α-Keto Acid Dehydrogenation Complexes

II. THE ROLE OF PROTEIN-BOUND LIPOMIC ACID AND FLAVIN ADENINE DINUCLEOTIDE

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Pyruvate and α-ketoglutarate dehydrogenation systems which catalyze Reaction 1 have been isolated from Escherichia coli extracts as structural units of high molecular weight (1). This reaction can be conveniently followed, with pyruvate as substrate, by coupling it with the phosphotransacetylase and lactic dehydrogenase reactions (Reactions 2 and 3) to give the pyruvate dismutation reaction (Reaction 4) (2). The pyruvate dehydrogenation complex, in the presence of appropriate supplements, also catalyzes reactions involving substrate amounts of lipoic acid or dihydrolipoic acid (Reactions 5 to 7), and the oxidation of pyruvate with ferricyanide as electron acceptor (Reaction 8) (1). Reaction 5 requires coenzyme A and phosphotransacetylase (3). The complete reaction mixture contained 100 pmoles of potassium phosphate buffer (pH 7.0), 50 μmoles of potassium pyruvate, 5 μmoles of DL-lipoamide, 5 μmoles of Fe(III)-cyanide, 5 μmoles of Fe(II)-cyanide, 5 μmoles of H2O, 2 units of phosphotransacetylase, 0.5 units of dihydrolipoic transacetylase, and a lipoic acid-activating enzyme system. Release of protein-bound lipoic acid resulted in a loss of enzymatic activities represented by Reactions 4 and 5, but did not affect those activities represented by Reactions 6 and 7. These results have been confirmed and extended in the present investigation with highly purified preparations of the pyruvate and α-ketoglutarate dehydrogenation complexes. Also, evidence indicating the participation of flavin adenine dinucleotide in Reactions 1, 4, 5, and 7 and pointing to its function as coenzyme of dihydrolipoic dehydrogenase is presented. Some of this work has been reported briefly (8).

EXPERIMENTAL PROCEDURE

The pyruvate and α-ketoglutarate dehydrogenation complexes were isolated from sonicated extracts of E. coli cells which had been grown in the presence of DL-lipoic acid-S,S'-disulfide, and contained bound, radioactive lipoic acid (1). Fractions A and B were obtained from the same extracts essentially as described by Hager and Gunsalus (4, 5). Fractions A and B were obtained from the same extracts essentially as described by Hager and Gunsalus (4, 5). In confirmation of their observation, bound lipoic acid concentrates in Fraction A, but not in Fraction B. The preparations of Fraction A used in this investigation correspond to the 0.36 to 0.48 and 0.36 to 0.40 saturated ammonium sulfate fractions and the calcium phosphate gel eluate. Fraction B, corresponding to the 0.6 to 0.7 saturated ammonium sulfate fraction, was purified by chromatography on a calcium phosphate gel-cellulose column as described in an accompanying paper (9).

The assay system for Reaction 5 is a modification of that described by Gunsalus (3). The complete reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 7.0), 50 μmoles of potassium pyruvate, 5 μmoles of DL-lipoamide (in 0.05 M of 95% ethanol), 0.3 μmole of MgSO4, 0.2 μmole of thiamine-PP, 0.02 μmole of CoA, 2 units of phosphotransacetylase,
Effect of release and reincorporation of lipoic acid on enzymatic activities of pyruvate dehydrogenation complex

A mixture of 2 ml of E. coli pyruvate dehydrogenation complex (17 mg of protein) and 2 ml of lipoic-X-hydrolase (48 mg of protein, specific activity 59 (7)) was incubated for 1 hour at 30°. The incubation mixture was diluted to 5 ml with 0.02 m phosphate buffer (pH 7.0) and centrifuged for 3 hours at 173,000 X g. The yellow pellet (15.9 mg of protein) was dissolved in 1.8 ml of phosphate buffer. This solution is designated inactivated sample. A mixture of 1 ml of the pyruvate dehydrogenation complex and 1 ml of 0.02 m phosphate buffer was treated in the same manner, and served as the control sample. One milliliter of the inactivated sample (8.8 mg of protein) was incubated for 1 hour at 30° with a mixture containing the lipoic acid-activating system (gel-treated E. coli Fraction T9'-1 (10); 20 mg of protein), 0.3 μmole of nL-lipoic acid-S2 (2.64 X 10^5 c.p.m. per μg.), 1 μmole of ATP, and 10 μmole of MgCl2, in a final volume of 3.6 ml. The incubation mixture was diluted to 5 ml with phosphate buffer and centrifuged for 3 hours at 173,000 X g. The yellow pellet was dissolved in 5 ml of phosphate buffer and the solution was recentrifuged for 3 hours at 173,000 X g. The pellet (7.2 mg of protein) was dissolved in 0.8 ml of phosphate buffer. This solution is designated reactivated sample. Repetition of the washing procedure with an aliquot (0.25 ml) of the latter sample did not change its specific radioactivity. The amounts of pyruvate dehydrogenation complex used in the assays were as follows: Reaction 1, 1 to 2 μg; Reaction 4, 1 to 3 μg; Reaction 5, 5 to 10 μg; Reaction 6, 6 to 20 μg; Reaction 7, 1 to 3 μg; Reaction 8, 100 to 200 μg; radioactivity, 80 μg. The preparations of lipoic-X hydrolase and the lipoic acid-activating system were centrifuged for 3 hours at 173,000 X g before use.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reaction 1</th>
<th>Reaction 2</th>
<th>Reaction 3</th>
<th>Reaction 4</th>
<th>Reaction 5</th>
<th>Reaction 6</th>
<th>Reaction 7</th>
<th>Bound lipoic acid</th>
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<tbody>
<tr>
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<td>0</td>
<td>109</td>
<td>1314</td>
<td>11</td>
<td>11</td>
<td>0.08</td>
<td></td>
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<tr>
<td>Reactivated</td>
<td>258</td>
<td>1000</td>
<td>169</td>
<td>104</td>
<td>1222</td>
<td>9</td>
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<tr>
<td>Control</td>
<td>288</td>
<td>1100</td>
<td>188</td>
<td>112</td>
<td>1326</td>
<td>11</td>
<td>1.87</td>
<td></td>
</tr>
</tbody>
</table>

and pyruvate dehydrogenation complex in a final volume of 1 ml. The mixture was incubated for 1 hour at 30° and then assayed for acetyl phosphate and sulphydryl. Pertinent information concerning other assay procedures, enzyme preparations, and source of materials is provided in previous papers (1, 7, 10). Crystalline alcohol dehydrogenase was obtained from the Worthington Biochemical Corporation. Unless specified otherwise, all specific activities are expressed as μmol of substrate utilized or product formed per hour per mg of protein.

Spectral changes of the flavin in the pyruvate and α-ketoglutarate dehydrogenation complexes were measured with a Beckman model DU spectrophotometer equipped with a micro attachment. The experiments were carried out under nitrogen in specially designed micro cuvettes (1 cm light path) equipped with a Y-shaped adapter provided with valves for gassing. Droplets (1 to 2 μl) of concentrated solutions of substrates were placed on the walls in the upper part of the cuvette, which were coated previously with Desicote. Additions were made by carefully tilting the cuvette to wash down the droplet (cf. (11)).

**RESULTS**

Release and Reincorporation of Lipoic Acid In the inactivation and reactivation experiments described below advantage was taken of the fact that the pyruvate and α-ketoglutarate dehydrogenation complexes sediment as pellets in the ultracentrifuge at 173,000 X g to separate the complexes from lipoic-X hydrolase and the lipoic acid-activating system, which remain in solution under these conditions. In preliminary experiments the minimum amounts of these enzyme preparations required to inactivate and reactivate the complexes were determined.

A preparation of the pyruvate dehydrogenation complex containing protein-bound, radioactive lipoic acid was incubated with lipoic-X hydrolase for 1 hour at 30° and the mixture was centrifuged for 3 hours at 173,000 X g in the No. 539L rotor of a Spinco model L ultracentrifuge. Approximately 96% of the radioactivity remained in the supernatant fluid. The yellow pellet was assayed for the various enzymatic activities associated with the pyruvate dehydrogenation complex. The data in Table I indicate that the dihydrodipicolinic transacetylase and dihydrolipoic dehydrogenase activities of the complex, as measured with substrate amounts of DL-dihydrolipoic acid (Reactions 6 and 7), were not affected by release of the protein-bound lipoic acid. These model reactions therefore are not mediated through the bound cofactor. In contrast to these results, oxidation of pyruvate with DPN as electron acceptor (Reactions 1 and 4) or with lipoylamine as electron acceptor (Reaction 5) did not proceed in the absence of protein-bound lipoic acid. It is also significant that oxidation of pyruvate with ferricyanide as electron acceptor (Reaction 8) was not affected by removal of the bound lipoic acid. Incubation of the lipoic acid-deficient complex with DL-lipoic acid-S2, ATP, and the lipoic acid-activating system restored those enzymatic activities represented by Reactions 1, 4, and 5. The reactivated complex contained essentially the same amount of bound lipoic acid as the original sample.

In a separate experiment the radioactive material released from the pyruvate dehydrogenation complex by lipoic-X hydrolase was examined by paper chromatography in three different solvent systems (Fig. 1). The major radioactive component migrated at the same rate as authentic nL-lipoic acid-S2. The minor radioactive component is presumably lipoic acid sulfoxide (β-lipoic acid), which is produced from lipoic acid after the latter is applied to the paper chromatograms (12).

Approximately 96% of the bound, radioactive lipoic acid in the α-ketoglutarate dehydrogenation complex was released by incubation with lipoic-X hydrolase (Table II). The dihydrolipoic dehydrogenase activity (Reaction 7) of the complex was not affected by removal of protein-bound lipoic acid, nor was its ability to oxidize α-ketoglutarate with ferricyanide as electron acceptor (Reaction 8). However, oxidation of α-ketoglutarate with DPN as electron acceptor (Reaction 1) did not proceed in the absence of the bound lipoic acid. This activity was restored by guest on October 20, 2017 http://www.jbc.org/ Downloaded from http://www.jbc.org/
FIG. 1. Radioautographs of radioactive material released from the pyruvate dehydrogenation complex by lipoyl-X hydrolase. A mixture of the pyruvate dehydrogenation complex (11 mg of protein; specific activity 1000 in dismutation assay; 1.67 µg of radioactive lipoic acid per mg of protein) and lipoyl-X hydrolase (9 mg of protein; specific activity 490) in 3.0 ml of 0.02 M phosphate buffer (pH 7.0) was incubated for 2 hours at 30°C and then centrifuged for 2 hours at 144,000 X g. Approximately 95% of the radioactivity remained in the supernatant fluid. The latter was lyophilized, the residue was extracted with 3 ml of 95% ethanol, and the extract was concentrated to a volume of 0.3 ml by means of a stream of nitrogen. Recovery of radioactivity was 74%. One-dimensional ascending chromatograms were prepared with the following solvent systems: I, 2,6-lutidine-water (65:35); II, butanol-acetic acid-water (4:1:1); III, butanol-0.5 N ammonium hydroxide. The samples used were: A, 0.6 µg of DL-lipoic acid-S³⁸ (1.6 X 10⁴ c.p.m. per µg); B, mixture of A and C; C, 0.02 ml of ethanol extract. The air-dried chromatograms were placed on x-ray-sensitive film for 58 hours.

By incubating the lipoic acid-deficient complex with DL-lipoic acid-S³⁸, ATP, and the lipoic acid-activating system. The amount of radioactive lipoic acid incorporated into the complex was essentially the same as that released by lipoyl-X hydrolase. These results are consistent with those obtained with the pyruvate dehydrogenation complex and provide additional evidence that the bound lipoic acid participates in Reaction 1, but not Reactions 7 and 8.

Reversible Dissociation of Flavin—When the pyruvate dehydrogenation complex was precipitated with ammonium sulfate at pH 3.6, up to 80% of the bound flavin was released. Concomitantly with the loss of flavin, there was a decrease in the following enzymatic activities: oxidation of pyruvate with DPN or with lipoamide as electron acceptor (Reactions 1 and 5), dihydrolipoic dehydrogenase activities (Reactions 1 and 7), and the DPN-linked oxidation of dihydrolipoic acid (Reaction 7). These activities were restored by addition of FAD, but not of FMN (Table III). Neither the dihydrolipoic transacetylase activity (Reaction 6) of the complex nor its ability to oxidize pyruvate with ferricyanide as electron acceptor (Reaction 8) was affected by removal of flavin. Since Hager and Gunsalus (5, 6) have shown that dihydrolipoic dehydrogenase is required in Reactions 1 and 4 the data strongly indicate that the flavin in the complex is associated with dihydrolipoic dehydrogenase. The requirement of FAD in Reaction 5 also suggests that dihydrolipoic dehydrogenase is involved in this reaction. Further support for this conclusion is furnished below.

Release of flavin from the α-ketoglutarate dehydrogenation complex resulted in a decrease in DPN reduction and dihydrolipoic dehydrogenase activities (Reactions 1 and 7), and these activities were restored by addition of FAD, but not of FMN.
Effect of release and reincorporation of lipoic acid on enzymatic activities of α-ketoglutarate dehydrogenation complex.

A mixture of 2 ml of E. coli α-ketoglutarate dehydrogenation complex (21 mg of protein) and 1 ml of lipoyl-α-ketoglutarate (24 mg of protein) was incubated for 75 minutes at 30°C. The incubation mixture was diluted to 5 ml with 0.02 M phosphate buffer (pH 7.0) and centrifuged for 3 hours at 173,000 × g. The yellow pellet (18 mg of protein) was dissolved in 1.8 ml of phosphate buffer. This solution is designated inactivated sample. A mixture of 1 ml of the α-ketoglutarate dehydrogenation complex and 0.5 ml of phosphate buffer was treated in the same manner, and served as the control sample. One milliliter of the inactivated sample (10 mg of protein) was incubated for 1 hour at 30°C with a mixture containing the lipoic acid-activating system (13 mg of protein), 0.19 μmole of DL-lipoic acid-S2H4, 1 μmole of ATP, and 10 μmoles of MgCl2, in a final volume of 2.1 ml. The incubation mixture was diluted to 5 ml with phosphate buffer and centrifuged for 3 hours at 173,000 × g. The yellow pellet (7.7 mg of protein) was dissolved in 0.9 ml of phosphate buffer. This solution is designated reactivated sample. The specific radioactivity of the latter sample was not changed by repetition of the washing procedure. The amounts of α-ketoglutarate dehydrogenation complex used in the assays were as follows: Reaction 1, 1 to 2 μg; Reaction 7, 1 to 3 μg; Reaction 8, 20 to 50 μg; radioactivity, 100 μg. Other conditions were as in Table I.

Effect of release and reincorporation of FAD on enzymatic activities of pyruvate dehydrogenation complex.

In Experiment 1, 225 mg of solid ammonium sulfate were added with stirring to a solution of the pyruvate dehydrogenation complex (1.84 mg of protein) in 1 ml of 0.02 M phosphate buffer (pH 7.0) at 4°C. The pH was adjusted to 3.6 with cold 1 N sulfuric acid containing 290 mg of ammonium sulfate per ml. Stirring continued for 5 minutes, the mixture was centrifuged, and the protein was dissolved in 0.5 ml of 0.02 M phosphate buffer (pH 7.0). A small amount of insoluble material was removed by centrifugation. Recovery of protein was 76%. Aliquots (0.03 ml) of the cleaved complex were preincubated at 30°C for 10 minutes with an equal volume of FMN or FAD solutions (1 μmole per ml) or phosphate buffer before assay. Final concentration of added flavin in the assays was 10⁻⁶ to 10⁻⁵ M. Experiment 2 was carried out in a similar manner with a different preparation of the pyruvate dehydrogenation complex.

<table>
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<tr>
<th>Specific activities</th>
<th>Before cleaving</th>
<th>After cleaving</th>
</tr>
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<tbody>
<tr>
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<td>With FAD</td>
</tr>
<tr>
<td>Reaction 1</td>
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<tr>
<td>Reaction 4</td>
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<td>Reaction 5</td>
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<td>Reaction 6</td>
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<tr>
<td>Reaction 7</td>
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<td>1040</td>
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<tr>
<td>Reaction 8</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

Effect of release and reincorporation of FAD on enzymatic activities of α-ketoglutarate dehydrogenation complex.

The procedure was similar to that described in Table III. Different preparations of the α-ketoglutarate dehydrogenation complex were used in Experiments 1 and 2.

<table>
<thead>
<tr>
<th>Specific activities</th>
<th>Before cleaving</th>
<th>After cleaving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay system</td>
<td>Without FAD</td>
<td>With FAD</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>192</td>
<td>204</td>
</tr>
<tr>
<td>Experiment 7</td>
<td>1530</td>
<td>1526</td>
</tr>
<tr>
<td>Experiment 8</td>
<td>90</td>
<td>88</td>
</tr>
</tbody>
</table>
alcohol dehydrogenase or pyruvate and lactic dehydrogenase (Figs. 2 and 3, Curve C). It is apparent, however, that the amount of DPNH formed is much larger than the amount of reduced flavin oxidized. From the absorbancy changes shown in Curves B and C it was calculated that the molar ratio of DPNH to reoxidized flavin is approximately 25:1. Furthermore, the ratio of DPNH to reduced flavin is approximately 2:1. It is to be noted that removal of DPNH was accompanied by essentially complete reoxidation of the flavin in both complexes (Figs. 2 and 3, Curve B). A possible explanation of these results is that all or part of the flavin turns over more than once and that the DPNH formed inhibits further oxidation of flavin by DPN. When the DPNH is removed by acetaldehyde and alcohol dehydrogenase or pyruvate and lactic dehydrogenase reoxidation of the flavin by DPN is observed. As shown in an accompanying paper (9) and independently by Notani and Gunsalus (14) DPNH apparently inhibits reduction of lipoic acid and lipoamide by E. coli dihydrolipoic dehydrogenase.

Under conditions similar to those used in Fig. 3, Curve B, Massey (13) observed a substantial reoxidation by DPN of the flavin in the pig heart α-ketoglutarate dehydrogenation complex. However, the amount of DPNH formed in the latter experiment was not reported. The different results obtained with the E. coli and pig heart complexes probably reflect differences in the properties of the bacterial and mammalian dihydrolipoic dehydrogenases (cf. (9)).

DISCUSSION

The results reported in this paper can be explained satisfactorily by means of the reaction sequence shown below. This scheme is a modification of that proposed previously by Gunsalus (5) and incorporates recent findings that the catalytically active lipoic acid is bound to protein in covalent linkage through its carboxyl group (7, 15) and that FAD is required in the overall reaction (8, 13).

\[
\text{RCOCoA} + \text{thiamine-PP} \rightarrow \text{RCOCoA} + \text{CO}_2 \quad (11)
\]

\[
\text{[RCHO—thiamine-PP] + thiamine-PP} \rightarrow \text{[RCHO—thiamine-PP] + CO}_2 \quad (12)
\]

\[
\text{HS} \quad \text{S—COCH}_3 \quad + \text{HS—CoA} \rightarrow \text{HS} \quad \text{S—COCH}_3 \quad + \text{CH}_2\text{CO—S—CoA} \quad (13)
\]

The amounts of Fraction A used in the dismutation assays (with Fraction B) were 5 to 20 μg. Approximately 3 times as much Fraction A was used in the lipoamide reduction assays. In the absence of Fraction B, 30 to 100 μg of Fraction A were used. Where indicated 2.9 μg of Fraction B (specific activity 4900 (9)) were added.

### TABLE V

<table>
<thead>
<tr>
<th>Fraction A preparation</th>
<th>Specific activities</th>
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<tr>
<td></td>
<td>Pyruvate dismutation</td>
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<tr>
<td></td>
<td>Without Fraction B</td>
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<td>1</td>
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<td>2</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
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</table>

Fig. 2. Anaerobic reduction and reoxidation of the flavin in the pyruvate dehydrogenation complex. Curve A, 1.8 mg of complex (specific activity 1138 in pyruvate dismutation assay) and 0.01 μmole of thiamine-PP in 0.25 ml of 0.05 M potassium phosphate buffer (pH 7.0); 0.1 μmole of potassium pyruvate was added at 1 minute, 0.02 μmole of CoA and 0.2 μmole of L-cysteine at 2 minutes, and 0.4 μmole of di-potassium lipoate at 10 minutes. Absorbancy was measured at 455 μm. Curves B and C, 1.6 mg of complex (specific activity 908) and 0.01 μmole of thiamine-PP in 0.25 ml of 0.05 M phosphate buffer (pH 7.0); 0.1 μmole of pyruvate was added at 1 minute, 0.02 μmole of CoA and 0.2 μmole of L-cysteine at 2 minutes, 0.1 μmole of DPN at 8 minutes, and 1 μmole of acetaldehyde and 20 units of crystalline alcohol dehydrogenase at 15 minutes. Absorbancy was measured at 455 μm (Curve B) and at 340 μm (Curve C). The temperature was 4°. Pyr, Ald, and ADH represent, respectively, pyruvate, acetaldehyde, and alcohol dehydrogenase.
FIG. 3. Anaerobic reduction and reoxidation of the flavin in the \( \alpha \)-ketoglutarate dehydrogenation complex. Curve A, 1.4 mg of complex (specific activity 86 in ferricyanide reduction assay) and 0.01 \( \mu \)mole of thiamine-PP in 0.20 ml of 0.05 M phosphate buffer (pH 7.0); 0.2 \( \mu \)mole of potassium \( \alpha \)-ketoglutarate was added at 1 minute, 0.02 \( \mu \)mole of CoA and 0.2 \( \mu \)mole of L-cysteine at 2 minutes, and 0.4 \( \mu \)mole of \( \alpha \)-potassium lipoate at 10 minutes. Absorbancy was measured at 353 nmu. Curves B and C, 0.84 mg of complex (specific activity 103) and 0.01 \( \mu \)mole of thiamine-PP in 0.25 ml of 0.05 M phosphate buffer (pH 7.0); 0.1 \( \mu \)mole of \( \alpha \)-ketoglutarate was added at 1 minute, 0.02 \( \mu \)mole of CoA and 0.2 \( \mu \)mole of L-cysteine at 2 minutes, 0.1 \( \mu \)mole of DPN at 8 minutes, and 0.1 \( \mu \)mole of pyruvate and 20 units of crystalline lactate dehydrogenase at 15 minutes. Absorbancy was measured at 355 nmu (Curve B) and at 340 nmu (Curve C). The temperature was 4 °C. KG, Pyr, and LDH represent, respectively, \( \alpha \)-ketoglutarate, pyruvate, and lactate dehydrogenase.

The availability of a method of releasing the catalytically active lipoic acid from protein and of reactivating the apoenzyme (7) has provided direct evidence of involvement of protein-bound lipoic acid in Reaction 1 and has permitted some clarification of the mechanism of model reactions catalyzed by the pyruvate and \( \alpha \)-ketoglutarate dehydrogenation complexes and enzymatic components thereof. Thus, it was shown in a previous paper (7), and corroborated in the present investigation, that release of protein-bound lipoic acid did not affect the dihydrolipoic transacetylase and dihydrolipoic dehydrogenase activities of the pyruvate dehydrogenation complex as measured in the model systems (Reactions 6 and 7) with substrate amounts of dihydrolipoic acid. The dihydrolipoic dehydrogenase activity of the \( \alpha \)-ketoglutarate dehydrogenation complex also was not affected by release of protein-bound lipoic acid. These results and the fact that highly purified \( E. \) coli dihydrolipoic dehydrogenase does not contain a detectable amount of bound lipoic acid (cf. (9)) indicate that the over-all model reactions (Reactions 6 and 7) and, therefore, the component reactions (Reactions 9 and 10) are not mediated through protein-bound lipoic acid. Apparently dihydrolipoic dehydrogenase and dihydrolipoic transacetylase can react with the catalytically active bound cofactor or, alternatively, with free dihydrolipoic acid. Since the available evidence indicates that the lipoic moiety in the pyruvate dehydrogenation complex is not attached to either dihydrolipoic transacetylase or dihydrolipoic dehydrogenase it would appear to be linked to an enzyme which catalyzes one of the earlier steps in pyruvate (and \( \alpha \)-ketoglutarate) dehydrogenation, presumably Reaction 12. As yet the individual enzymes presumed to catalyze Reactions 11 through 13 have not been isolated. The isolation of these enzymes from the complexes and reconstitution of active systems will be required for verification of the postulated reactions and further elucidation of mechanism.

Gunsalus et al. (3, 16) reported previously that a small amount of a thioester, presumed to be \( S \)-acetyl dihydrolipoic acid, was produced when a partially purified preparation of \( E. \) coli Fraction A was incubated with pyruvate, thiamine-PP, and a substrate amount of DL-lipoic acid. When the incubation mixture was supplemented with phosphotransacetylase, inorganic phosphate, and a catalytic amount of CoA, substantial and equivalent amounts of acetyl phosphate, dihydrolipoic acid, and CO\(_2\) were produced (Reaction 5). These results were interpreted as indicating a reductive acetylation of lipoic acid, followed by acetyl transfer to CoA and then to phosphate. The occurrence of Reaction 5 has been confirmed with preparations of the \( E. \) coli pyruvate dehydrogenation complex. However, attempts to produce hydroxylamine-reactive material in the absence of exogenous CoA were unsuccessful. Sanadi et al. (17) also reported failure to demonstrate synthesis of \( S \)-acetyl dihydrolipoic acid from pyruvate and free lipoic acid in the presence of \( E. \) coli Fraction A. In addition to the CoA requirement in Reaction 5, it was shown in a previous paper (7) and corroborated in the present investigation (Table I) that protein-bound lipoic acid participates in the reaction. These cofactor requirements were interpreted previously (7) and indicated that the sequence in Reaction 5 is Reactions 11 to 13 followed by the phosphotransacetylase reaction (Reaction 2). To account for the formation of free dihydrolipoic acid (or dihydrolipoamide) it was suggested that protein-bound dihydrolipoic acid, produced in Reaction 13, might be oxidized by free lipoic acid (or lipoamide) in a "disulfide interchange" type of reaction. The present finding that FAD is required in Reaction 5 renders unlikely a disulfide interchange reaction and suggests that the free dihydrolipoic acid (or dihydrolipoamide) is produced by reaction of reduced flavin formed in Reaction 14 with free lipoic acid (or lipoamide), i.e., Reaction 16. The flavin requirement in Reaction 5 can be attributed to participation of dihydrolipoic dehydrogenase in this reaction (cf. Table V). Direct evidence that \( E. \) coli dihydrolipoic dehydrogenase (Fraction B) is a flavoprotein.
tein is presented in an accompanying paper (9). The available evidence indicates that Reaction 5 is the sum of Reactions 11 to 14, involving protein-bound lipoic acid, and Reactions 2 and 16, the latter reaction involving free lipoic acid (or lipoamide). This proposed reaction sequence is consistent with the spectral changes observed in Fig. 2, Curve A, i.e. addition of both pyruvate and CoA reduced the flavin in the pyruvate dehydrogenation complex and reoxidation of the flavin was observed on subsequent addition of di-lipoic acid. Granted the reasonable -0.34 volt at pH 7.0. A note of caution is in order, however, since the observation of Hager and Gunsalus (4, 5) that bound lipoic acid is involved in Reaction 8. Apparently ferricyanide can function at still another site in the reaction of Sanadi et al. (17) that oxidation of α-ketoglutarate by the pig heart α-ketoglutarate dehydrogenation complex with ferri-cyanide as electron acceptor is not arsinite-sensitive is consistent with this suggestion, notwithstanding the implication of these authors that bound lipoic acid is involved in Reaction 8. Apparently ferri-cyanide can function at still another site in α-keto acid oxidation, possibly with a flavoprotein, in view of the observation of Hager and Gunsalus (4, 5) that lipoic acid stimulated markedly the oxidation of pyruvate by lipoic acid-deficient Streptococcus faecalis cells with ferri-cyanide as electron acceptor. It is to be noted (Tables I and II) that the rate of α-ketoglutarate oxidation by the α-ketoglutarate dehydrogenation complex with ferri-cyanide as electron acceptor is approximately 10 times as fast as the rate of pyruvate oxidation by the pyruvate dehydrogenation complex under similar conditions. Furthermore, the latter rate is only one-hundredth the dismutation rate. A similar difference in the ferri-cyanide reduction and pyruvate dismutation rates was noted by Hager and Gunsalus (4, 5) with purified E. coli Fraction A. Clarification of these differences in rate must await isolation from the complexes of the enzymes presumed to catalyze Reaction 11.

Since the reduction of FAD by DPNH is considered to be practically irreversible with respect to the free nucleotide, the direction of electron transfer observed with the flavoprotein in the complexes (cf. Reaction 15) is somewhat unexpected. However, it should be noted that the two systems linked by the flavoprotein, i.e. DPNH-DPN and LiplS2-LipS3, have approximately the same reduction potential in the free state and, therefore, interaction of those two systems would be expected to be readily reversible on thermodynamic grounds. The potential of the DPNH-DPN system is -0.320 volt at pH 7.0 (18) and that of the Lip(SH)2-LipS2 system is -0.294 volt at pH 7.1, based on equilibrium measurements of Reaction 10 (19), and -0.325 volt at pH 7.0 based on polarographic measurements (20). Also of significance with respect to Reaction 15 is the recent report of Searls and Sanadi (21) that the reduction potential of dihydrolipoic dehydrogenase prepared from the pig heart α-ketoglutarate dehydrogenation complex is approximately -0.34 volt at pH 7.0. A note of caution is in order, however, lest undue importance be attached to the physiological significance of Reaction 15. The fact that the mammalian and bacterial pyruvate and α-ketoglutarate dehydrogenation complexes can couple with DPN in vivo does not necessarily mean that DPNH is the physiological electron acceptor for these complexes. It is conceivable that in vivo the complexes are linked directly to the electron transport chain, possibly to a flavoprotein or cytochrome b, and that electron transport from α-keto acid to molecular oxygen is not mediated by DPNH. This possibility has yet to be investigated.

SUMMARY

The effect of release and reincorporation of lipoic acid and flavin adenine dinucleotide on the enzymatic activities of the Escherichia coli pyruvate and α-ketoglutarate dehydrogenation complexes was determined. The results indicate that both protein-bound lipoic acid and flavin adenine dinucleotide are required for oxidation of pyruvate and α-ketoglutarate with dihydrolipoic acid as electron acceptor. Both cofactors are also required for oxidation of pyruvate with free lipoic acid (or lipoamide) as electron acceptor. Flavin adenine dinucleotide, but not protein-bound lipoic acid, is required for dihydrolipoic acid dehydrogenase activity of both complexes as measured with free dihydrolipoic acid. Neither cofactor is required for oxidation of pyruvate or α-ketoglutarate with ferri-cyanide as electron acceptor, nor for dihydrolipoic transacetetylase activity of the pyruvate dehydrogenation complex as measured with free dihydrolipoic acid. It appears that the site of ferri-cyanide action is at the "aldehyde-thiamine pyrophosphate" level. The flavin in the pyruvate and α-ketoglutarate dehydrogenation complexes is reduced on addition of both pyruvate and coenzyme A and α-ketoglutarate and coenzyme A, respectively, and reoxidized on addition of lipoic acid. The data strongly indicate that the flavin in the complexes is associated with dihydrolipoic dehydrogenase, and that the sequence of electron transfer is pyruvate (α-ketoglutarate) → bound lipoic acid → FAD → DPNH (or free lipoic acid). A reaction sequence for the coenzyme A- and dihydrolipoic acid nucleotide-linked oxidative deacetylxylation of pyruvate and α-ketoglutarate which is consistent with the available data is presented.

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α-Keto Acid Dehydrogenation Complexes: II. THE ROLE OF PROTEIN-BOUND LIPOIC ACID AND FLAVIN ADENINE DINUCLEOTIDE
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