Modification of Bovine Kidney Pyruvate Dehydrogenase Kinase Activity by CoA Esters and Their Mechanism of Action*

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The activation of pyruvate dehydrogenase, kinase activity by CoA esters has been further characterized. Half-maximal activation of kinase activity was achieved with about 1.0 \( \mu \text{M} \) acetyl-CoA after a 20-s preincubation in the presence of NADH. More than 80% of the acetyl-CoA was consumed during this period in acetyating sites in the pyruvate dehydrogenase complex as a result of the transacytlation reaction proceeding to equilibrium. At 1.0 \( \mu \text{M} \) acetyl-CoA, this resulted in more than a 4-fold higher level of CoA than residual acetyl-CoA. Activation of kinase activity could result either from acetylation of specific sites in the complex or tight binding of acetyl-CoA. Removal of CoA enhanced both acetylation and activation, suggesting acetylation mediates activation. For allosteric binding of acetyl-CoA to elicit activation, an activation constant, \( K_a < 50 \text{ nM} \) would be required.

To further distinguish between those mechanisms, the effects of other CoA esters as well as the reactivity of most of the effective CoA esters were characterized. Several short-chain CoA esters enhanced kinase activity including (in decreasing order of effectiveness) malonyl-CoA, acetoacetyl-CoA, propionyl-CoA, and methylmalonyl-CoA. Butyryl-CoA inhibited kinase activity as did high concentrations of long-chain acyl-CoAs. Inhibition by long-chain acyl-CoAs may result, in part, from detergent-like properties of those esters. Malonyl-CoA, propionyl-CoA, butyryl-CoA, and methylmalonyl-CoA, obtained with radiolabeled acyl groups, were shown to acylate sites in the complex.

Propionyl-CoA and butyryl-CoA were tested, in competition with acetyl-CoA or pyruvate, as alternative substrates for acylation of sites in the complex and as competitive effectors of kinase activity. Propionyl-CoA alone rapidly acylated sites in the complex at low concentrations, and low concentrations of propionyl-CoA were effective in activating kinase activity although only a relatively small activation was observed. When an equivalent level (20 \( \mu \text{M} \)) of acetyl-CoA and propionyl-CoA was used, marked activation of kinase activity due to a dominant effect of acetyl-CoA was associated with acetylation of a major portion of sites in the complex and with a small portion undergoing acylation with propionyl-CoA. Those results were rapidly achieved in a manner independent of the order of addition of the two CoA esters. That indicates that tight slowly reversible binding of acetyl-CoA is not involved in kinase activation. High levels of propionyl-CoA greatly reduced acetylation by acetyl-CoA and nearly prevented activation of kinase activity by acetyl-CoA.

In marked contrast to propionyl-CoA, high concentrations of butyryl-CoA were required to acylate sites in the complex and to inhibit kinase activity. Furthermore, acylation and deacylation of protein components were slow processes with butyryl-CoA. Consistent with this pattern for the reactivity, only when butyryl-CoA was added first did it cause a large decrease in the capacity of acetyl-CoA or pyruvate either to acylate sites in the complex or to activate kinase activity. Thus we have observed a strong correlation between the pattern of acylation and effects on kinase activity with a diverse group of CoA esters.

Several effectors have been shown to modulate the activity of the pyruvate dehydrogenase, kinase which inactivates the pyruvate dehydrogenase complex by phosphorylation of the pyruvate dehydrogenase (PDH)\(^*\) component of the complex. A major group of effectors of PDH, kinase are the substrates and products of the overall reaction catalyzed by the complex. Kinase activity is stimulated by acetyl-CoA, NADH, and low concentrations of pyruvate and inhibited by CoA, NAD\(^*\), and high concentrations of pyruvate (1-5). In addition to being products of the pyruvate dehydrogenase reaction, NADH and acetyl-CoA are also products of fatty acid oxidation as well as amino acid degradation. The activation of kinase activity by NADH and acetyl-CoA constitutes an important, albeit indirect, mechanism for feedback inhibition of the pyruvate dehydrogenase complex.

The stimulatory effect of acetyl-CoA is maximized in the presence of reductants, NADH, dihydrolipoamide (0.05 mM), or dithiothreitol (2.0 mM), and is approximately equivalent to nonadditive with stimulation by low levels of pyruvate (6, 7). It has been of interest to determine whether stimulation of kinase activity, particularly by acetyl-CoA, requires catalytic utilization of that effector (3, 6-8) or simply its allosteric interaction with the kinase (9). We have removed PDH kinase from the transacetylase core by the procedure of Stepp et al. (10), but we have not detected activation of resolved kinase by acetyl-CoA.\(^*\) However, we have occasionally observed some inhibition by CoA which would appear to require an allosteric binding site, and we were concerned that the resolution procedure while not decreasing kinase activity might damage a regulatory site. We felt that the apparently conflicting results might be clarified by a detailed analysis of

\(^*\) This work was supported by National Institutes of Health Grant AM 18320 and by the Kansas State Agricultural Experiment Station (Contribution No. 85-180-5). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the concentration dependence for the effects of acetyl-CoA on kinase activity and by testing the effects of a variety of other CoA esters on kinase activity as well as the capacity of such esters to alter acetyl-CoA and pyruvate stimulation of the kinase. Several CoA esters were found to alter kinase activity. We present evidence that a variety of regulatory effects occur in association with catalytic utilization of those thioesters.

**EXPERIMENTAL PROCEDURES**

**Materials**—Kidney pyruvate dehydrogenase complex (13–15 μmol of NADH min⁻¹mg⁻¹) and α-ketoglutarate dehydrogenase complex were prepared by the procedure of Roche and Cate (11) and stored at −70 °C. NADH, NAD⁺, CoA, acetyl-CoA, succinyl-CoA, β-hydroxy-butyryl-CoA, acetoacetyl-CoA, L-methylmalonyl-CoA, and all long-chain CoA esters (C₆-C₁₆) were purchased from Sigma. Propionyl-CoA and malonyl-CoA were from Pharmacia-P-L Biochemicals. New England Nuclear supplied [1-⁴⁰C]acetyl-CoA, [β-⁴⁰C]methyl-2-⁴⁰C]malonyl-CoA, [2-⁴⁰C]malonyl-CoA, and [2-⁴⁰C]pyruvate. [1-³⁵S]Propionyl-CoA and [1-³⁵S]butyryl-CoA were from Amersham Corp.

**Assays of PDH Kinase Activity**—The initial rate of incorporation of ³⁵P into protein from [γ³⁵P]ATP was determined at least in duplicate at 30 °C. Results are representative of data obtained in multiple experiments. Reproducibility averaged within ±5% with any particular enzyme preparation. Some batch to batch variations in activity profile were noted but usually were within ±15%. Freshly thawed complex was equilibrated at 5.0 mg/ml in 50 mM Mops–K plus 20 mM potassium phosphate (pH 7.3), 60 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.10 mM dithiothreitol, 0.5 mM ADP, 0.1 mM [γ³⁵P]ATP (70,000–100,000 cpm/nmol), the indicated levels of effectors, and 1.0 mg/ml complex in a final volume of 50 μl. To temperature-equilibrated test tubes, the order of addition was buffers and salts followed by effectors, and then the endogenous kinase was added for 20 s (unless otherwise indicated) followed by a mixture of ADP and ATP to initiate kinase activity. After 20 s, the reaction was terminated by applying 35 μl of reaction mixture to a dry paper disc (Whatman 3MM, 22 mm) previously soaked in 10% (w/v) trichloroacetic acid plus 10 mM pyrophosphate. Three 50-min washes and a fourth 1-h wash with 10% (w/v) trichloroacetic acid plus 10 μM pyrophosphate were conducted and the assay completed as described (4).

In some experiments long-chain CoA esters and acetoacetyl-CoA were pretreated with 60 s with 1.0 μg of phosphotransacetylase (specific activity, 220 μmol/min/mg) to convert any contaminating acetyl-CoA to acetylphosphate.

**Acylation of Protein**—Assays were conducted in duplicate at 30 °C in the same buffer as PDH kinase assays. The extent of acylation by labeled CoA esters (60,000–100,000 cpm/nmol) or by pyruvate (40,000–50,000 cpm/nmol) was determined after a 20- or 40-s reaction period at 30 °C. These conditions optimize the extent of activation of kinase (data not shown). The reaction was terminated by addition of aliquots to dry paper discs previously soaked in trichloroacetic acid. Papers were washed and radioactivity determined as previously described (8). Performic acid lability of protein-bound acyl groups was evaluated by placing washed paper discs in a desiccator over performic acid (96 ml of 90% formic acid and 0.5 ml of 30% H₂O₂) for 24 h at room temperature. Free acyl groups were removed, either by treatment in a vacuum desiccator over solid KOH for 24 h or by additional washes of papers in 10% (w/v) trichloroacetic acid.

**Purity of CoA Esters**—The purity of nonradioactive short-chain CoA esters was evaluated by a high pressure liquid chromatography procedure with a 25×1.6-cm Ultrasphere ODS 5-μm column (Alltech). Chromatography was developed with an isocratic system: 160 mM KH₂PO₄ (pH 5.2) plus 20% (v/v) methanol (adapted based on Ref. 12). That system separated CoA and acetyl-CoA from all other short-chain CoA esters except acetoacetyl-CoA. The purity of radioactive CoA esters was analyzed using descending paper chromatography on Whatman P81 papers (movement of front, ~32 cm) using 1-butanol-acetic acid:H₂O (5:2:3). There was no radioactive acetyl-CoA contamination in other short-chain CoA esters.

**RESULTS AND DISCUSSION**

**Effects of Thioesters on PDH Kinase Activity**—To our initial surprise, we found that a structurally diverse group of CoA esters altered PDH kinase activity. Table I shows the effects of a series of straight chain thioesters on PDH kinase activity in the presence or absence of NADH. Thioesters at 10 or 250 μM were incubated with the kidney complex for 20 s prior to initiation of catalytic turnover by the endogenous PDH kinase, and turnover was allowed to proceed for 20 s. Kinase activity was inhibited by a 5:1 ratio of ADP:ATP. These conditions optimize the extent of activation of kinase activity by acetyl-CoA. No other thioester was as effective as acetyl-CoA in increasing kinase activity. Propionyl-CoA gave a small but reproducible increase in kinase activity with greater activation at 10 than at 250 μM in the presence of NADH. With a further increase in chain length to butyryl-CoA, only inhibition of kinase activity was observed.

Longer straight chain thioesters were tested after a pretreatment with phosphotransacetylase which converted contaminating acetyl-CoA to acetylphosphate which was ineffective in altering PDH kinase activity. Small activation effects (not shown) with 10 μM C-6 to C-16 acyl-CoAs were reduced or eliminated by treatment with phosphotransacetylase. At the 250 μM level, C-10 or longer acyl-CoAs inhibited kinase activity. It is difficult to evaluate whether that constitutes a specific effect or detergent-like properties of high levels of the long-chain acyl-CoAs. Pretreatment with low levels of long-chain acyl-CoAs did not change the capacity of acetyl-CoA to activate the kinase (data not shown).

In Table II, the effects of other CoA esters on kinase activity are presented. β-Hydroxybutyryl-CoA had little effect on kinase activity, but acetoacetyl-CoA gave a modest but reproducible increase in kinase activity. We were unable to establish conditions using high performance liquid chromatography that adequately separated acetoacetyl-CoA from acetyl-CoA to ensure that slight contamination was not present. Pretreatment with phosphotransacetylase to remove contaminating acetyl-CoA failed to prevent activation by acetoacetyl-CoA. Subsequent studies revealed that very low levels of CoA were generated by treatment of commercial acetoacetyl-CoA with either phosphotransacetylase or pyruvate dehydrogenase complex.

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3 CoA was detected by a cyclic reaction catalyzed by the pyruvate dehydrogenase complex and phosphotransacetylase in the presence of saturating pyruvate, NAD⁺, and phosphate (M. Cary, M. Rahmatullah, and T. E. Roche, unpublished data). That assay is linear with CoA concentration from low nanomolar to micromolar levels of CoA.
Surprisingly, despite the lack of effect of succinyl-CoA on kinase activity, its structural relative malonyl-CoA was a very effective stimulator of kinase activity, and methylmalonyl-CoA gave a small but reproducible activation. Elsewhere, we established that the effect of malonyl-CoA did not result from contamination of malonyl-CoA with acetyl-CoA, and similarly methylmalonyl-CoA was free of propionyl-CoA. The following CoA esters had no effect on kinase activity: tiglyl-CoA, crotonyl-CoA, glutaryl-CoA, and DL-3-hydroxy-3-methylglutaryl-CoA; acetylcarnitine was also ineffective. Benzoyl-CoA appeared to give a slight activation.

Thus we found significant and apparently specific effects on PDH kinase activity due to propionyl-CoA, butyryl-CoA, acetoacetyl-CoA, malonyl-CoA, and L-methylmalonyl-CoA. We wished to evaluate whether those effects reflected simply the binding or the reactivity of those thioesters. With the exception of acetoacetyl-CoA, we were able to obtain those thioesters with ¹⁴C-labeled acyl groups. We found that all of those thioesters acylated sites in the pyruvate dehydrogenase complex. As reported elsewhere, two protein components that are also rapidly acylated by pyruvate and acetyl-CoA underwent acylation. These were the transacylase core and a structurally distinct protein (designated X) of unknown function that remained associated with the transacylase-kinase subcomplex upon resolution of the pyruvate dehydrogenase complex. Further evidence for acylation by propionyl-CoA and butyryl-CoA will be presented below. Performing acid treatment led to release of greater than 95% of acyl groups incorporated into protein from all of these CoA esters. Further studies reported elsewhere demonstrated that malonyl-CoA stimulation resulted from enzyme-catalyzed decarboxylation to generate acetyl-CoA. We have found that activation by methylmalonyl-CoA was nearly equivalent, using high levels of this CoA ester, to the optimum level of activation achieved with low levels of propionyl-CoA. Following acylation of sites in the complex by [2-¹⁴C]methylmalonyl-CoA, performic acid oxidation released a volatile product (data not shown) which is characteristic of propionic acid but not of methylmalonic acid. Thus it would appear that acylation by methylmalonyl-CoA also proceeds by decarboxylation to form propionyl-CoA prior to its acylation of sites in the complex.

The production of CoA from acetoacetyl-CoA (noted above) may result in part from low levels of acylation of sites in the complex. However, it is not a very effective acylating agent, since it was ineffective in preventing acylation by pyruvate and gave only a small decrease in acylation by acetyl-CoA. On the other hand, our studies presented herein and with malonyl-CoA indicate that very low levels of acylation may be effective.

**Concentration Dependence for Acetyl-CoA and Analysis of Free Acetyl-CoA—The concentration dependencies for activation of PDH kinase by acetyl-CoA in the presence or absence of NADH are shown in Fig. 1. Acetyl-CoA enhanced kinase activity at very low concentrations with half-maximal activation achieved with 1.0 μM effector in the presence or absence of NADH. At concentrations above 200 μM, even with the short pretreatment (20 s) acetyl-CoA gave decreased stimulation of kinase activity. That effect was more pronounced at longer times of pretreatment and was reduced by the simultaneous presence of CoA. It should be noted that complex was pretreated with 0.5 mM dithiothreitol before being diluted 5-fold into assay mixtures. That treatment was important for acetyl-CoA activation in the absence of NADH.

To further analyze what the above concentration dependence means we evaluated, at low levels of acetyl-CoA, what portion of the acetyl-CoA was utilized to acylate sites in the complex and what portion was still available for potentially binding at an allosteric site (Table III). Assays were done

![Graph](image-url)

**Fig. 1.** Concentration dependence for activation of PDH kinase activity by acetyl-CoA in the presence or absence of NADH plus NAD⁺. When added (●), NADH and NAD⁺ were each present at 250 μM. Other conditions were as described under “Experimental Procedures.”

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4 Rahmatullah, M., Roche, T. E., Jilka, J., and Kazemi, M. (1985) Eur. J. Biochem., in press. Decarboxylation was a slow reaction catalyzed by trace levels (<0.1% of protein) of a contaminating enzyme.

5 M. Rahmatullah, J. Jilka, and T. E. Roche, unpublished data (cf. (1986) Fed. Proc. 44, 683). Besides the transacylase core another protein (referred to as protein X) undergoes acylation. As many as 6 acetyl groups can be incorporated into protein X which is tightly associated with the transacylase-kinase subcomplex. We have established that protein X is structurally unrelated to the transacylase core and is not the catalytic subunit of the kinase.

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**TABLE II**

Effects of modified CoA esters on PDH kinase activity in the presence or absence of 250 μM NADH

Kinase activity was measured as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Acyl-CoA added</th>
<th>Acyl-CoA concentration</th>
<th>NADH (250 μM) plus acyl-CoA concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>138</td>
</tr>
<tr>
<td>Acetocetyl-CoA</td>
<td>109</td>
<td>131</td>
</tr>
<tr>
<td>β-Hydroxybutyryl-CoA</td>
<td>102</td>
<td>93</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>99</td>
<td>93</td>
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<tr>
<td>Malonyl-CoA</td>
<td>119</td>
<td>147</td>
</tr>
<tr>
<td>L-Methylmalonyl-CoA</td>
<td>103</td>
<td>109</td>
</tr>
</tbody>
</table>
CoA Ester Effects on Kinase Activity

CoA Ester Effects on Kinase Activity

TABLE III
Correlation of changes in kinase activity with the distribution of acetyl-CoA and its reaction products when kinase activity is initiated

All assays were conducted in the presence of 0.25 mM NAD+ and 0.25 mM NADH. Activation of kinase activity represents an increase in activity beyond the activation by NADH. For the row of data designated,* CoA was removed by conversion to succinyl-CoA by the α-ketoglutarate dehydrogenase complex (4.4 μg/50 μl reaction mixture). α-Ketoglutarate was also included in that incubation mixture at 0.4 mM. CaCl₂ (0.10 mM) was also present in all reaction mixtures and served to lower the Km of the α-ketoglutarate dehydrogenase complex for α-ketoglutarate (13). Other conditions were as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Initial level of acetyl-CoA added</th>
<th>Acetyl-CoA consumed in acetylation</th>
<th>Free acetyl-CoA available</th>
<th>Increase in kinase activity</th>
<th>Mol [%]Acetyl group incorporated/mol complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>μM</td>
<td>μM</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.227</td>
<td>0.013</td>
<td>9</td>
<td>1.58</td>
</tr>
<tr>
<td>0.50</td>
<td>0.456</td>
<td>0.045</td>
<td>15</td>
<td>3.25</td>
</tr>
<tr>
<td>1.00</td>
<td>0.87</td>
<td>0.13</td>
<td>40</td>
<td>6.1</td>
</tr>
<tr>
<td>2.50</td>
<td>2.16</td>
<td>0.34</td>
<td>59</td>
<td>15.1</td>
</tr>
<tr>
<td>5.00</td>
<td>3.40</td>
<td>1.60</td>
<td>79</td>
<td>23.8</td>
</tr>
<tr>
<td>5.00*</td>
<td>4.95</td>
<td>0.06</td>
<td>102</td>
<td>34.7</td>
</tr>
</tbody>
</table>

* CoA was removed by conversion to succinyl-CoA.

with 1.0 mg/ml complex in the presence of 250 μM NADH plus 250 μM NADH*. The acetylation of sites in the complex utilized most of the added [1-14C]acetyl-CoA. That reaction was made favorable by the high concentration of reduced lipoic moieties and other sites available in the complex (≥8.5 μM)8 which can undergo acylation. The result is found to closely approximate what would be expected for a transacylation reaction approaching equilibrium with an equilibrium constant of about 1. Since in 10 s at 4 °C we find that similar levels of acylation occur,9 equilibrium should be attainable by 20 s at 30 °C.

With 1.0 μM acetyl-CoA, which gave in this experiment slightly less than half-maximal activation, only about 0.13 μM acetyl-CoA was not used to acylate sites in the complex, while 0.87 μM CoA was generated by the acetylation reactions. Thus half-maximal activation occurred at an available acetyl-CoA concentration of 0.02 μM in the face of about a 6-fold excess of CoA, a weak inhibitor (1) of kinase activity (see later). Thus very tight binding of acetyl-CoA would be required for activation to result from binding at an allosteric site.

On the other hand, activation may be mediated through acetylation of specific sites in the complex. Half-maximal activation occurred in association with <9.0 acetyl groups incorporated into sites in the complex. As described elsewhere,6 both the transacylase core and a distinct protein are being acetylated.

CoA formed can be converted to succinyl-CoA by simultaneous catalysis by low levels of the α-ketoglutarate dehydrogenase complex (see legend to Table III). With 5.0 μM acetyl-CoA, acetylation was further enhanced by removal of CoA, and activation slightly beyond that achieved in the absence of CoA removal was observed. These conditions greatly reduce the free acetyl-CoA level. Thus, while removing any competitive effects of CoA, these data suggest that for activation to result from allosteric binding of acetyl-CoA to the kinase that very tight binding (Kd <50 nM) with a slow rate of dissociation would be required. Even assuming very rapid association, very slow dissociation and, therefore, tight binding must be invoked because removal of CoA pulls the transacylation reaction in the direction of acetylation of sites in the complex. Thus, under these conditions, the level of free acetyl-CoA should continue to be reduced with time. Thus, these data do not eliminate the potential for regulation by binding at an allosteric site, but they are more easily explained by acetylation of specific sites in the complex mediating activation. Studies with other thioesters were conducted to aid in distinguishing those possibilities.

Analysis of the Effects of Propionyl-CoA and Butyryl-CoA on PDH kinase Activity—In the remaining sections of this paper we present a detailed analysis of the effects of propionyl-CoA and butyryl-CoA on PDH kinase activity. Propionyl-CoA has previously been shown to inhibit the pyruvate dehydrogenase complex from various sources (14, 15). As shown below, propionyl-CoA was a very effective substrate for acetylating sites in the kidney pyruvate dehydrogenase complex, but, while effective at low concentrations, it was a much poorer activator of PDH kinase activity than acetyl-CoA or pyruvate.

Although propionyl-CoA gave a much lower maximal level of activation, half-maximal activation was observed at 1.0 μM propionyl-CoA (Fig. 2, left panel). The fact that, with 1.0 mg/ml complex, propionyl-CoA gave half-maximal activation at about 1.0 μM which was equivalent to acetyl-CoA may reflect achievement of a near equilibrium for the transacylation reaction. As the level of propionyl-CoA was increased stimulation decreased until at near millimolar levels inhibition was observed in the presence of NADH. After longer periods of pretreatment with propionyl-CoA, there was a rapid loss in activation even with fairly low levels of propionyl-CoA.

In contrast, butyryl-CoA had little effect at low concentrations and inhibited kinase activity at higher concentrations with a more pronounced fall-off in activity in the presence of NADH (Fig. 2, right panel). Half-maximal inhibition occurred at butyryl-CoA levels ≥100 μM. The inhibitory effects of butyryl-CoA made it particularly suitable for evaluating the effect of order of addition of that compound on the effects of acetyl-CoA, as well as pyruvate, which activates the kinase to an extent equivalent to acetyl-CoA plus NADH (7).
CoA alone weakly inhibits kinase activity (1). With 10 preparations of complex, we have observed between 5 and 25% inhibition with 250 μM CoA with an average of 15% inhibition. However, the effect was very specific since 3'-dephospho-CoA gave no inhibition. As reported elsewhere (11) CoA inhibition is overcome by acetyl-CoA at low (<0.05) acetyl-CoA:CoA ratios. Fig. 3 shows the effects on kinase activity of varying propionyl-CoA:CoA and butyryl-CoA:CoA. The pool size of thiol ester plus CoA was held constant at 0.75 mM, and all assays were conducted in the presence of a 1:1 NADH:NAD+ ratio (each 250 μM). Also plotted above the appropriate ratio in Fig. 3 are the effects of the same level of acetyl-CoA, but in the absence of CoA.

A propionyl CoA:CoA ratio of about 0.4 gave half-maximal activation (Fig. 3, Panel A). Though low, that is about 3-fold higher than the acetyl-CoA:CoA ratio required for half-maximal activation (11). The maximum stimulation by propionyl-CoA was about half that achieved with the optimal acetyl-CoA:CoA ratio and somewhat lower than the maximal activation achieved with 50 μM propionyl-CoA. At high ratios where near millimolar levels of propionyl-CoA were present, CoA counteracted the fall-off in inactivation observed with propionyl-CoA alone. A similar effect was observed with acetyl-CoA but at lower acetyl-CoA:CoA ratios.

Interestingly, at all butyryl-CoA:CoA ratios (Fig. 3, Panel B), CoA reduced the level of inhibition of kinase activity relative to that by butyryl-CoA alone. As with acetyl-CoA and propionyl-CoA, effects of CoA might result from competition for its binding at the same site, which in this case would result in lesser inhibition by CoA than by butyryl-CoA. However, other effects of CoA, such as its facilitating deacylation of sites in the complex, must be considered.

**Competitive Effect of Propionyl-CoA with Acetyl-CoA or Pyruvate in Altering Acylation of the Complex and Kinase Activity**—As shown in Table IV, 20 μM propionyl-CoA or acetyl-CoA gave similar extents of acylation of sites in the complex after 20- or 40-s incubations at 30 °C which are the same conditions used in studies on kinase activity below. These levels of acylation clearly reflect equilibrium values since slightly higher levels of acylation were observed after 10 s at 4 °C.

The effects of 20 or 250 μM propionyl-CoA on acylation by Pyruvate in Altering Acylation of the Complex and Kinase Activity—As shown in Table IV, 20 μM propionyl-CoA or acetyl-CoA gave similar extents of acylation of sites in the complex after 20- or 40-s incubations at 30 °C which are the same conditions used in studies on kinase activity below. These levels of acylation clearly reflect equilibrium values since slightly higher levels of acylation were observed after 10 s at 4 °C.

**Table IV**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mol acyl group incorporated/ mol complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 s</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>20</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>20</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>20</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>250</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Competitive CoA ester (concentration)</th>
<th>Mol [1-14C]acyl group incorporated/mol complex when acetyl-CoA was added</th>
<th>First</th>
<th>Second</th>
<th>Simultaneously</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionyl-CoA (20 μM)</td>
<td>39</td>
<td>39</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>Propionyl-CoA (250 μM)</td>
<td>17</td>
<td>9</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Butyryl-CoA (20 μM)</td>
<td>54</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Butyryl-CoA (250 μM)</td>
<td>46</td>
<td>11</td>
<td>41</td>
<td>34</td>
</tr>
</tbody>
</table>

20 μM acetyl-CoA were evaluated when [1-14C]acetyl-CoA was added first (for 40 s) or second (for 20 s) or simultaneously for those times (Table V). When both CoA esters were present at 20 μM, near equilibrium levels of acetylation were rapidly achieved at about 39 mol incorporated/mol of complex, sug-
Propionyl groups were incorporated (data not shown). At a level of 100% control, when the [l-14C]propionyl-CoA was added first or second. When added first, propionyl-CoA greatly reduced acylation and, surprisingly, lowered kinase activity to a level below that observed in the presence of propionyl-CoA or pyruvate alone. That would appear to reflect not only elimination of activation of the kinase by pyruvate but also the presence of pyruvate inhibition as discussed below. When pyruvate was added first, higher but still greatly diminished acetylation was observed, and only a small activation of kinase activity beyond that by NADH was observed.

**Competitive Effects of Butyryl-CoA or Pyruvate in Altering Acylation of the Complex and Kinase Activity**—In contrast with acetyl-CoA or propionyl-CoA, acylation by 20 μM butyryl-CoA was slower and increased between 20 and 40 s (Table IV). A much higher concentration (250 μM) gave high levels of acylation. Interestingly, that matched the concentration dependence for butyryl-CoA in inhibiting PDH kinase in Fig. 2. As shown in Table V, addition of even a low level of butyryl-CoA prior to acetyl-CoA reduced the level of acetylation versus that observed when the opposite order of addition was used. That implies that deacylation was slow. The slow rate of acylation and deacylation by butyryl-CoA was confirmed in studies with 250 μM butyryl-CoA. When added first, it greatly reduced acetylation by acetyl-CoA, but when added second or simultaneously, high levels of acetylation were observed. Under the latter conditions a slow time-dependent reduction in acetylation occurred. Acylation by pyruvate was similarly influenced by the order of addition of 250 μM butyryl-CoA (Table VII). The above conclusions were supported by studies on acylation by [1-14C]butyryl-CoA in competition with unlabeled acetyl-CoA and pyruvate (data not shown).

As shown in Tables VI and VII, there were large differences in PDH kinase activity that depended on the order of addition of 250 μM butyryl-CoA and either acetyl-CoA (Table VI) or pyruvate (Table VII). Pretreatment with butyryl-CoA completely eliminated activation by these effectors whereas post-treatment lowered, but did not eliminate, activation. Clearly a close parallel between effects on acylation and kinase activity was observed.

When added first, not only did 250 μM levels of propionyl-CoA and butyryl-CoA prevent activation by pyruvate, but inhibition beyond that due to the CoA esters alone was observed. It seems likely that reflects pyruvate inhibition...
which occurs by allosteric binding of pyruvate to PDH kinase (4, 9) and is enhanced by ADP (16). When pyruvate activation was saturated by pretreatment of complex with a low level of pyruvate prior to dilution into assays, Pratt and Roche (16) detected inhibition with 0.1 mM pyruvate. Interestingly, addition of pyruvate after acetyl-CoA gave lower activity than the opposite order or simultaneous addition (data not shown). When dichloroacetate (which inhibits at the same regulatory site on the kinase as pyruvate) was used in place of pyruvate, additive effects were observed (data not shown). Somewhat lower activities were observed when dichloroacetate was added after acetyl-CoA, but that appeared to reflect decay in the stimulation by acetyl-CoA rather than enhanced inhibition. The lower activity observed when pyruvate was added after acetyl-CoA probably resulted from an enhanced rate of decay of stimulation.  

As noted above, both transacetylase core and a protein designated protein X undergo acylation. Based on peptide maps, it would appear that multiple sites are acetylated in both the transacetylase core and protein X. Activation of kinase activity appears to be associated with low and intermediate levels of acetylation whereas decay in activation appears to occur with time in association with high levels of acetylation with the kidney PDH kinase.

Thus the pattern of change in activation effects is made more complicated by time and concentration-dependent changes in the effects of activators. However, our data show a strong correlation between changes in activation and changes in levels of acylation of sites in the complex. We are unable to explain the various effects of CoA esters on the kinase based on a mechanism involving reversible binding at an allosteric site on the kinase.

Finally, the portion of the pyruvate dehydrogenase complex present in the active form has been shown in various tissues to be affected by long-chain fatty acids as well as by short-chain fatty acids (e.g. Refs. 17-23). Some of these effects were proposed to result from changes in the intramitochondrial acyl-CoA:CoA ratio which, in turn, might lead to an alteration of PDH kinase activity. Our results suggest that the acetyl-CoA:CoA ratio would dominate the latter effects. However, under selected conditions, effects of other CoA esters may be important. For instance, Patel et al. (23) observed that infusion of low levels of propionate into rat liver decreased metabolic flux through the pyruvate dehydrogenase complex, and the fraction of active complex was decreased when liver mitochondria were treated with low levels (<1.0 mM) of propionate. When they infused high propionate levels (e.g. 20 mM), inhibition of the pyruvate dehydrogenase complex was alleviated, and treatment of mitochondria with 2-10 mM propionate activated the complex. Although clearly not the entire explanation (e.g. propionate is a weak inhibitor of the kinase), the activating effects of low levels of propionyl-CoA on kinase activity and the lack of activation (and even slight inhibition) of kinase activity at high levels of propionyl-CoA could contribute to those results.

Acknowledgments—We thank Joseph Jilka and Mohammed Kazemi for their skilled technical assistance. We would also like to thank Alice Clements for drawing the figures and helping with many other aspects.

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