A Kinetic Study of Dihydrolipoyl Transacetylase from Bovine Kidney*

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The mammalian pyruvate dehydrogenase complex contains a core, consisting of dihydrolipoyl transacetylase, to which pyruvate dehydrogenase and dihydrolipoyl dehydrogenase are joined. This report describes studies on the kinetic mechanism of the transacetylase-catalyzed reaction between [1-14C]acetyl-CoA and dihydrolipoyl. This reaction appears to be a model of the physiological reaction, in which the acetyl group is transferred from the S-acetyldihydrolipoyl moiety, bound covalently to the transacetylase, to CoA. The model reaction is not affected by pyruvate dehydrogenase or dihydrolipoyl dehydrogenase, their substrates and products, or by removal of the covalently bound lipoyl moiety. These findings, together with the results of initial velocity, product inhibition, and dead-end inhibition studies, indicate that the model reaction and, apparently, the physiological reaction as well, proceeds via the Random Bi Bi (rapid equilibrium) mechanism. It appears that at the catalytic center of the transacetylase there are two adjacent sites, one that binds CoA and acetyl-CoA and another that binds dihydrolipoyl and S-acetyldihydrolipoyl (or the corresponding forms of the covalently bound lipoyl moiety).

The multienzyme pyruvate dehydrogenase complex catalyzes a coordinated sequence of reactions (Reactions 1 to 5) (2).

1. \[ \text{CH}_3\text{CO-CH}_2\text{CH}_3 + \text{thiamine-PF} \rightarrow \text{E}_1 + \text{CO}_2 \]
2. \[ \text{[CH}_3\text{CHOR-thiamine-PF]} + \text{E}_1 + \text{S-acetyl-lipoyl} \rightarrow \text{[CH}_3\text{CO-S-lipoyl]} + \text{[thiamine-PF]} + \text{E}_1 \]
3. \[ \text{[CH}_3\text{CO-S-lipoyl]} + \text{CoA} + \text{SN} \rightarrow \text{[S-acetyl-lipoyl]} + \text{E}_1 + \text{CoA} + \text{SN} \]
4. \[ \text{[S-acetyl-lipoyl]} + \text{FAO} \rightarrow \text{[FAO]} + \text{E}_1 + \text{NAD}^+ + \text{H}^+ \]
5. \[ \text{[FAO]} + \text{NAD}^+ + \text{H}^+ \rightarrow \text{[FAO]} + \text{E}_1 + \text{NADH} + \text{H}^+ \]
6. \[ \text{[S-acetyl-lipoyl]} + \text{CoA} + \text{SN} \rightarrow \text{[CoA]} + \text{SN} + \text{H}_2 \text{O} \]

Reactions 1 and 2 are catalyzed by pyruvate dehydrogenase (E_1), Reaction 3 is catalyzed by dihydrolipoyl transacetylase (E_2), and Reactions 4 and 5 are catalyzed by dihydrolipoyl dehydrogenase (E_3). E_1 forms the core of the complex, and E_3 and E_2 are attached to it.

Recent studies of the kinetics of the pyruvate dehydrogenase complex from bovine kidney mitochondria indicated that over-all Reaction 6 proceeds via a ping-pong mechanism (1, 3). Presumably, the lipoyl moiety (lipoyl), which is bound covalently to the transacetylase (E_2), shuttles between the catalytic centers of the three different enzymes that comprise the complex.

This investigation was undertaken to gain insight into the kinetic mechanism of the transacetylase-catalyzed reaction (Reaction 3). To facilitate the kinetic studies, a model reaction (Reaction 7) was used.

\[ \text{[CH}_3\text{CO-S-CoA]} + \text{lipoyl} \rightarrow \text{[CoA]} + \text{SN} + \text{S-acetyl-lipoyl} \]

In this model reaction, which is the reverse of the physiological reaction (Reaction 3), exogenous dihydrolipoyl substitutes for the protein-bound dihydrolipoyl moiety (4). The results indicate that Reaction 7 and, apparently, the physiological reaction (Reaction 3) as well, proceeds via a random mechanism.

EXPERIMENTAL PROCEDURE

Materials—Acetyl-CoA, palmitoyl-CoA, and CoA were purchased from P-L Biochemicals, and [1-14C]acetyl-CoA and [1-14C]palmitoyl-CoA were obtained from New England Nuclear. dl-Dihydrolipoamide was prepared by reduction of dl-lipoamide (Sigma) with sodium borohydride (5). S-Acetyldihydrolipoyl was prepared enzymatically by coupling Reaction 7 with the phosphotransacetylase-catalyzed reaction as described by Gunsalus et al. (6). The product was analyzed by measuring its absorbance at 236 to 238 nm (7) and by determining its content of heat-stable thioester groups (8). 1,2-Diiselenolane-3-valeric acid, the selenium analog of lipoic acid, was prepared by treatment of ethyl 6,8-dichlorooctanoate with sodium diselenide followed by...
saponification (9, 10); reddish brown needles, m.p. 88.5-90°, λmax 441 nm. All other chemicals were of the purest grade available commercially.

The pyruvate dehydrogenase complex was isolated from bovine kidney mitochondria and separated into its component enzymes as described previously (11). Lipoamidase was isolated from Streptococcus faecaliis by the method of Suzuki and Reed (12), with the following modifications. The cells were ruptured in a Manton-Gaulin laboratory homogenizer (four cycles at 6000 p.s.i.). In the DEAE-cellulose chromatography step (12), the column was equilibrated and developed with 0.2 M potassium phosphate buffer, pH 7.0, containing 0.05 M KCl, and lipoamidase was eluted with the same buffer, containing 0.15 M KCl. The lipoic acid-activating enzyme was isolated from Escherichia coli (13).

**Assay Procedure**—Dihydrolipoamide transacetylase catalyzes the transfer of the acetyl group of [1-14C]acetyl-CoA to dihydrolipoamide (Reaction 7). The radioactive S-acetyldihydrolipoamide is extractable into benzene, leaving the unreacted [1-14C]acetyl-CoA in the aqueous phase. Fig. 1 shows the time course of the reaction. The reaction mixture contained 0.25 ml of 0.05 M potassium phosphate-0.005 M cysteine buffer, pH 7.4, 0.1 ml of 2.5 mM dihydrolipoamide in 25% ethanol, 0.1 ml of [1-14C]acetyl-CoA (9 x 10^6 cpm/ml), 4 to 10 µg of dihydrolipoamide transacetylase or pyruvate dehydrogenase complex, and water to make a total volume of 0.5 ml. The reaction was started by the addition of [1-14C]acetyl-CoA, and the mixture was incubated at room temperature (24°) for 2 min. One milliliter of benzene was added to stop the reaction, and the mixture was shaken for 5 to 10 min in a Vortex mixer to extract the radioactive S-acetyldihydrolipoamide. A 0.2 ml aliquot of the benzene layer was withdrawn, and radioactivity was measured in a Beckman liquid scintillation spectrometer. Enzyme or dihydrolipoamide was omitted from the control tubes. Typically, the controls gave values of 50 to 60 cpm. All assays were made in duplicate. Initial reaction velocities are expressed as micromoles of product formed per min. In kinetic studies, the concentrations of substrates ranged from 0.05 to 0.5 mM, and the amount of transacetylase was limited to about 4 µg. The terminology used to describe kinetic data, particularly product inhibition plots, is that of Cleland (14, 15).

**Release and Reincorporation of Protein-bound Lipoyl Moiety**—The conditions were similar to those used in previous studies (4) with the E. coli pyruvate dehydrogenase complex. Release of protein-bound lipoic acid from the dihydrolipoamide transacetylase component of the complex by incubation with lipoamidase is accompanied by loss of activity in over-all reaction 6. This activity is restored by reincorporating lipoic acid into the transacetylase by incubation with lipoic acid, ATP, and the lipoic acid-activating enzyme. In the present investigation, bovine kidney dihydrolipoamide transacetylase (0.4 mg) or pyruvate dehydrogenase complex (0.9 mg) was incubated at 30° with 5 to 10 units of lipoamidase (0.02 M potassium phosphate buffer, pH 7.4, containing 0.005 M cysteine). At various time intervals, samples were withdrawn for assay as indicated below. The loss of activity of the pyruvate dehydrogenase complex in Reaction 6 was monitored by spectrophotometric assay of NAD reduction (11). Transacetylase activity was assayed by reconstituting the full complex. The transacetylase (5 µg) was incubated at room temperature for 10 min with 16 µg of pyruvate dehydrogenase and 16 µg of dihydrolipoamide dehydrogenase in 0.1 ml of 0.06 M phosphate buffer, pH 7.5, containing 2 mM dithiothreitol and 1 mM MgCl2. A 0.05-ml sample was removed for spectrophotometric assay of NAD reduction activity.

Reincorporation of lipoic acid was achieved by incubating 0.1 to 0.2 µg of the radioactive lipoic acid-deficient transacetylase or pyruvate dehydrogenase complex with 0.8 unit of lipoic acid-activating enzyme, 0.01 µmol of [3H]lipoic acid, 0.1 µmol of ATP, 0.08 µmol of thiamine pyrophosphate, 2 µmol of cysteine hydrochloride, 1.6 µmol of MgSO4, and 12 µmol of K2HPO4 in a reaction volume of 0.5 ml at pH 7.4 and 30°. Reactivation of the pyruvate dehydrogenase complex, or of the reconstituted complex in the case of the transacetylase, was monitored by spectrophotometric assay of NAD reduction (Reaction 6).

**RESULTS**

**Initial Velocity Experiments**—Double reciprocal plots of initial velocity data for Reaction 7 catalyzed by dihydrolipoamide transacetylase at varying concentrations of both substrates are shown in Fig. 2. Some scatter in the points representing the lowest substrate concentrations was unavoidable using the radiochemical assay. However, for both the uncomplexed transacetylase and the intact pyruvate dehydrogenase complex (data not shown), the plots were linear and converged at a point close to the base-line. Secondary plots in which the slopes and intercepts of the lines in Fig. 2 were plotted against the reciprocal of the fixed substrate concentration were also linear. The maximum velocity and the Kₘ values for each substrate were calculated from the secondary plots (16). The Kₘ values obtained in different experiments ranged from 0.03 to 0.1 mM for acetyl-CoA and from 0.13 to 0.4 mM for dihydrolipoamide. There was no significant difference in the values found for the transacetylase in the uncomplexed state (E₂) and in association with pyruvate dehydrogenase and dihydrolipoyl dehydrogenase as an integral part of the pyruvate dehydrogenase complex (E₁-E₂-E₃). The intersecting double reciprocal plots (Fig. 2) suggest that the bisubstrate model reaction (Reaction 7) proceeds via a sequential mechanism.

**Product Inhibition Experiments**—Product inhibition studies showed that CoA is competitive with acetyl-CoA and noncom-
petitive with dihydrolipoamide (Fig. 3), whereas S-acetyldihydrolipoamide is competitive with dihydrolipoamide and non-
competitive with acetyl-CoA (Fig. 4). These results are compatible with either the Random Bi Bi (rapid equilibrium)
mechanism or the Theorell-Chance mechanism (14, 17).

**Dead-end Inhibition Experiments**—To distinguish between
the random and ordered mechanisms, dead-end competitive inhibitors of the substrates were used (18). Palmityl-CoA is
competitive with acetyl-CoA and noncompetitive with dihy-
drolipoamide (Fig. 5). Control experiments showed that the
transacetylase catalyzes little, if any, transfer of the palmityl
group of [1-14C]-palmityl-CoA to dihydrolipoamide. A survey of
analogs and derivatives of lipoic acid revealed that the
selenium analog, 1,2-diselenolane-3-valeric acid, is a competi-
tive inhibitor of dihydrolipoamide (Fig. 6A). This analog is
noncompetitive with acetyl-CoA (Fig. 6B). These results
support a random mechanism involving a separate site for each
substrate.

**Effects of Release and Reincorporation of Covalently Bound Lipoyl Moiety**—Since dihydrolipoyl transacetylase contains
covaently bound lipoic moieties that function in the physio-
logical reaction (Reaction 3), it seemed essential to determine
whether or not these endogenous lipoic moieties participate in
the model reaction (Reaction 7). The protein-bound lipoic
moieties are reductively acetylated in the presence of pyruvate
dehydrogenase, [2-14C]pyruvate, thiamine pyrophosphate, and
Mg2+ (Reactions 1 and 2) (19). Also, the bound lipoic moieties
are reduced in the presence of NADH and dihydrolipoyl
dehydrogenase (20). In preliminary experiments we observed
that concentrations of NADH up to 2 mM did not inhibit the
model reaction catalyzed by either the intact pyruvate dehy-
drogenase complex or the uncomplexed dihydrolipoyl trans-
acetylase. Pyruvate at concentrations up to 2 mM plus 0.2 mM
thiamine pyrophosphate and 0.2 mM Mg2+ also failed to show
any inhibition of the model reaction. Since reduction or
reductive acetylation of the protein-bound lipoic moieties did
not affect the model reaction, it seemed unlikely that the
endogenous lipoic moiety participates in the model reaction.
Direct support for this conclusion was obtained with preparations of the transacetylase and the pyruvate dehydrogenase complex that had been treated with lipoamidase to release the protein-bound lipoyl moieties. Almost total inactivation of the pyruvate dehydrogenase complex in the physiological reaction (Reaction 6) occurred during 2.5 to 3 hours of incubation with lipoamidase (Fig. 7B). By contrast, activity in the model reaction (Reaction 7) remained unchanged throughout the incubation period (Fig. 7A). During the long incubation period, some loss in the activity of the control sample in Reaction 6 occurred, but this loss was slight compared with the loss in activity in the presence of lipoamidase. A slight increase in the physiological activity was often seen at the beginning of the incubation period, due possibly to stimulation by some component of the incubation medium.

Similar results were obtained with the uncomplexed transacetylase. Incubation of the transacetylase with lipoamidase for 3 hours resulted in a complete loss of activity in the physiological reaction (Reaction 6), but no significant change in the maximum velocity of the model reaction or the $K_m$ values for the substrates was observed. The transacetylase activity was determined after addition of pyruvate dehydrogenase and dihydrolipoyl dehydrogenase to reconstitute the pyruvate dehydrogenase complex. Fig. 8 shows double reciprocal plots of data obtained with the apotransacetylase in the model reaction. The value for $V$ was identical with that of a control sample of transacetylase that had been incubated without lipoamidase for 3 hours. From secondary plots of the data shown in Fig. 8, $K_m$ values of 0.1 and 0.36 mM were calculated for acetyl-CoA and dihydrolipoamide, respectively. For the control, the corresponding values were 0.08 and 0.32 mM.

That the loss of activity of the transacetylase in the physiological reaction was due to release of the protein-bound lipoyl moiety, and not to proteolysis or to nongeneric denaturation, was proved by restoration of 90 to 100% of the catalytic activity of the transacetylase (in the reconstitution assay) after incubation of the apotransacetylase for 20 to 30 min with the lipoic acid-activating enzyme, lipoic acid, and ATP (see "Experimental Procedure"). Observation of reactivation of the apopyruvate dehydrogenase complex under similar conditions was masked by the presence of an endogenous kinase, which, in the presence of ATP, phosphorylates and inactivates the pyruvate dehydrogenase component of the complex (21). Using $[\gamma-32P]ATP$, we observed that the rate of phosphorylation of the pyruvate dehydrogenase component of the complex was not affected by removal of the protein-bound lipoyl moieties (data not shown). Also, phosphorylation of the pyruvate dehydrogenase component of the complex had no effect on the model reaction.

**DISCUSSION**

The initial velocity patterns obtained with the uncomplexed dihydrolipoyl transacetylase and with the intact pyruvate dehydrogenase complex indicate that the transacetylation reaction between acetyl-CoA and exogenous dihydrolipoamide (Reaction 7) proceeds via a sequential mechanism. The product inhibition data, showing competitive-noncompetitive linear patterns, fit the Random Bi Bi (rapid equilibrium) mechanism as well as the Theorell-Chance mechanism. However, the patterns obtained with dead-end substrate inhibitors, showing that in each case the inhibitor is competitive with its analogous substrate and noncompetitive with the unrelated substrate, are consistent with the random rather than the ordered mechanism. These results suggest that at the catalytic center of the transacetylase there are two adjacent sites, one that binds CoA and acetyl-CoA, and another that binds dihydrolipoamide and S-acetyldihydrolipoamide. The noncompetitive inhibitions presumably arise from the formation of inactive ternary complexes of enzyme, CoA, and dihydrolipoamide, and enzyme, S-acetyldihydrolipoamide, and acetyl-CoA (14).

Reductive acetylation of the covalently bound lipoyl moieties in the bovine kidney pyruvate dehydrogenase complex with pyruvate and thiamine pyrophosphate, or reduction of the bound lipoyl moieties with NADH, had no effect on the model reaction (Reaction 7). Furthermore, removal of the protein-bound lipoyl moieties by incubating the uncomplexed trans-

**Fig. 7.** Effect of release of protein-bound lipoyl moiety on reactions catalyzed by the bovine kidney pyruvate dehydrogenase complex. A 0.9-mg sample of the complex was incubated with 8 units of lipoamidase at 30° and pH 7.4. Samples were withdrawn at the indicated time intervals for assay of activity in the model reaction (Reaction 7) (A) and in the physiological reaction (Reaction 6) (B) as described under "Experimental Procedures." Lipoamidase was omitted from the control sample (O).

**Fig. 8.** Initial velocity patterns obtained with apotransacetylase (Reaction 7). Dihydrolipoyl transacetylase was incubated with lipoamidase for 3 hours to release the protein-bound lipoyl moieties. The apotransacetylase showed no activity in the physiological reaction (Reaction 6) after reconstitution of the pyruvate dehydrogenase complex. A, the concentration of [1-14C]acetyl-CoA was varied at fixed concentrations of dihydrolipoamide (DHL) as indicated; B, the concentration of dihydrolipoamide was varied at fixed concentrations of [1-14C]acetyl-CoA as indicated. Assay conditions were as in Fig. 2.
acetylase or the pyruvate dehydrogenase complex with lipoamidase did not affect the model reaction. Thus, it is clear that none of the three possible forms of the protein-bound lipoyl moiety, i.e., oxidized, acetylated, and reduced, which are generated in the oxidative decarboxylation of pyruvate (Reactions 1 to 5), is involved in the model reaction. These findings are consistent with the results of previous studies with lipoic acid-deficient preparations of the E. coli and S. faecalis pyruvate dehydrogenase complexes (4, 5). Exclusion of a role for the covalently bound lipoyl moiety in the model reaction is consistent with a random or ordered mechanism, since it might be envisaged that a mechanism involving acetylation of the protein-bound dihydrolipoamyl moiety as an obligatory intermediate step would be likely to proceed by a ping-pong scheme. At an early stage of this investigation it appeared that acetyl groups were incorporated into the bovine kidney dihydrolipoyl transacetylase when the enzyme was incubated with [3H]acet-CoA, and that subsequent treatment of the acetylated transacetylase when the enzyme was incubated with [3H]acet-CoA, and that subsequent treatment of the acetylated enzyme with dihydrolipoamide released the bound acetyl groups (22). However, further investigation revealed that these earlier results were due to incomplete separation of [3H]acetyl-CoA from the transacetylase. Acetyl groups from acetyl-CoA were incorporated into the transacetylase only when the protein-bound lipoyl moieties were in the reduced state, e.g., by a reversal of Reactions 5, 4, and 3.

In view of the substrate specificity of lipoamidase (12) and the lipoic acid-activating enzyme (4), and by analogy with the results obtained with the E. coli dihydrolipoyl transacetylase (23), we assume that the lipoyl moiety is bound in amide linkage to the ε-amino group of a lysyl residue in the bovine kidney dihydrolipoyl transacetylase. This attachment would provide a flexible arm of about 14 Å for the reactive dithiolane ring, conceivably enabling the lipoyl moiety to rotate between the various catalytic centers in the pyruvate dehydrogenase complex (24). The simplest interpretation of the results presented in this communication is that the catalytic center on the transacetylase at which the model reaction (Reaction 7) occurs is identical with the catalytic center where acetylation of CoA occurs in the physiological reaction (Reaction 3) and that both reactions proceed by the same random mechanism. In the model reaction, some degree of competition between the endogenous lipoyl moiety and exogenous dihydrolipoamide might be expected under some circumstances, even though the concentration of exogenous dihydrolipoamide far exceeds that of the covalently bound lipoyl moiety during the enzyme assays. However, removal of the covalently bound lipoyl moiety with lipoamidase produced no change in the K_m value for dihydrolipoamide. We envisage that the protein-bound lipoyl moiety is not "fixed" at the catalytic center on the transacetylase, but can move freely in and out of the site.

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