p$, dihydrolipoyl dehydrogenase (a flavoprotein); $LTA$, dihydrolipoyl transacetylase; $Ac-CoA$, acetyl-CoA.

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1. T. E. Roche, unpublished observation.
dehydrogenase complex. In addition to our observation that very low ratios of NADH or dihydrolipoamide to bound lipoyl moieties can stimulate PDH kinase activity, we have observed that PDH kinase activity increases appreciably with the level of acetylation at lower levels of acetylation but is increased to a lesser degree by higher levels of acetylation. These observations, together with consideration of the low stoichiometry of PDH kinase associated with the pyruvate dehydrogenase complex, are consistent with the possibility that PDH kinase can be maximally stimulated by reduction or acetylation of a few or possibly specific dihydrolipoyl residues.

An interesting possibility is that PDH kinase is associated with a specific and unique subunit of the dihydrolipoyl transacetylase core that contains a lipoyl moiety and that this subunit functions as a regulatory subunit of the PDH kinase. We have prepared highly phosphorylated (completely inactive) pyruvate dehydrogenase complex which allows only a low level of acetylation of lipoyl moieties. With a specific and unique subunit of the dihydrolipoyl transacetylase core to another.

In conclusion, our data support a regulatory mechanism involving the following sequence: (a) substrates and products of the overall reaction bind and undergo conversion at topographically distinct catalytic sites; (b) these diverse catalytic events alter the distribution of the covalently bound lipoyl moiety between the oxidized, acetylated, and reduced forms; and (c) this changes the degree of interaction by the acetylated and reduced forms of this mobile carrier at a specific site (or sites), thereby modifying PDH kinase activity.

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