Bovine Heart Pyruvate Dehydrogenase Kinase Stimulation by α-Ketoisovalerate*

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Purified bovine heart pyruvate dehydrogenase complex was used to investigate the effects of monovalent cations and α-ketoisovalerate on pyruvate dehydrogenase (PDH) kinase inhibition by thiamin pyrophosphate. Initial velocity patterns for thiamin pyrophosphate inhibition were consistent with hyperbolic non-competitive and hyperbolic uncompetitive inhibition at various K+ concentrations between 0 and 120 mM. The Km, Km, and Km for thiamin pyrophosphate were in the range of 0.009 to 6.1 μM over the range of K+ concentrations tested. In the absence of K+, 1 mM α-ketoisovalerate had no effect on PDH kinase inhibition by thiamin pyrophosphate, whereas in the presence of 20 mM K+, α-ketoisovalerate stimulated PDH kinase activity almost 2-fold over the range of 0–80 μM thiamin pyrophosphate. Half-maximal stimulation by α-ketoisovalerate occurred at about 200 μM in the presence of 100 μM thiamin pyrophosphate and 20 mM K+. Similar but less extensive changes occurred in the presence of 100 μM thiamin pyrophosphate and 1 mM NH4+. Initial velocity patterns for PDH kinase inhibition by thiamin pyrophosphate in the presence of 2 mM α-ketoisovalerate were mixed non-competitive, but α-ketoisovalerate increased the Vm and Km for adenosine 5′-triphosphate in the presence of inhibitor. In the presence of thiamin pyrophosphate, PDH kinase remained stimulated after chromatography on Sephadex G-25 to remove α-ketoisovalerate. The results indicate that acylation of pyruvate dehydrogenase complex by α-ketoisovalerate results in PDH kinase stimulation but only in the presence of monovalent cations and thiamin pyrophosphate.

Mammalian pyruvate dehydrogenase complex is a multienzyme complex containing three enzymes, pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase, that catalyzes the conversion of pyruvate to acetyl-CoA and CO2, with the concomitant production of 1 equivalent of NADH (1–3). In addition to allosteric regulation by cofactors and end products, a protein kinase and protein phosphatase regulate activity of the enzyme complex through a cycle of phosphorylation and dephosphorylation. Tightly bound PDH kinase phosphorylates the α subunit of PDH and inactivates the enzyme complex, while PDH phosphatase dephosphorylates PDH and reactivates the enzyme complex.

Bovine PDH kinase has been purified from kidney enzyme complex and contains two dissimilar subunits of Mr 48,000 and 45,000 (4). Kinase activity resides in the larger subunit, and it has been suggested that the smaller subunit serves a regulatory role. In contrast, heart PDH kinase has not been purified. The kinase phosphorylates 3 serine residues on the α subunit of PDH and is highly specific for PDH, although it also has been shown to phosphorylate casein and several synthetic peptides at much lower rates (5). The phosphorylation sites on the α subunit have been identified and sequenced, and it has been shown that PDH inactivation correlates closely with phosphorylation at site 1 (6). The role of phosphorylation at the other two sites has not been elucidated.

In general, substrates, end products, and cofactors all modulate PDH kinase activity. In addition, several other non-physiological molecules such as 5,5′-dithiobis(2-nitrobenzoic acid) (7) and dichloroacetate (8) have also been shown to inhibit PDH kinase. Furthermore, data from separate laboratories support at least two regulatory schemes involving either direct or indirect control of PDH kinase activity. For instance, experiments with isolated components of the kidney enzyme complex show that acetyl-CoA, NADH, pyruvate, and ADP directly regulate PDH kinase activity in the absence of dihydrolipoyl transacetylase (9), whereas experiments with intact kidney enzyme show that regulatory effects may be transmitted indirectly via reduction or acetylation of lipoyl groups on the dihydrolipoyl transacetylase component (10, 11). Moreover, indirect regulatory effects depend on the K+ concentration, and high K+ concentrations alone inhibit kidney PDH kinase (10).

Several studies have shown that TPP also inhibits PDH kinase, in addition to its role as a cofactor in the overall PDH reaction. Binding studies with [35S]TPP (12, 13) demonstrate a stochiometry of two TPP sites per PDH tetramer and a Km in the range of 6–17 μM, but the actual subunit location of the TPP-binding site on PDH has not been determined. Therefore its proximity to any of the three phosphorylation sites also is not known.

Steady-state kinetic experiments demonstrate that TPP causes uncompetitive inhibition of PDH kinase with respect to ATP (14). However, TPP does not inhibit PDH kinase with casein as substrate, indicating that its effect is on the substrate rather than on the kinase (15). Uncompetitive inhibition indicates that PDH-bound TPP only interacts with the kinase-ATP complex and not with the kinase alone. However, this is an unusual case because there are two substrates in the reaction and the inhibitor actually binds to one of the substrates rather than to the enzyme. Moreover, we recently showed that K+ stimulates heart PDH kinase, in contrast to kidney PDH kinase, and that TPP blocks K+ stimulation (16). We also observed that α-ketoisovalerate...
reverses the effect of TPP (17). In this sense, α-ketoisovalerate stimulates PDH kinase. These observations suggested alternative mechanisms for TPP inhibition and therefore we have examined the effects of K' and α-ketoisovalerate on TPP inhibition in more detail.

**MATERIALS AND METHODS**

Reagents—Dithiothreitol, CoA, NAD+, and ATP were purchased from P-L Biochemicals, and [γ-32P]ATP was from Du Pont-New England Nuclear. Pyruvate was from Boehringer Mannheim. Behring Diagnostics was the source of TPP. Bovine serum albumin fraction V, imidazole, MOPS, and Na,EDTA were from Sigma. Trichloroacetic acid, MgCl2, NH4Cl, and MgATP were from Fisher. Sodium pyrophosphate and α-ketoisovalerate were from Aldrich. Liquiscint was from National Diagnostics. Prepackaged PD-10 columns (Sephadex G-25) were from Pharmacia Inc.

Purification of PDH—Bovine heart PDH was purified as described previously (18), with several modifications (16). All pH adjustments during purification were made with NaOH to exclude K’ from the enzyme preparation. Imidazole MOPS, pH 7.4, was used to resuspend the final enzyme pellet and was used as the buffer in all subsequent manipulations of the enzyme. Enzyme activity was assayed as described previously (16). Specific activities of the preparations used in this study were 19.2, 17.2, and 13.8 pmol of NADH/min/mg of PDC.

Kinetic Analysis—Activity of PDH kinase was measured by following 32P incorporation into trichloroacetic acid-precipitable PDC, as described previously (16). Reactions contained, in a final volume of 150 μl, 6.9 mM MOPS, pH 7.4, 1.5 mM MgCl2, 0.5 mM Na,EDTA, 0.5 mM [γ-32P]ATP (2000-50,000 dpm/μmol), 0.5 mg/ml PDC, and additions noted in the figures. Enzyme was equilibrated with the reaction mixture at 30 °C for 1 min, and the reaction was started by addition of [γ-32P]ATP. At 30 and 60 s after [γ-32P]ATP addition, aliquots of the reaction were spotted on squares of Whatman 8MM filter paper, the papers were quenched in 500 μl of 10% trichloroacetic acid, 20 mM sodium pyrophosphate, and 32P incorporation was quantitated by liquid scintillation counting of dried filter papers in 10 ml of Liquiscint.

Preparation of Acylated PDC—Two Pharmacia PD-10 columns were equilibrated and stored at 4 °C in 6.9 mM MOPS, 20.6 mM imidazole, pH 7.4. Enzyme was incubated for 0 min at 30 °C in reaction mixture containing 1.73 mM MOPS, 5.15 mM imidazole, pH 7.4, 0.5 mM MgCl2, 0.5 mM Na,EDTA, 0.5 mM [γ-32P]ATP (2000-50,000 dpm/μmol), 0.5 mg/ml PDC, and additions noted in the figures. Enzyme was equilibrated with the reaction mixture at 30 °C for 1 min, and the reaction started by addition of [γ-32P]ATP. At 30 and 60 s after [γ-32P]ATP addition, aliquots of the reaction were spotted on squares of Whatman 8MM filter paper, the papers were quenched in 500 μl of 10% trichloroacetic acid, 20 mM sodium pyrophosphate, and 32P incorporation was quantitated by liquid scintillation counting of dried filter papers in 10 ml of Liquiscint.

**RESULTS**

Initial Velocity Patterns for TPP Inhibition of PDH Kinase at Different K’ Concentrations—Studies have shown that K’ stimulates a number of different enzymes including heart PDH kinase (16). Recently, we also demonstrated that TPP prevents heart PDH kinase stimulation by K’, and that TPP has very little inhibitory effect on PDH kinase in the absence of K’. Other studies have indicated that TPP is an uncompetitive inhibitor of pig heart PDH kinase with respect to ATP (14), although the actual data for the initial velocity patterns were not shown in this study and there were no comparisons of data fit to different kinetic equations. In order to gain additional mechanistic information on the interactions of K’, TPP, and ATP with bovine heart PDH kinase, and to provide comparisons of data fit to different equations, the steady-state inhibition pattern for TPP as a function of ATP concentration was determined at four K’ concentrations.

Preliminary inhibition data were collected at 0, 1, 2, 5, and 50 μM TPP. This data suggested that distinct inhibition lines could be generated at even higher TPP concentrations. Consequently, extensive data were collected at 0, 0.002, 0.005, and 1 mM TPP.

In Fig. 1, eight points comprising the substrate saturation curve at 0 μM TPP and 0 mM K’ were fit to Equation 1, and the best fit was used to draw the line through the data. Subsequently, each set of eight data points in each panel of Fig. 1 was fit separately to Equation 1 and the best fitting line was drawn through the data. By this method, the patterns of intersecting lines obtained in Fig. 1 clearly demonstrate that the inhibition pattern for TPP changed from a form of noncompetitive inhibition to a form of uncompetitive inhibi-
be related to the fact that TPP binds to PDH rather than to
3 to calculate the relevant inhibition constants.

produce additional incremental inhibition (16), which is con-
high TPP concentrations do not provide total inhibition may
mechanism.

Data determined at 0, 0.002, 0.005, and 1 mM thiamin pyrophosphate. Data from 8
determinations at each ATP concentration were averaged and plotted, as shown in Fig. 1. The combined data from each panel in Fig. 1 then were fit to Equations 2 and 3.

An additional effort was made to determine inhibition

Previous studies showed that near-maximal TPP inhibition
occurs at \( \approx 10 \mu M \) TPP, but that higher TPP concentrations
produce additional incremental inhibition (16), which is con-
consistent with the data in this study showing that distinct
inhibition lines can be obtained at 1 mM TPP. The fact that
high TPP concentrations do not provide total inhibition may
be related to the fact that TPP binds to PDH rather than to
PDH kinase and that the E-S-I complex produces products,
as will be discussed below in the context of the inhibition
mechanism.

An additional effort was made to determine inhibition
constants by fitting data to the equations for hyperbolic
noncompetitive and hyperbolic uncompetitive inhibition.

Table I shows the comparisons for hyperbolic noncom-

Table I

<table>
<thead>
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<th>KCl</th>
<th>Hyperbolic noncompetitive</th>
<th>Hyperbolic uncompetitive</th>
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<tr>
<td>0 mM</td>
<td>( K_{ih} ) ( 0.0086 \pm 0.0012 )</td>
<td>( K_{ih} ) ( 0.69 \pm 1.1 )</td>
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<td></td>
<td>( K_{hn} ) ( 0.36 \pm 0.015 )</td>
<td>( K_{hn} ) ( 0.078 \pm 0.11 )</td>
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<tr>
<td></td>
<td>( K_{id} ) ( 0.065 \pm 0.00014 )</td>
<td>( K_{id} ) ( 0.065 \pm 0.00014 )</td>
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<tr>
<td></td>
<td>( K_{id} ) ( 0.53 \pm 0.075 )</td>
<td>( K_{id} ) ( 0.53 \pm 0.075 )</td>
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<tr>
<td></td>
<td>( V_{m} ) ( 0.1 \pm 0.026 )</td>
<td>( V_{m} ) ( 0.1 \pm 0.017 )</td>
</tr>
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<td></td>
<td>SS ( 0.141 )</td>
<td>SS ( 0.143 )</td>
</tr>
<tr>
<td>2 mM</td>
<td>( K_{ih} ) ( 2.4 \pm 0.66 )</td>
<td>( K_{ih} ) ( 2.4 \pm 0.64 )</td>
</tr>
<tr>
<td></td>
<td>( K_{hn} ) ( 2.8 \pm 0.67 )</td>
<td>( K_{hn} ) ( 5.1 \pm 1.4 )</td>
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<tr>
<td></td>
<td>( K_{id} ) ( 4.7 \pm 1.1 )</td>
<td>( K_{id} ) ( 54 \pm 6.1 )</td>
</tr>
<tr>
<td></td>
<td>( K_{id} ) ( 39 \pm 5 )</td>
<td>( K_{id} ) ( 2.99 \pm 0.099 )</td>
</tr>
<tr>
<td></td>
<td>( V_{m} ) ( 1.54 \pm 0.079 )</td>
<td>SS ( 0.177 )</td>
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<td></td>
<td>SS ( 0.240 )</td>
<td>SS ( 0.287 )</td>
</tr>
<tr>
<td>120 mM</td>
<td>( K_{ih} ) ( 0.76 \pm 0.23 )</td>
<td>( K_{ih} ) ( 0.76 \pm 0.23 )</td>
</tr>
<tr>
<td></td>
<td>( K_{hn} ) ( 0.54 \pm 0.15 )</td>
<td>( K_{hn} ) ( 0.51 \pm 0.16 )</td>
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<tr>
<td></td>
<td>( K_{id} ) ( 1.2 \pm 0.31 )</td>
<td>( K_{id} ) ( 1.1 \pm 0.30 )</td>
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<tr>
<td></td>
<td>( K_{id} ) ( 45 \pm 4.4 )</td>
<td>( K_{id} ) ( 54 \pm 4.4 )</td>
</tr>
<tr>
<td></td>
<td>( V_{m} ) ( 3.57 \pm 0.12 )</td>
<td>( V_{m} ) ( 3.749 \pm 0.12 )</td>
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<td>SS ( 0.347 )</td>
<td>SS ( 0.287 )</td>
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Concentration. By this method, the inhibition constants for TPP varied between 0.0086 and 5.1 \( \mu M \), but did not show any linear correlation to the \( K^+ \) concentration. As previously shown (16), the \( K_{hn} \) and \( V_{m} \) increased and then decreased as the \( K^+ \) concentration increased over the range of 0 to 120 mM. At 2 and 20 mM \( K^+ \), the data fit the hyperbolic noncom-

PDH Kinase Stimulation by \( \alpha \)-Ketoisovalerate in the Presence of TPP—The TPP inhibition in the presence and absence of \( \alpha \)-ketoisovalerate was measured to determine the extent of TPP inhibition under potential regulatory condi-
tions. Furthermore, the experiment was performed at two \( K^+ \)
concentrations, 0 and 20 mM, to represent both minimal and
maximal TPP effects on PDH kinase activity.

In the absence of \( K^+ \), data in Fig. 2 show that there is
essentially no difference in TPP inhibition in the absence or
presence of 1 mM α-ketoisovalerate. Over the range of 0–80 μM TPP, activity in the absence of K⁺ declined from 3.3 to 1.9 nmol of 32P/min/mg of PDC in the absence of α-ketoisovalerate, and from 3.4 to 2.2 nmol of 32P/min/mg of PDC in the presence of α-ketoisovalerate, representing 35 and 37% decreases, respectively. However, at 20 mM K⁺, TPP inhibited activity 67% in the absence of α-ketoisovalerate, from 6.1 to 2.2 nmol of 32P/min/mg of PDC, whereas it inhibited activity only 32% in the presence of α-ketoisovalerate, from 6.0 to 4.1 nmol of 32P/min/mg of PDC. The increase in activity from 1.9 nmol of 32P/min/mg of PDC in the absence of ω-ketoisovalerate decreases, respectively. However, at 20 mM α-ketoisovalerate, representing 35 and 37% of the turnover number for α-ketoisovalerate is =5.7/min. The concentration of α-ketoisovalerate in Fig. 2 was 1 mM, in Fig. 4 was 2 mM, and in Fig. 3 α-ketoisovalerate was varied between 1 μM and 10 mM. The incubation and assay time in all reactions totaled 2 min, 1 min for enzyme equilibration in the presence of effectors, and 1 min for the PDH kinase reaction with ATP. The Kₐ for TPP in the pig heart pyruvate dehydrogenase reaction is ~0.76 μM (14).

From these values, it can be seen that the PDH kinase reactions in Figs. 2 and 4 were carried out near or above the Kₐ for α-ketoisovalerate and well above the Kₐ for TPP when TPP was included in or varied in the reaction. The 1-min equilibration before ATP addition indicates that at least several turnovers of α-ketoisovalerate can occur before the start of the PDH kinase assay. Therefore, in the experiments reported here, any resonance stabilization of TPP due to α-ketoisovalerate decarboxylation should occur prior to the start of the assays by ATP addition. Consequently, the binding constant for TPP may be assumed not to change during the course of these experiments.

In Fig. 3, the α-ketoisovalerate concentration was varied from 1 μM to 10 mM at both 20 mM K⁺ and 1 mM NH₄⁺ to determine its half-maximal stimulatory concentration at a maximal inhibitory concentration of 100 μM TPP. From the data in Fig. 3, the half-maximal stimulatory concentrations of α-ketoisovalerate were estimated to be 200 and 500 μM in the presence of 20 mM K⁺ and 1 mM NH₄⁺, respectively.
Initial Velocity Patterns for TPP in the Presence and Absence of α-Ketoisovalerate—Data in Fig. 4 show the results of additional experiments performed to determine the effect of α-ketoisovalerate on the initial velocity pattern for TPP inhibition. All assays were done at 20 mM K⁺ in order to measure the maximum TPP inhibitory effect. In addition, high inhibitor concentrations were used in order to discriminate as clearly as possible between separate inhibition lines in the reciprocal plot.

Data in the upper panel of Fig. 4 correspond to the data in Fig. 1 at 20 mM K⁺, except that the TPP concentrations in Fig. 4 were 0.1 and 4 mM. At these higher inhibitor concentrations, however, both inhibition lines were parallel with respect to each other (slopes = 0.03 ± 0.002 and 0.031 ± 0.001), but not with the control line in the absence of inhibitor (slope = 0.023 ± 0.001), demonstrating a form of mixed noncompetitive inhibition. In the presence of 2 mM α-ketoisovalerate, the inhibition pattern also was mixed noncompetitive, and the effect of α-ketoisovalerate was to increase both Vₐ and Kₐ in the presence of inhibitor. In the presence of 0.1 mM TPP, the Vₐ increased from 1.7 to 2.1 nmol of ³²P/min/mg of PDC when α-ketoisovalerate was added and the Kₐ increased from 50 to 67 μM. Similarly, at 4 mM TPP, the Vₐ increased from 1.2 to 1.7 nmol of ³²P/min/mg of PDC when α-ketoisovalerate was added and the Kₐ increased from 36 to 51 μM. However, in the absence of TPP, α-ketoisovalerate decreased Vₐ from 2.5 to 2.1 nmol of ³²P/min/mg of PDC, and decreased Kₐ from 68 to 44 μM. This indicates that α-ketoisovalerate alone had a slight inhibitory effect at 20 mM K⁺.

TPP Inhibition of PDH Kinase in Acylated and Nonacylated Enzyme Complex—Previous experiments have shown that α-ketoisovalerate acylates two subunits of bovine heart PDH (17). Data in Figs. 2–4 therefore suggested the possibility that changes in PDH kinase inhibition by TPP might be due to PDC acylation by α-ketoisovalerate. To provide experimental support for this idea, enzyme complex was incubated with either buffer or α-ketoisovalerate, and then was chromatographed on a desalting column to remove reactants. Both enzyme samples were assayed for PDH kinase inhibition by TPP in the presence and absence of α-ketoisovalerate. Assays also contained 20 mM K⁺ in order to observe the maximum effects of TPP and α-ketoisovalerate.

Data in Fig. 5 show that TPP inhibited PDH kinase in nonacylated enzyme as it did in Fig. 2, and that α-ketoisovalerate stimulated PDH kinase in nonacylated enzyme as it did in Fig. 2. Data in Fig. 5 also show that acylated enzyme had the same PDH kinase activity as nonacylated enzyme stimulated with α-ketoisovalerate, indicating that acylation can account for the stimulatory effect of α-ketoisovalerate. Furthermore, addition of α-ketoisovalerate to acylated enzyme caused only a slight increase in the stimulatory effect, and therefore further supports the idea that acylation can account for PDH kinase stimulation in the presence of TPP.

DISCUSSION

Kinetic studies indicate that PDH kinase proceeds through an ordered sequential mechanism where ATP binds before PDC and PDH dissociates before ADP (25). Previous studies also have indicated that TPP causes competitive inhibition of PDH kinase with respect to ATP. However, the molecular details of TTP inhibition have been difficult to determine.

Changes in the apparent Kₐ of TPP for phosphorylated and nonphosphorylated PDH suggest that the phosphorylation site and the TPP-binding site influence each other (15). Phosphorylation also inhibits the formation of α-hydroxyethyl TPP from TPP and pyruvate, but not the subsequent reaction of α-hydroxyethyl TPP with lipoic acid, and this suggests that there are different conformations or possibly more than one binding site for the two forms of the cofactor. Also, the transition state analog thiamin thiazolone pyrophosphate inhibits the kinase reaction but not the phosphatase reaction, and this has been interpreted to mean that thiamin thiazolone pyrophosphate binding causes a conformational change that buries the phosphorylatable serine residue, but that once the serine residue has been phosphorylated it remains accessible to the phosphatase even in the presence of thiamin thiazolone pyrophosphate (12).

In contrast, our data show that TPP inhibition depends on K⁺ (16), and the data in Fig. 1 show further specific effects of K⁺ on TPP inhibition. Other workers have shown that K⁺ does not alter TPP binding to PDH (13), and therefore the data in Fig. 1 cannot be explained simply as K⁺ causing TPP to bind to different forms of PDH. We propose that K⁺ provides an additional positive charge at the active site of the kinase and that the additional positive charge contributes to stabilization of negative charges on either ATP or thiamin pyrophosphate, depending on which molecules are present. In this mechanism, K⁺ stabilizes the kinase ATP complex in the absence of any TPP and this effect results in high kinase activity. Conversely, in the presence of the negative charges on the pyrophosphate moiety of TPP, there is less K⁺ stabilization of the kinase-ATP complex and hence lower activity.

Assuming an ordered sequential mechanism (25), Scheme 1 can be proposed to describe the results of TPP inhibition. The upper part of the pathway represents the ordered sequential addition of substrates and assumes no TPP bound to PDH. Alternatively, when PDH contains bound TPP, the mechanism states that the PDH-TPP complex may bind to either the kinase or the kinase-ATP complex via steps k₅ or k₆. The mechanism also states that both k₅ and k₆ ultimately lead to formation of a kinase-ATP-PDH-TPP complex. Data in Fig. 2 show that TPP does not cause complete inhibition at high concentrations and these data are consistent with the breakdown of the kinase-ATP-PDH-TPP complex to products. Hence, steps k₅ and k₆ describe product release. The order of release has been assumed to be the same as in the absence of TPP.
Inhibitor binding via $k_0$ exclusively would represent uncompetitive inhibition, and with breakdown to products would represent hyperbolic uncompetitive inhibition. Binding via $k_0$ also is consistent with an ordered sequential mechanism because PDH (in the form of PDH-TPP) would still be binding to the kinase after formation of the kinase-ATP complex. However, binding of PDH-TPP to both the kinase and kinase-ATP complex would represent noncompetitive inhibition, and with breakdown to products would represent hyperbolic noncompetitive inhibition. Hyperbolic noncompetitive inhibition by TPP has not been described in previous reports and indicates that ATP can bind to the kinase after formation of a kinase-PDH-TPP complex.

Data in Fig. 1 and Table 1 show that increasing the $K'$ concentration from 0 to 2 mM or 20 mM converts hyperbolic uncompetitive inhibition to hyperbolic noncompetitive inhibition, while increasing the $K'$ concentration from 20 to 120 mM converts hyperbolic noncompetitive inhibition back to hyperbolic uncompetitive inhibition. According to Scheme 1, $K'$ therefore must shift the inhibitor binding steps from a single step, represented by $k_0$, to a combination of steps, represented by $k_0$ and $k_0$, and back to a single step, represented by $k_0$. This most likely could occur by a change in the rate constants such that $k_0$ becomes much faster than $k_0$. In turn, changes in rate constants suggest that the $K'$ concentration influences the relative rates of PDH-TPP binding to the kinase and to the kinase-ATP complex.

According to Scheme 1 and our postulate that $K'$ stabilizes charges at the active site of the kinase, there are two effects of $K'$. First, there is a concentration-dependent stabilization of charge that stimulates kinase activity (16), and, second, there is a concentration-dependent effect on the binding of PDH-TPP to the kinase. Concomitant with the second effect, there is a decrease in $K'$ stimulation due to the presence of additional negative charges on TPP.

Such a scheme avoids the problems inherent in a conformational change mechanism for TPP inhibition. First, for instance, it is not thought that TPP concentrations in the mitochondria change, and the enzyme turns over pyruvate even under a variety of inhibitory conditions such as during fatty acid oxidation (26) and flow-induced ischemia (27). Therefore, presumably, TPP remains bound to PDH under all metabolic conditions. It 'TPP causes a conformational change that alters the accessibility of phosphorylation sites, evidence would suggest that no phosphorylation could occur because of the bound TPP. Yet it is clear that PDH undergoes extensive phosphorylation under different metabolic conditions. Second, a conformational change mechanism would seem to imply complete inhibition by TPP, which is inconsistent with the results in Fig. 1. Postulating TPP inhibition as a decrease in the charge stabilization by $K'$ avoids these inconsistencies.

The results presented here also show that $\alpha$-ketoisovalerate stimulates PDH kinase in the presence of TPP. Inhibition by TPP remains a form of noncompetitive inhibition in the presence of $\alpha$-ketoisovalerate, as shown in Fig. 4, but $\alpha$-ketoisovalerate increases the $V_m$ and the $K_m$ for ATP. Data in Fig. 5 show that $\alpha$-ketoisovalerate stimulation persists through gel filtration to remove free $\alpha$-ketoisovalerate. This is similar to other experiments showing that acetylation stimulates kidney PDH kinase (10, 11).

Previously we showed that $\alpha$-ketoisovalerate acylates the dihydrolipoyl transacetylase component of PDC (17), and therefore the effect of $\alpha$-ketoisovalerate might be interpreted simply as higher kinase activity due to the acylated state of the enzyme complex, rather than to a direct regulatory effect on TPP inhibition. However, $\alpha$-ketoisovalerate alone does not stimulate heart PDH kinase, as shown in Fig. 2 at 0 mM TPP. Furthermore, in the absence of $K'$, there is no difference between TPP inhibition in the absence of $\alpha$-ketoisovalerate and TPP inhibition in the presence of $\alpha$-ketoisovalerate, as shown in Fig. 2. Thus, the argument cannot be made that acylation by $\alpha$-ketoisovalerate alone causes higher PDH kinase activity.

Similarly, it is not the case that acylation by $\alpha$-ketoisovalerate simply requires high TPP concentrations, as shown in Figs. 2 and 5 where the stimulatory effect is evident at TPP concentrations as low as 5 mM. In addition, acylation in Fig. 5 was performed at 1 mM TPP prior to chromatography to remove $\alpha$-ketoisovalerate. The effect is specific for a combination of $\alpha$-ketoisovalerate, TPP, and $K'$, and suggests an effect of $\alpha$-ketoisovalerate at the TPP site.

In the overall PDC reaction mechanism, lipoyl groups transfer acetyl moieties from TPP to CoA, and lipoyl groups may be acylated with $\alpha$-ketoisovalerate in the forward direction (17) or by acyl-CoA's in the reverse direction (28). Therefore, it is plausible that the role of $\alpha$-ketoisovalerate is to form acyl-lipoate that subsequently interacts at TPP sites on different PDH subunits to alter sterically the ability of TPP to cause kinetic inhibition.

Alternatively, other chemical intermediates may be envisioned. For instance, it has been shown that 2-acetylthiamin pyrophosphate may be a viable intermediate in the PDH reaction (29). It is possible to speculate that a stable 2-acetylthiamin pyrophosphate might form with $\alpha$-ketoisovalerate as substrate, and that the 2-acylthiamin pyrophosphate would not be an inhibitor due to altered steric interactions. If such a stable intermediate forms with $\alpha$-ketoisovalerate as substrate, this also would explain why kinase stimulation persists after gel filtration to remove $\alpha$-ketoisovalerate. Moreover, if such an intermediate is not as reactive with lipoyl groups as $\alpha$-hydroxyethyl TPP, the effect would not be washed out by turnover in the presence of pyruvate, and this is consistent with the observed $\alpha$-ketoisovalerate inhibition of the overall PDC reaction (17).

In summary, the results presented here indicate that TPP inhibition occurs through interaction at a $K'$ site and that $\alpha$-ketoisovalerate specifically alters this interaction. The role of...
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K⁺ may be to increase the charge stabilization of ATP bound to PDH kinase, and this effect may subsequently promote PDH binding, thereby stimulating PDH kinase activity. Inhibition by TPP would occur by providing additional negative charge that reduces K⁺ stabilization, and acylation by α-ketoisovalerate would prevent the normal inhibition by TPP.

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