Function and Regulation of Mammalian Pyruvate Dehydrogenase Complex

ACETYLATION, INTERLIPOYL ACETYL TRANSFER, AND MIGRATION OF THE PYRUVATE DEHYDROGENASE COMPONENT*

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We have presented evidence that stimulation of pyruvate dehydrogenase (PDH) kinase activity by pyruvate or by acetyl-CoA is mediated through acetylation of lipoil moieties (Cate, R. L., and Roche, T. E. (1978) J. Biol. Chem. 253, 496-503). In accord with this indirect mechanism for the action of these effectors, we now find that the degree of stimulation of PDH, kinase increases with the level of acetylation with either [3-14C]pyruvate or [1-14C]acetate-CoA as substrate until about 30 acetyl groups are incorporated per molecule of complex. Half-maximal stimulation is observed at about 5 to 6 acetyl residues incorporated. At ratios of effector to pyruvate dehydrogenase complex less than 40:1, virtually all the added effector (pyruvate or acetyl-CoA) is converted to protein-bound acetyl groups under the conditions selected for these experiments. These results clearly support a common mechanism involving acetylation of lipoil moieties for stimulation of the activity of this converter enzyme.

With both [3-14C]pyruvate and [1-14C]acetate-CoA, about 100 mol of acyl-stable acetyl residues are incorporated per mol of complex. Similar levels are also incorporated from pyruvate and acetyl-CoA into resolved dihydrolipoyl transacetylase. Since there are only 60 dihydrolipoyl transacetylase subunits/molecule of complex, more than one lipoil moiety is acetylated per transacetylase subunit.

High levels of acetylation with pyruvate are achieved under conditions in which only a few pyruvate dehydrogenase subunits are functional. Acetyl transfer between lipoil moieties and interchange of the pyruvate dehydrogenase component among sites on the dihydrolipoyl transacetylase can contribute to acetylation under these conditions. The rate of acetylation due to movement of pyruvate dehydrogenase subunits is estimated and is not large enough to contribute to steady state catalysis by the pyruvate dehydrogenase complex. However, we estimate that movement of the pyruvate dehydrogenase component is fast enough to participate in, and possibly be an essential step for, the interconversion of PDH by a fixed PDH kinase.

Pyruvate dehydrogenase complexes from Escherichia coli and mammalian sources contain three catalytic components that act in sequence to convert pyruvate, CoA, and NAD to acetyl-CoA, NADH, and CO₂. The pyruvate dehydrogenase and dihydrolipoyl dehydrogenase components are attached to a core dihydrolipoyl transacetylase component. This last component is a large oligomer consisting of 24 or 60 subunits for the E. coli complex or mammalian complex, respectively (1). The cofactor lipoic acid is covalently attached to the dihydrolipoyl transacetylase component and has been postulated to serve as a swinging arm in the transfer of electrons and acetyl groups between catalytic sites (2). Many features of the structure and function of the complex are consistent with this hypothesis, including: (a) model reactions catalyzed by the resolved components (2-6); (b) detection of expected stable reaction intermediates (7, 8); (c) steady state kinetics of the overall reaction (9); (d) structural organization of the complex (1); and (e) studies with spin-labeled lipoil moieties (10).

However, fluorescence energy transfer experiments with E. coli pyruvate dehydrogenase complex have suggested that the active sites for binding TPP, CoA, and FAD are too far apart to be served by one lipoil moiety (1-14). This problem has apparently been resolved by finding that each of the 24 subunits of E. coli transacetylase contains two functionally active lipoil moieties and that acetyl groups and electrons can be transferred between these moieties (8, 15, 16).

We have presented evidence that, in addition to its roles in the catalytic reactions, lipoic acid has an important role in the regulation of mammalian pyruvate dehydrogenase complex (17, 18). Mammalian pyruvate dehydrogenase complex is regulated by interconversion between an active, nonphosphorylated form and an inactive, phosphorylated form. Phosphorylation is catalyzed by a MgATP requiring kinase, and dephosphorylation by a Mg²⁺- and Ca²⁺-requiring phosphatase. We have proposed that NADH, acetyl-CoA, and pyruvate stimulations of PDH, kinase activity are mediated through the reduction and acetylation of the lipoil moieties (17, 18). This mechanism predicts that there would be a correlation between the stoichiometry of incorporation of acetyl groups from pyruvate or acetyl-CoA and the degree of stimulation of PDH, kinase. We have investigated this correlation using conditions under which virtually all the added pyruvate or acetyl-CoA are converted to acid-stable groups bound to protein except at very high ratios of substrate to complex. Based on previous studies, these acid-stable groups are assumed to be acetylated lipoil moieties (7, 8, 15).

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We have presented evidence that, in addition to its roles in the catalytic reactions, lipoic acid has an important role in the regulation of mammalian pyruvate dehydrogenase complex (17, 18). Mammalian pyruvate dehydrogenase complex is regulated by interconversion between an active, nonphosphorylated form and an inactive, phosphorylated form. Phosphorylation is catalyzed by a MgATP requiring kinase, and dephosphorylation by a Mg²⁺- and Ca²⁺-requiring phosphatase. We have proposed that NADH, acetyl-CoA, and pyruvate stimulations of PDH, kinase activity are mediated through the reduction and acetylation of the lipoil moieties (17, 18). This mechanism predicts that there would be a correlation between the stoichiometry of incorporation of acetyl groups from pyruvate or acetyl-CoA and the degree of stimulation of PDH, kinase. We have investigated this correlation using conditions under which virtually all the added pyruvate or acetyl-CoA are converted to acid-stable groups bound to protein except at very high ratios of substrate to complex. Based on previous studies, these acid-stable groups are assumed to be acetylated lipoil moieties (7, 8, 15).

1 The abbreviations used are: TPP, thiamin pyrophosphate; PDH, active nonphosphorylated form of the pyruvate dehydrogenase component; Mops, 3-(N-morpholino)propanesulfonic acid; TTPP, thiamin thiazolone pyrophosphate.
Stoichiometries of acetylation observed in the present study suggest that in the mammalian pyruvate dehydrogenase complex as in E. coli complex (8, 15) there are two lipoyl moieties per dihydrolipoyl transacetylase and that acetyl transfer between lipoyl moieties may occur. Further studies are described that are consistent with interlipoyl transfer of acetyl groups. However, in contrast with studies on E. coli pyruvate dehydrogenase complex (8, 16), we have also obtained evidence for movement of pyruvate dehydrogenase subunits between dihydrolipoyl transacetylase cores. In addition to possibly contributing to acetylation, this movement of pyruvate dehydrogenase subunits may be important in the regulatory interconversion process.

**EXPERIMENTAL PROCEDURES**

Materials—Imidazole (grade III), 3-(N-morpholino)propanesulfonic acid, NAD (grade III), NADH (grade III), potassium pyruvate (grade III), and thiamin pyrophosphate were purchased from Sigma. Acetyl-CoA and adenosine nucleotides came from P-L Biochemicals. [3H]Acetyl-CoA and [14C]acetyl-S-CoA were obtained from New England Nuclear. Thiamin thiazolone pyrophosphate was prepared by the procedure of Butler et al. (19).

The pyruvate and α-ketoglutarate dehydrogenase complexes were isolated from bovine kidney mitochondria as described previously (20) and stored at −70°C in the presence of 0.5 mM dithiothreitol. Acetyl-CoA and adenine nucleotides came from P-L Biochemicals. In all experiments the effectors used were pyruvate, acetyl-CoA, and ADP.

To determine protein-bound acetyl groups in association with the kinase assays described above, the reaction mixture was the same except for the addition of [3H]pyruvate (20,000 cpm/nmol) or [14C]acetyl-CoA (8000 cpm/nmol). Reaction conditions were identical to those used for kinase assay reaction mixture. The precise pyruvate and acetyl-CoA concentrations were determined enzymatically as described below. Acetylation reactions were terminated after 15 or 3 min, for the acetyl-CoA or pyruvate assays, respectively, and a 0.05-ml aliquot was assayed for protein-bound radioactivity (21). Paper discs, presoaked with 10% trichloroacetic acid (w/v) and then dried, were used in these assays.

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The concentrations of labeled and unlabeled solutions were matched based on these assays.

**Inactivation of Pyruvate Dehydrogenase Complex with Thiamin Thiazolone Pyrophosphate—Pyruvate dehydrogenase complex was inactivated by slow addition of TTP (24) to a reaction mixture containing 2 mg/ml of pyruvate dehydrogenase complex, 0.5 mM EDTA, and 50 mM potassium phosphate (pH 7.2). The mixture was incubated at 30°C and assayed after 10 min in an NAD reduction assay (22). TTP at concentrations of 7.0 and 9.0 μM reduced control pyruvate dehydrogenase complex activity to 24 and 6%, respectively.

Inactivated pyruvate dehydrogenase complex used in the mixing experiment with active enzyme was prepared by incubation of 60 nmoles of TTP with 5.6 mg of pyruvate dehydrogenase complex in 50 mM potassium phosphate (pH 7.2), 0.2 mM EDTA, and 0.5 mM dithiothreitol in a total volume of 1.06 ml. After 10 min at 30°C, enzyme activity was more than 99% inactivated. The treated pyruvate dehydrogenase complex was then dialyzed twice against 500 ml of 50 mM potassium phosphate (pH 7.2), 0.2 mM EDTA, and 0.5 mM dithiothreitol for 12 h, followed by dialysis against 500 ml of 50 mM potassium phosphate (pH 7.2) containing 0.2 mM EDTA for 12 h. Protein concentration was determined by the biuret method (25) and incubated for 12 h. Acetyl-CoA resulted in an increase in enzyme activity to 4% but this enzyme had no activity when assayed in the absence of added TTP. Incubation of inactivated enzyme with an equivalent amount of active enzyme for 6 min at 30°C did not result in the loss of activity of the active enzyme.

**Inactivation of Pyruvate Dehydrogenase Complex with ATP—Kidney pyruvate dehydrogenase complex, 5.6 mg in 50 mM potassium phosphate (pH 7.2), 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM MgCl₂ were incubated with 0.1 μmol of ATP in a final volume of 1.0 ml for 5 min at 30°C. Inactivation to less than 1% activity was verified by an NAD reduction assay (22). The enzyme was then dialyzed against four 500-ml volumes of 50 mM potassium phosphate (pH 7.2) containing 0.2 mM EDTA and 0.5 mM dithiothreitol for 12 h each. Protein was quantitated and diluted to 2 mg/ml. Dialysis did not increase the activity of the enzyme; and incubation of the phosphorylated enzyme with an equivalent amount of active enzyme for 6 min at 30°C yielded no perceptible loss in activity of the active enzyme.

**RESULTS**

**Correlation of the Level of Acetylation by Acetyl CoA or Pyruvate with the Degree of Stimulation of PDH, Kinase—Previously we have shown that stimulation of PDH, kinase activity persists following acetylation with pyruvate of the lipoyl moieties and removal of excess pyruvate by gel filtration (18). To investigate the dependence of kinase stimulation on the level of acetylation of the complex, conditions were found that would allow acetylation of the complex using low concentrations of pyruvate or acetyl-CoA. These conditions were such that essentially all pyruvate or acetyl-CoA was converted to acid stable residue bound to protein at molar ratios of substrate to complex less than 40:1 (Table I). Table I and Figs. 1 and 2 show levels of stimulation of PDH, kinase activity obtained at various levels of acetylation of the pyruvate dehydrogenase complex by pyruvate or acetyl-CoA. The observed stimulation in the absence of free pyruvate or acetyl-CoA (Table I) clearly supports our model for lipoic acid-mediated stimulation of PDH, kinase activity. The close similarity in the curves for pyruvate and for acetyl-CoA is also consistent with this mechanism (Figs. 1 and 2). Maximal stimulation of PDH, kinase activity was achieved at about 30 acetyl groups/molecule of complex, whether pyruvate or acetyl-CoA was the acetylating agent. Half-maximal stimulation of PDH, kinase activity was achieved at 5 or 6 acetyl groups/molecule of complex for the pyruvate or acetyl-CoA curves.
Correlation of the level of acetylation with the degree of stimulation of PDH, kinase activity under conditions of complete depletion of free pyruvate or acetyl-CoA

Each assay was performed in duplicate, once with unlabeled pyruvate or acetyl-CoA, in order to determine kinase activity, and once with [3-14C]pyruvate or [1-14C]acetyl-CoA in order to determine the number of protein-bound acetyl groups. The concentration of pyruvate or acetyl-CoA added to each assay is indicated in the table. Values for PDH, kinase activity are in units of nanomoles of 32P incorporated/mg protein/min. Other conditions for assays were as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Concentration of effector added</th>
<th>Mol effector/mol complex</th>
<th>[14C]Acetyl groups/mol complex</th>
<th>PDH, kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>0.3</td>
<td>2.1</td>
<td>2.1</td>
<td>6.6</td>
</tr>
<tr>
<td>0.9</td>
<td>6.3</td>
<td>5.5</td>
<td>7.2</td>
</tr>
<tr>
<td>1.8</td>
<td>12.6</td>
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<td>8.6</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>10.1</td>
</tr>
<tr>
<td>0.4</td>
<td>2.8</td>
<td>2.8</td>
<td>10.6</td>
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<tr>
<td>0.8</td>
<td>5.6</td>
<td>5.5</td>
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<td>1.6</td>
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<td>35.0</td>
<td>34.4</td>
<td>12.7</td>
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</table>

FIG. 1. Correlation of the level of acetylation by [3-14C]pyruvate with the degree of stimulation of PDH, kinase activity. Pyruvate was added at concentrations ranging from 0.3 to 100 μM. Assay conditions were as described in Table I and under “Experimental Procedures.”

respectively. The lower level of stimulation observed with acetyl-CoA than with pyruvate results from the requirement for a reductant, NADH, to acetylate lipoyl moieties with acetyl-CoA (17). NADH stimulated PDH, kinase activity and, thus, stimulation at each level of acetylation by acetyl-CoA was above an already stimulated rate.

For the same preparations of the pyruvate dehydrogenase complex, the same absolute level of PDH, kinase activity was achieved for acetylation with pyruvate or acetyl-CoA plus NADH to greater than 30 acetyl groups/molecule of complex (18). These levels differ in Fig. 1 and 2 because different preparations of complex with different levels of endogenous PDH, kinase activity were used.

FIG. 2. Correlation of the level of acetylation by [1-14C]acetyl-S-CoA with the degree of stimulation of PDH, kinase activity. Acetyl-CoA was added at concentrations ranging from 0.4 to 20 μM. Assay conditions were as described in Table I and under “Experimental Procedures.”

It was important to ascertain whether plateauing of the stimulatory effect on PDH, kinase activity at 30 acetyl groups/molecule of complex was due to saturation of stimulatory sites or to depletion of sites for phosphorylation. Both PDH and PDH, kinase are bound to the dihydrolipoyl transacetylase and the ratio is fixed in the purified complex. The experiment shown in Fig. 3 was conducted under conditions that allowed reduced rates of phosphorylation in order to minimize depletion of the protein substrate. Conditions included using a lower ATP concentration (0.02 mM) and a

FIG. 3. Rates of phosphorylation of pyruvate dehydrogenase complex at various levels of acetylation by pyruvate. Assay conditions for PDH, kinase activity were as described under “Experimental Procedures,” except that [γ-32P]ATP and ADP concentrations were 0.02 and 0.2 mM, respectively (ADP/ATP = 10). The reaction volume was increased to 0.5 ml, and a 0.05-ml aliquot was assayed for protein-bound radioactivity at the indicated times following initiation of kinase activity. Pyruvate was added after the 60-s incubation, at the following concentrations: ○, none; □, 1.5 μM; ●, 3.0 μM; △, 4.5 μM; ▲, 7.5 μM. The number of protein-bound acetyl groups was determined in a duplicate experiment in which [3-14C]pyruvate was used and assayed at times corresponding to the initiation and termination of the kinase assay. The moles of acetyl groups per mol of complex were constant throughout the duration of the kinase assay and were as follows: ○, none; □, 7.9; ●, 16.8; △, 25.8; ▲, 44.4.
higher ADP/ATP ratio (10:1). As shown in Fig. 3, differences between the rates of phosphorylation were maintained for several time points, both with complex lacking acetylated residues and with enzyme with low or higher levels of acetylation. However, an increase in the level of acetylation from 26 to 44 acetyl groups/molecule of complex did not increase the rate of phosphorylation. Thus, it can be concluded that saturation of the stimulatory effect for acetylation above 30 acetyl groups/molecule of complex does not result from a substrate-limited rate.

The decrease in stimulation of PDH kinase activity observed at values of acetylation greater than 60 acetyl groups/molecule of complex was repeatedly observed but somewhat variable. One factor appears to be the time that the enzyme is allowed to remain at the high acetylation level before initiation of kinase activity since larger decreases in stimulation were observed at longer times.

Acetylation at Low Levels of Active Pyruvate Dehydrogenase and Evidence for Movement of Pyruvate Dehydrogenase Subunits. Recently, it has been shown that acetyl transfer can occur between lipoic moieties in the E. coli pyruvate dehydrogenase complex (8, 16). This process allows one pyruvate dehydrogenase monomer to serve more than one dihydrolipoyl transacetylase subunit. An obvious possibility is that this mechanism may also occur in the pyruvate dehydrogenase complexes from mammalian sources. As in the bacterial system (8, 15), this may utilize more than one lipoic moiety per dihydrolipoyl transacetylase subunit. Using [3-14C]pyruvate or [1-14C]acetate-S-CoA, we have observed values as high as 100 mol of acetyl groups incorporated per mol of kidney pyruvate dehydrogenase complex (e.g. Fig. 4). This is a ratio of 1.66 acetyl groups/dihydrolipoyl transacetylase subunit and suggests that there are two lipoic moieties per transacetylase subunit. That only the dihydrolipoyl transacetylase component undergoes acetylation was established by studies with resolved dihydrolipoyl transacetylase which was acetylated to 83 or 110 acetyl groups/60-subunit core with pyruvate or acetyl-CoA, respectively. These values were not corrected for a small amount of dihydrolipoyl dehydrogenase present in the resolved dihydrolipoyl transacetylase. Resolved pyruvate dehydrogenase (1.0 mg/mg of transacetylase) was added for acetylation with pyruvate. No acetylation was obtained with pyruvate dehydrogenase component alone. Acetylation with acetyl-CoA was conducted as described under “Experimental Procedures” with contaminating dihydrolipoyl dehydrogenase catalyzing the reduction of bound lipoic moieties and with CoA being removed by formation of succinyl-CoA.

In several studies, including results shown in Fig. 1, we have observed high levels of acetylation with kidney pyruvate dehydrogenase complex that had very low activity in the absence of added TPP. This acetylation could result from TPP movement between pyruvate dehydrogenase subunits, exchange of the pyruvate dehydrogenase component between sites on the dihydrolipoyl transacetylase component, or interlipoyl transfer of acetyl groups entering at limited sites from the fixed pyruvate dehydrogenase subunits. Experiments were conducted to test these possibilities. The experiment described below eliminates the possibility that acetylation is due solely to TPP movement.

Kidney pyruvate dehydrogenase complex was prepared with various levels of inactivation by treatment with thiamin thiazolone pyrophosphate. TTPP is a transition state analog that can be used to titrate and selectively inhibit thiamin pyrophosphate binding sites of the pyruvate dehydrogenase component (24). Inhibition by this analog is essentially irreversible. In Fig. 4, the level of acetylation at various times is shown for fully active complex and for two samples that were inactivated to 24 and 6% activity by treatment with TTPP. These assays were conducted with 0.1 mM TPP to ensure that all pyruvate dehydrogenase subunits not inactivated by TTPP would be active. Only a slight decrease was apparent in the extent of acetylation of pyruvate dehydrogenase complex with 24% activity remaining as compared to the control. For complex inactivated to 6% activity remaining, which on the average would have only about one-twentieth of the pyruvate dehydrogenase component in an active form (equivalent to about one active tetrameric unit, αβ2, per complex (11)), more than 80 acetyl groups/molecule of complex were incorporated by 2 min.

To determine whether exchange of the pyruvate dehydrogenase component contributes to this high level of acetylation, we conducted “mixing experiments” using active pyruvate dehydrogenase complex and complex that had been inactivated by treatment with TTPP or by phosphorylation with ATP. Excess TTPP or ATP was removed by extensive dialysis. In Figs. 5A and 6A, the levels of acetylation are shown at various times for active pyruvate dehydrogenase complex, inactive pyruvate dehydrogenase complex, and mixtures of the two complexes. The preincubation period before addition of pyruvate was 1 min and twice as much inactive complex as active complex was present in the indicated assays. The top curve (A) shows the total number of bound acetyl groups, while the bottom curve (B) is normalized with respect to the amount of protein present. When enzyme was inactivated by either ATP or TTPP, the mixtures of the two complexes produced a level of acetylation greater than the additive levels measured with active and inactive pyruvate dehydrogenase complex assayed individually (Figs. 5A and 6A). This result is most easily explained by migration of pyruvate dehydrogenase subunits from one complex to another but it might also result from TPPP transfer to a pyruvate dehydrogenase subunit on the inactivated complex that lacks this prosthetic group. To evaluate the latter possibility, the experiment was repeated, with 0.1 mM TPP present in the preincubation mixture. If TPPP were indeed transferring to an active pyruvate dehydro-
Acetylation in Mammalian Pyruvate Dehydrogenase Complex

Acetylation component that did not contain a TPP or was displac-
ing a TTPP group (in the case of dialyzed enzyme inactivated
with TTPP), the presence of TPP should eliminate any dif-
ference in the level of acetylation produced by the mixture of
active and inactive enzyme and the additive level produced
with active and inactive enzyme assayed individually. As
shown in Figs. 5C and 6C, that was not the case. Although the
presence of TPP increased the number of protein-bound ace-
ty groups in the TTPP-treated and phosphorylated enzyme,
there was still a pronounced increase in the level of acetylation
produced by the mixture of complexes beyond the additive
levels produced when assayed separately. Thus, the evidence
presented supports the hypothesis that a fairly rapid exchange
of pyruvate dehydrogenase subunits can occur between cores.

This conclusion predicts that a mixture of resolved dihydro-
lipoyl transacetylase component and intact complex would
yield a product that migrates in a mass transport experiment
with an intermediate molecular weight. This was observed
(Fig. 7) in sedimentation velocity studies on samples mixed

Fig. 5. Time course of acetylation of active pyruvate dehydrogenase complex, pyruvate dehydrogenase complex inactivated with
TTPP, and mixtures of active and inactive complex. Inactivation of
pyruvate dehydrogenase complex with TTPP was performed as de-
scribed under "Experimental Procedures." For the determination of
protein-bound acetyl groups, incubation mixtures contained 50
mM potassium phosphate (pH 7.2), 0.1 mM MgCl₂, 0.2 mM EDTA,
and either 0.12 mg of active pyruvate dehydrogenase complex (〇)
or 0.24 mg of TTPP-treated pyruvate dehydrogenase complex (□)
or a combination of these levels of active and inactive complex (△).
In Curves C and D, 0.1 mM TPP also was included in the preincubation
mixture. [3-14C]Pyruvate was added at a concentration of 0.2 mM after a 90-s
incubation at 30°C. The total volume of the reaction mixture was 0.3
ml. The reaction was terminated at the indicated time, and a 0.05-ml
aliquot was assayed for protein-bound radioactivity. ---, sum of the
levels of protein-bound acetyl groups for active and inactive enzyme
assayed individually (〇 + □).

Fig. 6. Time course of acetylation of active pyruvate dehydrogenase complex, pyruvate dehydrogenase complex inactivated with ATP,
and mixtures of active and inactive complex. Inactivation of pyruvate
dehydrogenase complex with ATP was performed as described under
"Experimental Procedures." Other conditions were as described in
Fig. 5 except as indicated below. The incubation mixtures contained
either 0.12 mg of active pyruvate dehydrogenase complex (〇) or 0.24
mg of phosphorylated pyruvate dehydrogenase complex (□) or a
combination of these levels of active and inactive complex (△). ---,
sum of the levels of protein-bound acetyl groups for active and
inactive enzyme assayed individually (〇 + □).

Fig. 7. Sedimentation velocity patterns for kidney pyruvate dehydrogenase complex, resolved dihydrolipoyl transacetylase, and a
mixture of these enzymes. Experiments were conducted at 30,000 rpm
in 50 mM potassium phosphate, pH 7.2, 1 mM dithiothreitol, 0.1 mM
MgCl₂, 0.1 mM EDTA at 20.9°C. Other conditions are described under
"Experimental Procedures." The first pattern (far left) shows the
pyruvate dehydrogenase complex (6.8 mg/ml) and resolved dihydrolipoyl transacetylase (5.0 mg/ml) in separate sectors. The sample
with the pyruvate dehydrogenase complex had a slightly higher
meniscus and contained a small amount of the α-ketoglutarate de-
hydrogenase complex. In the frame shown, the α-ketoglutarate de-
hydrogenase complex is almost directly under the sample peak for
the dihydrolipoyl transacetylase but it did have a higher sedimenta-
tion coefficient than the transacetylase since it passed the latter
during the course of this velocity experiment. The second pattern
shows a mixture of the pyruvate dehydrogenase complex (5.4 mg/ml)
and dihydrolipoyl transacetylase (2.8 mg/ml) at close to the same
sedimentation time as the first pattern. The last two patterns show
this mixture at 12 and 24 min after the second pattern, respectively.
The contaminating α-ketoglutarate dehydrogenase complex can be
seen to have a slower sedimentation velocity than the single peak
produced by the mixture.
just prior to addition to the centrifuge cell. Schlieren patterns
for the control pyruvate dehydrogenase complex and resolved
dihydrolipoyl transacetylase are also shown. These have $S_{20, w}$
values of 32 S for the dihydrolipoyl transacetylase and 70 S
for the intact complex (26). An $S_{20, w}$ value of 49 S was obtained
for a complex of intermediate size produced by the mixture.
Although the centrifugation experiment takes several minutes
prior to observation of the sedimentation pattern, it not only
gives strong support to the above conclusion that pyruvate
dehydrogenase exchange occurs, but the results also indicate
that exchange must be complete within a few minutes.

**Discussion**

Our mechanism (17, 18) for pyruvate, acetyl-CoA, NADH,
or dihydrolipamide stimulations of PDH, kinase activity
predicts a correlation between the number of bound acetyl
groups per complex and the level of stimulation of kinase
activity. Our results demonstrate that the level of stimulation
increases with the level of acetylation. Furthermore, as shown
in Table I, stimulations were observed when virtually all of
the pyruvate or acetyl-CoA was depleted prior to initiation
of kinase activity. This strongly indicates that these molecules
are not directly stimulating PDH, kinase. Furthermore, the
dependence of PDH, kinase activity on the level of acetylation
was the same regardless of whether pyruvate or acetyl-CoA
was the acetylating agent. This implies that acetylated en-
zyme, presumably through acetylated lipoyl moieties, me-
diates the stimulation of PDH, kinase activity.

The results (Figs. 4 to 6) clearly show that mammalian
pyruvate dehydrogenase complex can attain a high level of
acetylation when only a few pyruvate dehydrogenase subunits
are functional. Our data provide direct support for transfer of
the pyruvate dehydrogenase component between binding sites
on the dihydrolipoyl transacetylase (Figs. 5 to 7). In addition
to indicating that nearly two sites are acetylated per trans-
acetylase subunit, the results in Fig. 4 suggest a rapid process
for introduction of acetyl groups into protein from a limited
number of active pyruvate dehydrogenase units. In recent
studies, the rate profiles for acetylation were characterized by
quench-flow rapid reaction experiments with enzyme in
activated to different extents with TTPP. Acetylation of more
than one site per $\alpha \beta$ pyruvate dehydrogenase unit proceeds at
a constant rate, equivalent (for the fraction of active enzyme)
to the rate-limiting step in the overall reaction catalyzed by
the pyruvate dehydrogenase complex. This is consistent with
a mechanism in which acetyl groups are being transferred at
a rate faster than a preceding rate-limiting step. The initial
rate is faster than the rate of pyruvate dehydrogenase move-
ment as described below but it is followed by a slower rate for
which movement of pyruvate dehydrogenase unit may con-
tribute to the rate of acetylation. Thus, the incorporation
of several acetyl groups per pyruvate dehydrogenase subunit at
the initial time points in Fig. 4 probably results to a significant
degree from a rapid transfer of acetyl groups between trans-
acetylase subunits. It seems likely that the mechanism for this
transfer is an interlipoyl transfer of acetyl groups as has been
shown for *E. coli* pyruvate dehydrogenase complex (8, 16).

An estimate of the rate of pyruvate dehydrogenase transfer
is possibly with data in Fig. 5B. When assayed in the absence
of added TTPP, the active complex used in these experiments
had about 2.5% of the activity measured with a complete
assay mixture. The rate of the overall reaction catalyzed in
the absence of added TTPP was constant with time and varied
in proportion to enzyme concentration, indicating that TPP

3 R. L. Cate, L. C. Davis, and T. E. Roche, unpublished observa-
tions.

movement does not occur during catalytic turnover. Thus, it
can be estimated that per active complex about 1 $\alpha \beta$ unit is
functional in the absence of added TTPP. Under these condi-
tions, the rate of acetylation of TTPP-inactivated complex
mixed with active pyruvate dehydrogenase complex should
reflect the rate of pyruvate dehydrogenase transfer between
transacetylase cores. Consistent with this proposal, decreasing
the ratio of active pyruvate dehydrogenase component to
potential sites of acetylation by a factor of 3, by the addition
of TTPP-treated complex, reduced the rate of acetylation per
mg of complex by approximately 3-fold. The absence of a
biphasic curve indicates that, under these conditions, the rate-
limiting step for acetylation of active and inactive pyruvate
dehydrogenase complex is the same and must, therefore, be
limited by pyruvate dehydrogenase transfer. Certainly, acetyl
transfer could contribute to the extent of this acetylation. The
acetylation rate calculated for active pyruvate dehydrogenase
complex in the absence of added TTPP (from Fig. 5B) is about
30 mol of acetyl groups incorporated/mol of complex/min.
That is at least 70-fold slower than the steady state rate for
the overall reaction catalyzed by this sample of active complex
in the absence of added TTPP.

The slow rate of acetylation resulting from pyruvate dehy-
rogenase transfer indicates that this process cannot make a
significant rate contribution to steady state catalysis by the
pyruvate dehydrogenase complex. However, pyruvate dehy-
rogenase transfer may make a significant contribution to
regulation of pyruvate dehydrogenase complex by intercon-
version. PDH, kinase remains tightly bound to the dihydro-
lipoyl transacetylase during a resolution process that removes
the other components (22). As previously suggested, pyruvate
dehydrogenase subunits may migrate to the kinase to be
phosphorylated (18). Heart PDH, kinase has a much lower
molecular activity than kidney or liver PDH, kinase, which
might reflect a slower movement of heart pyruvate dehydro-
genase subunits. In this regard, it is interesting to note that
heart complex is isolated with a much larger complement of
pyruvate dehydrogenase subunits than are the liver or kidney
complexes (20, 22). This might reflect a tighter association
for the pyruvate dehydrogenase component. Rapid reaction
studies with active and inactive enzymes will permit us in future
studies to characterize the rate of pyruvate dehydrogenase
transfer under conditions in which TTPP is not limiting.

Collins and Reed (8) and Bates et al. (16) both concluded
that pyruvate dehydrogenase transfer does not occur with *E.
coli* pyruvate dehydrogenase complex. Bates et al. (16) have
shown that with reconstituted pyruvate dehydrogenase com-
plex containing on the average only one pyruvate dehy-
rogenase dimer per transacetylase core, only about one-half of
the potential sites are acetylated. However, Collins and Reed
(8) have shown that at longer reaction times all 48 sites can
be acetylated at the same ratio of components. Reconstitution
at that ratio should result in a significant fraction of dihydro-
lipoyl transacetylase cores without pyruvate dehydrogenase
subunits. Thus, acetylation of all 48 sites apparently would
require at least a very slow pyruvate dehydrogenase transfer.
Clearly, pyruvate dehydrogenase transfer in the bacterial
complex must be much slower than we have observed with
mammalian complex.

Studies on *E. coli* pyruvate dehydrogenase complex have
also indicated that electrons may be transferred between
lipoyl moieties (8). This might be particularly important in
mammalian pyruvate dehydrogenase complex for which the

4 Heart and kidney pyruvate dehydrogenase complex appear to
have the same number of moles of PDH, kinase based on moles of
[^1-14]C]ATP bound per mol of complex (T. E. Roche, unpublished
observation).
ratio of dihydrolipoyl dehydrogenase component to subunits of the dihydrolipoyl transacetylase is very low (1). With acetylated kidney pyruvate dehydrogenase complex, from which the excess acetylating substrate (pyruvate) has been removed by gel filtration, addition of CoA and NAD + leads to a rapid production of NADH that is stoichiometric with the number of acetyl groups. Further studies are needed to determine whether this observation is a result of interlipoyl transfer of electrons or movement of the dihydrolipoyl dehydrogenase component.

Thus, we have presented further evidence that pyruvate and acetyl-CoA stimulations of PDH, kinase activity occur as a consequence of acetylation and that the degree of stimulation is a function of the level of acetylation of lipoyl moieties. Related to this regulatory process, transacetylation may shuttle acetyl groups between subunits of the transacetylase. It seems likely that this process involves interlipoyl transfer of reduced and oxidized lipoyl moieties. These mechanisms would allow a rapid equilibration of the lipoyl moiety between the oxidized, reduced, and acetylated forms in response to changes in product to substrate ratios. Such cofactor-mediated mechanisms in affecting the activity of a converter enzyme would be efficient and also economical in terms of binding sites on the converter enzyme. Furthermore, transfer of acetyl groups and electrons within the transacetylase core would allow the activity of a converter enzyme to be altered when associated with a transacetylase subunit, even when that subunit is not directly associated with other catalytic components. Finally, we have demonstrated exchange of the pyruvate dehydrogenase component between sites on the dihydrolipoyl transacetylase core. The rate appears to be adequate for pyruvate dehydrogenase movement to participate in a distinct process—interconversion of this component by a mechanism involving movement to and conversion by, a small number of fixed regulatory enzymes.

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