Reconstitution of the *Escherichia coli* pyruvate dehydrogenase complex

(multienzyme complex/subunit composition/structural model)


Clayton Foundation Biochemical Institute and Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712

Contributed by Lester J. Reed, June 6, 1975

**ABSTRACT** The binding of pyruvate dehydrogenase and dihydrolipoyl dehydrogenase (flavoprotein) to dihydrolipoyl transacetylase, the core enzyme of the *E. coli* pyruvate dehydrogenase complex [EC 1.2.4.1; pyruvate:diaphorase 2-oxidoreductase (decarboxylating and acceptor-acetylating)], has been studied using sedimentation equilibrium analysis and radioactive enzymes in conjunction with gel filtration chromatography. The results show that the transacetylase, which consists of 24 apparently identical polypeptide chains organized into a cube-like structure, has the potential to bind 24 pyruvate dehydrogenase dimers in the absence of flavoprotein and 24 flavoprotein dimers in the absence of pyruvate dehydrogenase. The results of reconstitution experiments, utilizing binding and activity measurements, indicate that the transacetylase can accommodate a total of only about 12 pyruvate dehydrogenase dimers and six flavoprotein dimers and that this stoichiometry, which is the same as that of the native pyruvate dehydrogenase complex, produces maximum activity. It appears that steric hindrance between the relatively bulky pyruvate dehydrogenase and flavoprotein molecules prevents the transacetylase from binding 24 molecules of each ligand. A structural model for the native and reconstituted pyruvate dehydrogenase complex is proposed in which the 12 pyruvate dehydrogenase dimers are distributed symmetrically on the 12 edges of the transacetylase cube and the six flavoprotein dimers are distributed in the six faces of the cube.

The *Escherichia coli* (Crookes strain) pyruvate dehydrogenase complex [EC 1.2.4.1; pyruvate:diaphorase 2-oxidoreductase (decarboxylating and acceptor-acetylating)] has been separated into three enzymes—pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase (1). These three enzymes, acting in sequence, catalyze overall Reaction 1. The pyruvate dehydrogenase and the dihydrolipoyl dehydrogenase (a flavoprotein) are joined to the transacetylase by noncovalent bonds. The appearance of the transacetylase in the electron microscope is that of a cube, and its design appears to be based on octahedral (432) symmetry (2, 3). It consists of 24 apparently identical polypeptide chains of molecular weight 65,000 to 70,000 (4). Each chain apparently contains one molecule of covalently bound lipoic acid. The molecular weight of pyruvate dehydrogenase isolated from the complex is about 192,000, and it consists of two apparently identical polypeptide chains. The isolated flavoprotein contains two apparently identical polypeptide chains and two molecules of flavin adenine dinucleotide per molecule of enzyme of molecular weight about 112,000. Our data (4) show that the native pyruvate dehydrogenase complex (molecular weight about 4.6 million) consists of about 24 pyruvate dehydrogenase chains (or 12 dimers), 24 transacetylase chains, and about 12 flavoprotein chains (or six dimers). The native complex does not bind additional pyruvate dehydrogenase or flavoprotein. This subunit stoichiometry is supported by recent thiamine pyrophosphate binding measurements (5) and flavin determinations (6). However, Vogel et al. (7) have reported a subunit composition of 16 pyruvate dehydrogenase chains, 16 transacetylase chains, and 16 flavoprotein chains for the *E. coli* K-12 pyruvate dehydrogenase complex. It is apparent from our data that the *E. coli* pyruvate dehydrogenase complex does not contain equimolar amounts of its component enzymes. The present investigation was undertaken to determine the molecular basis of this phenomenon and its functional significance.

**MATERIALS AND METHODS**

Enzyme Preparations. The basic procedures for growing cells of *E. coli* (Crookes strain) and for purification and resolution of the pyruvate dehydrogenase complex were carried out as described (1, 4). Radioactive enzymes were isolated from cells grown in the presence of L-[U-14C]leucine (Schwartz/Mann, 0.27 Ci/mmole), L-[4,5-3H]leucine (Amer- sham/Searle, 51 Ci/mmmole), or DL-[35S]lipoic acid (8 x 10^4 cpm/μg). When labeling was done with [14C]leucine or [3H]leucine, 100 μCi of radioactive leucine and 1.5 mg of unlabeled L-leucine were added per liter of culture medium. The radioactive cells were mixed with unlabeled cells in a ratio of 1:9 (w/w) before processing. When labeling was with [35S]lipoic acid, 102 μg of the radioactive lipoic acid in 10 μl of 5% NaHCO₃ was added per liter of sterilized culture medium. To prevent proteolysis of enzymes during purification and storage, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride were included in the phosphate buffer. The enzyme preparations showed little or no evidence of degradation or contamination.

Sedimentation Equilibrium Experiments. Mixtures containing transacetylase and pyruvate dehydrogenase or transacetylase and flavoprotein in a molar ratio of 1:30 were incubated in 0.05 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA (phosphate-EDTA) for 20 min at 20°C. The transacetylase–pyruvate dehydrogenase subcomplex was separated from uncomplexed pyruvate dehydrogenase by sedimentation in a 10–30% sucrose gradient containing phosphate-EDTA for 160 min at 59,000 rpm (Beckman SW65 Ti rotor). The transacetylase–flavoprotein subcomplex was separated from uncomplexed flavoprotein by sedimentation in a 5–20% sucrose gradient for 70 min at 65,000 rpm. The gradients were analyzed with an Isco density gradient.
fractionator and absorbance recorder. Peak fractions were pooled and dialyzed against three changes of phosphate-EDTA-0.002% sodium azide. Molecular weights were determined by the meniscus depletion technique of Yphantis, with the modifications described previously (4, 8). The rotor speed was 3000 rpm, and the temperature was 5°. The data were evaluated by a program written for a CDC-6600 computer, and the results were analyzed according to the method of Munk and Cox (8). The partial specific volumes of the transacetylase–pyruvate dehydrogenase and transacetylase–flavoprotein subcomplexes were estimated from their amino-acid compositions (4) to be 0.735 and 0.746 ml/g, respectively.

Chromatography on Sepharose 6B. A 1 × 20 cm column of Sepharose 6B (Pharmacia) was treated at 23° with a solution containing 5 mg of crystalline bovine serum albumin in phosphate-EDTA-0.5 mM phenylmethylsulfonyl fluoride and then equilibrated with the buffer solution. A few drops of glycerol were added to the sample (0.5-0.4 ml) and the mixture was carefully layered on the column. The flow rate was about 11 ml/hr, and fractions of about 0.3 ml were collected. Radioactivity was measured with a Beckman LS-230 liquid scintillation system.

Crosslinking of Flavoprotein. A solution containing 1.5 mg of flavoprotein (isolated from the pyruvate dehydrogenase complex) and 0.71 mg of diethyl malonimide hydrochloride (9) in 1.5 ml of 0.2 M triethanolamine buffer, pH 8.5, and 1 mM EDTA was allowed to stand for 50 min at 23° and then dialyzed exhaustively in the cold against phosphate-EDTA. Analysis by polyacrylamide gel electrophoresis (5% polyacrylamide gels) in the presence of dodecyl sulfate (4) showed the presence of about 86% crosslinked flavoprotein dimer, about 4% noncrosslinked flavoprotein, about 8% crosslinked flavoprotein tetramer, and trace amounts of larger oligomeric species. The dihydrolipoyl dehydrogenase activity (10) of the preparation was about 90% of that of untreated flavoprotein.

RESULTS

Binding Capacity of Dihydrolipoyl Transacetylase for Pyruvate Dehydrogenase and for Dihydrolipoyl Dehydrogenase Determined by Sedimentation Equilibrium Analysis. The apparent molecular weights of the transacetylase–pyruvate dehydrogenase and transacetylase–flavoprotein subcomplexes, as determined by sedimentation equilibrium analysis, are given in Table 1. From these data, the known molecular weights of the transacetylase (1,700,000), pyruvate dehydrogenase (192,000), and flavoprotein (112,000), and the flavin content (11) of the transacetylase–flavoprotein subcomplex, we estimate that a molecule of the transacetylase bound about 22 pyruvate dehydrogenase dimers in the absence of the flavoprotein and about 19 flavoprotein dimers in the absence of pyruvate dehydrogenase. It should be noted that essentially identical results were obtained with flavoprotein isolated from the pyruvate dehydrogenase complex, crosslinked flavoprotein dimer, flavoprotein isolated from the α-ketoglutarate dehydrogenase complex. This finding is consistent with evidence that the flavoprotein components of the two complexes are very similar, if not identical (12, 13). Since we found previously (14) that dihydrolipoyl transsuccinylase, the core enzyme of the α-ketoglutarate dehydrogenase complex, also binds about 18 flavoprotein dimers, the binding sites for flavoprotein on the transacetylase and on the transsuccinylase are likely to be very similar, if not identical.

Table 1. Binding capacity of transacetylase for pyruvate dehydrogenase and for flavoprotein determined by sedimentation equilibrium

<table>
<thead>
<tr>
<th>Subcomplex*</th>
<th>Mapp</th>
<th>E₁</th>
<th>E₂</th>
<th>E₃</th>
</tr>
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</tr>
<tr>
<td>E₁-E₂</td>
<td>3,850,000</td>
<td>1</td>
<td>19</td>
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</tr>
</tbody>
</table>

*Subcomplexes consisting of pyruvate dehydrogenase (E₁) and dihydrolipoyl transacetylase (E₂) or transacetylase and dihydrolipoyl dehydrogenase (E₃) were prepared as described in Materials and Methods.

Binding Stoichiometry Determined with Radioactive Enzymes in Conjunction with Gel Filtration Chromatography. Mixtures containing 14C-labeled transacetylase and 3H-labeled pyruvate dehydrogenase in a molar ratio of 1:36 and 14C-labeled transacetylase and 3H-labeled flavoprotein in a molar ratio of 1:48 were chromatographed on columns of Sepharose 6B. The elution profiles are shown in Fig. 1. The subcomplexes emerged within 30 min after application of the samples to the columns. From the radioactivity of the peak fractions and the known specific activities of the individual enzymes, we calculate that a molecule of the transacetylase bound about 22 pyruvate dehydrogenase dimers in the absence of flavoprotein and about 21 flavoprotein dimers in the absence of pyruvate dehydrogenase.

Inhibition of Binding of Pyruvate Dehydrogenase to Transacetylase by Bound Flavoprotein. A mixture containing 35S-labeled transacetylase and 3H-labeled flavoprotein in a molar ratio of 1:24 was incubated for 20 min at 23°. The binding stoichiometry, determined by chromatography on Sepharose 6B, showed that about 18.5 flavoprotein dimers were bound per molecule of transacetylase. Pyruvate dehydrogenase was added to the incubation mixture at a molar ratio to transacetylase of 13:1, and the rate of appearance of NAD reduction activity (15) was followed. At 2 min the specific activity of the mixture was 4.6 μmol of NADH produced/min per mg of transacetylase, and at 3 hr, the specific activity was 34.8. By comparison, the specific activity of the native pyruvate dehydrogenase complex is 85–100 μmol of NADH/min per mg of transacetylase (equivalent to 34–37 μmol of NADH/min per mg of complex). In a parallel experiment, 35S-labeled transacetylase and 3H-labeled flavoprotein in a molar ratio of 1:7 were incubated for 20 min at 23°, and then pyruvate dehydrogenase was added at a molar ratio to transacetylase of 13:1. At 2 min the specific activity of this mixture was 100. The simplest interpretation of these results is that binding of flavoprotein in "excess" of about six flavoprotein dimers per molecule of transacetylase inhibits binding of pyruvate dehydrogenase to the transacetylase. That binding of pyruvate dehydrogenase to the transacetylase–flavoprotein subcomplex and concomitant appearance of NAD reduction activity involves displacement of "excess" flavoprotein was indicated by the results of a second set of experiments. 35S-Labeled transacetylase.
Inhibition of Binding of Flavoprotein to Transacetylase by Bound Pyruvate Dehydrogenase. In a related set of experiments $^{35}$S-labeled transacetylase (8991 cpm/nmol) and $^3$H-labeled pyruvate dehydrogenase (4784 cpm/nmol) in a molar ratio of 1:25 (Solution A) and nonradioactive transacetylase and $^3$H-labeled pyruvate dehydrogenase in a molar ratio of 1:25 (Solution B) were incubated for 20 min at 23°. Analysis of a portion of Solution A by chromatography on Sepharose 6B showed that about 19 pyruvate dehydrogenase dimers were bound per molecule of transacetylase. Nonradioactive flavoprotein was added to Solution A at a molar ratio to transacetylase of 15:1, and $^{14}$C-labeled flavoprotein (1186 cpm/nmol) was added to Solution B at a molar ratio to transacetylase of 13:1. After 2 min of incubation at 23° the specific activities of the two mixtures were 38 and 47 μmol of NADH/min per mg of transacetylase, respectively. The specific activities increased to 92 and 97, respectively, after 20 min of incubation. Analysis of the two incubation mixtures by chromatography on Sepharose 6B showed that the reconstituted pyruvate dehydrogenase complex contained about 13 pyruvate dehydrogenase dimers and about four flavoprotein dimers per molecule of transacetylase. Since about 19 pyruvate dehydrogenase dimers were bound per molecule of transacetylase prior to addition of flavoprotein, about six pyruvate dehydrogenase dimers were displaced from the transacetylase–pyruvate dehydrogenase subcomplex during incubation with flavoprotein. Flavoprotein apparently displaces “excess” pyruvate dehydrogenase from the transacetylase more readily than pyruvate dehydrogenase displaces “excess” flavoprotein. The results also indicate that near maximum activity is obtained with a reconstituted pyruvate dehydrogenase complex containing about 12 pyruvate dehydrogenase dimers and only about four flavoprotein dimers. This finding was confirmed and extended in the titration experiments described below.

Reconstitution of Pyruvate Dehydrogenase Complex Activity. A series of experiments was performed to determine the molar ratio of pyruvate dehydrogenase, transacetylase, and flavoprotein and the conditions required to reconstitute a pyruvate dehydrogenase complex with maximum activity. Preliminary experiments indicated that near maximum NAD reduction activity was obtained when the molar ratio of the three enzymes in the incubation mixture was about 16:1:7, respectively. At this molar ratio, the order of addition of pyruvate dehydrogenase and flavoprotein to the transacetylase did not affect significantly the recovery of NAD reduction activity, and near maximum activity was obtained within 2 min of incubation of these ligands with the transacetylase. The data presented in Fig. 2 (Curve 1) show representative results obtained when a mixture containing transacetylase and pyruvate dehydrogenase in a molar ratio of 1:16 was exposed to increasing amounts of flavoprotein. Curve 2 shows the results obtained when a mixture containing transacetylase and flavoprotein in a molar ratio of 1:7 was incubated with increasing amounts of pyruvate dehydrogenase. To determine the binding stoichiometry associated with near maximum activity of the reconstituted pyruvate dehydrogenase complex, a mixture containing 0.42 mg (0.25 nmol) of nonradioactive transacetylase...
and 0.77 mg (4.0 nmol) of $^{14}$C-labeled pyruvate dehydrogenase (544 cpm/nmol) was incubated for 15 min at 23°, and then 0.20 mg (1.8 nmol) of $^{3}$H-labeled flavoprotein (2558 cpm/nmol) was added. After further incubation for 2 min, the mixture showed a specific activity of 93 µmol of NADH/min per mg of transacytase. The reconstituted pyruvate dehydrogenase complex was separated from uncomplexed pyruvate dehydrogenase and flavoprotein by chromatography on Sepharose 6B. Radioactivity measurements showed that the molar ratio of $^{14}$C-labeled pyruvate dehydrogenase to $^{3}$H-labeled flavoprotein in the reconstituted complex was 1.85. This ratio is close to the ratio (about 2.0) found for the native pyruvate dehydrogenase complex and corresponds to an average of about 11 pyruvate dehydrogenase dimers and six flavoprotein dimers per molecule of transacytase.

The transacytase can apparently accommodate a total of about 12 pyruvate dehydrogenase dimers and six flavoprotein dimers, and this stoichiometry produces maximum activity. In view of these findings, we might expect each pyruvate dehydrogenase dimer bound to the transacytase to produce $\frac{1}{2}$ of maximum activity and each flavoprotein dimer bound to produce $\frac{1}{2}$ of maximum activity. The results obtained by titrating the transacytase–flavoprotein subcomplex with pyruvate dehydrogenase (Curve 2) are in reasonable agreement with this expectation. However, it appears that the initial flavoprotein dimers bound to the transacytase–pyruvate dehydrogenase subcomplex produce proportionally greater NADH reduction activity than do subsequent flavoprotein dimers (Curve 1). A possible explanation of this phenomenon is presented in the Discussion.

Functional Identity of Crosslinked and Native Flavoproteins. The binding data presented in Table 1 show that crosslinking the two polypeptide chains comprising the isolated flavoprotein does not affect the binding of the flavoprotein to the transacytase. Since overall Reaction 1 apparently involves a coordinated sequence of reactions between protein-bound intermediates, reconstitution of the NAD-linked oxidation of pyruvate provides a sensitive test of the functional identity of the crosslinked and native flavoproteins. The results obtained by titrating the transacytase–pyruvate dehydrogenase subcomplex with crosslinked flavoprotein (Fig. 2, Curve 3) are similar to those obtained with the native flavoprotein (Curve 1). The differences can be attributed to the somewhat less than optimal molar ratio of transacytase to pyruvate dehydrogenase used in the experiment with crosslinked flavoprotein and to the fact that the purity of the latter preparation was only about 86%. These observations indicate that flavoprotein monomers are not essential for pyruvate dehydrogenase complex activity and that flavoprotein dimers per se are present in the native complex.

DISCUSSION

The data reported in this communication show that the isolated transacytase can bind about 22 pyruvate dehydrogenase dimers in the absence of the flavoprotein and 19 to 21 flavoprotein dimers in the absence of pyruvate dehydrogenase. Since in all experiments the binding was measured after separation of the subcomplex from free ligand rather than under saturation conditions, the numbers of molecules bound to the transacytase are not necessarily saturation values. The possibility also exists that a few binding sites on the transacytase were impaired during its isolation and purification. In view of these considerations and the fact that the transacytase consists of 24 apparently identical polypeptide chains, we interpret the binding data as indicating that there is a binding site (domain) on each of the 24 transacytase chains for a pyruvate dehydrogenase dimer and a separate binding site on each transacytase chain for a flavoprotein dimer.

Although the transacytase has the potential to bind 24 pyruvate dehydrogenase dimers and 24 flavoprotein dimers, the results of the reconstitution experiments indicate that the transacytase can accommodate a total of only 12 pyruvate dehydrogenase dimers and six flavoprotein dimers and that this stoichiometry, which is the same as that of the native pyruvate dehydrogenase complex, produces maximum activity. Because enzymatic function requires a particular spatial arrangement of reacting groups, it is likely that attachment of the 12 pyruvate dehydrogenase dimers and six flavoprotein dimers to the transacytase follows a definite pattern. Electron microscopic studies (16) indicate that the pyruvate dehydrogenase molecules in the native complex are located near the 2-fold positions (i.e., on the edges) of the transacytase cube and that the flavoprotein molecules are located at the fourfold positions (i.e., in the faces). Apparently, the "excess" molecules of either ligand bound to the transacytase lie close to its surface, since the transacytase–pyruvate dehydrogenase and transacytase–flavoprotein subcomplexes have essentially the same dimensions as the native pyruvate dehydrogenase complex (R. M. Oliver...
Since binding of pyruvate dehydrogenase in excess of 12 dimers or of flavoprotein in excess of six dimers reduces the activity of the pyruvate dehydrogenase complex, it seems likely that the amount of pyruvate dehydrogenase and flavoprotein synthesized in vivo is regulated. It should be noted in this connection that the structural genes for pyruvate dehydrogenase and dihydriopolyl transacetylase are closely linked on the E. coli chromosome, and that biosynthesis of the complex begins with the biosynthesis of the pyruvate dehydrogenase component (17). It appears that there is only one gene controlling the synthesis of dihydriopolyl dehydrogenase for both the pyruvate and α-ketoglutarate dehydrogenase complexes (18). Our in vitro studies indicate that any "excess" pyruvate dehydrogenase bound to the transacetylase would be relatively easily displaced by the flavoprotein.

That the amount of flavoprotein synthesized by E. coli Crookes cells may be restricted to that amount required for maximal activity of the pyruvate and α-ketoglutarate dehydrogenase complexes is suggested by our previous observation (14) that the native α-ketoglutarate dehydrogenase complex contains only six flavoprotein dimers (or 12 chains), although the complex is capable of binding at least 12 additional flavoprotein dimers.

We thank Karen Hobson, Fred Hoffman, and William Lee for skilled technical assistance, Dr. Peter Munk for advise and assistance with the sedimentation equilibrium analyses, and Dr. Marvin Hackert and Dr. David DelRosier for valuable discussion. This work was supported in part by Grant GM06590 from the United States Public Health Service.