Mammalian α-Keto Acid Dehydrogenase Complexes

VII. RESOLUTION AND RECONSTITUTION OF THE PIG HEART 2-OXOGLUTARATE DEHYDROGENASE COMPLEX*

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SUMMARY

The nonionic detergent Triton X-100 has been found to solubilize readily the pig heart 2-oxoglutarate dehydrogenase complex from the protamine precipitate eluate. A highly active preparation was obtained from the Triton X-100 extract by calcium phosphate gel-cellulose column chromatography.

The complex was first separated into lipoamide dehydrogenase and a colorless fraction by fractionation on a calcium phosphate gel-cellulose column in the presence of 2.5 M urea. Then, the latter fraction was further dissociated into two additional components, 2-oxoglutarate dehydrogenase and lipoate succinyltransferase, by Sepharose 6B gel chromatography at pH 7.0 in the presence of 0.7 M guanidine hydrochloride, 0.5 % Triton X-100, and 2 mM dithiothreitol. When mixed at neutral pH, the three isolated enzymes reassociated spontaneously to produce a large unit functionally and structurally resembling the native complex. The complex was also reconstituted from the colorless fraction and lipoamide dehydrogenase. Reconstitution experiments indicated that 2-oxoglutarate dehydrogenase and lipoamide dehydrogenase did not combine with each other but each of these enzymes did combine with lipoate succinyltransferase.

Electron micrographs of the complex negatively stained with 0.25% sodium phosphotungstate (pH 7.2) showed a polyhedral structure with a diameter of about 260 Å. This suggests symmetrical distribution of morphological subunits around the core of a polyhedron with dimensions and appearance similar to that of the isolated lipoate succinyltransferase molecule, which is 117 Å in diameter.

Biochemical and electron microscopic data indicate that the complex is a mosaic comprising 1 molecule of lipoate succinyltransferase, 6 molecules of 2-oxoglutarate dehydrogenase, and 6 molecules of lipoamide dehydrogenase.

As reported in previous papers of this series (1–4), the enzyme systems which catalyze a coenzyme A- and nicotinamide adenine dinucleotide-linked oxidative decarboxylation of pyruvate and 2-oxoglutarate (Reaction 1) in pig heart tissue have been isolated from the Keilin-Hartree preparation as soluble multienzyme complexes with molecular weights of several million.

\[
R-CO-COOH + CoA-SH + NAD^+ \rightarrow R-CO-S-CoA + CO_2 + NADH + H^+ \quad (1)
\]

where \( R = \text{CH}_3 \) or \( \text{HOOC-} \text{(CII,)} \).

Subsequently, the pyruvate dehydrogenase complex has been separated into three component enzymes and reassembled from these isolated enzymes (4). Recently, the isolation procedure for the 2-oxoglutarate dehydrogenase complex previously reported (3) has been improved by a solubilization of the complex from the protamine precipitate eluate with Triton X-100 instead of the freezing and thawing procedure and ultracentrifugation. This complex has been further separated into three component enzymes and reassociated from these enzymes. The present paper deals with the improved isolation procedure, resolution and reconstitution studies of the 2-oxoglutarate dehydrogenase complex. Some of this work has already been reported in brief (5).

EXPERIMENTAL PROCEDURE

Materials—Triton X-100, alkylaryl polyether alcohol, was a gift from Rohm & Haas, Pa. Guanidine hydrochloride (guaranteed reagent) and dithiothreitol were obtained from Nakarai Chemicals, Kyoto; mercaptoethanol from Hayashi Pure Chemical Industries, Osaka; and Sepharose 6B from Pharmacia AB, Uppsala. Takadiastase was a gift from Doctors G. Sunagawa and T. Yusa, Sankyo Company, Tokyo. dl-Lipoamide was a gift from Dr. H. Nakano, Fujisawa Pharmaceutical Company, Osaka. Butvar B and 72A were generously furnished by Monsanto Co., Mass. The sources of other materials are described in previous papers (1–6). All solutions were prepared with distilled and deionized water; 0.05 M potassium phosphate buffer used for the solvent and dialyses of enzyme preparations contained 0.5 mM EDTA.

Methods—Protein was determined by the phenol method (7) with crystalline bovine serum albumin as the standard.
phosphate gel suspended on neutral cellulose powder (Whatman Chromata CF 1 or CF II) was prepared as described by Koike and Hata (8). Protein bound lipoic acid content was determined after alkaline hydrolysis (2) as described by Gansalus, Dolin, and Struglia (9). Flavin content was determined by the method of Beinert and Page (10) and thiamine-PP content by the method of Kanzawa (11) with pyruvate decarboxylase (2-oxo-acid carboxylate-lyase, EC 4.1.1.1), which was prepared by the modified procedure (12) of Green, Herbert, and Subrahmanyan (13). Thiamine-PP was also assayed spectrophotometrically after conversion to thiamine by takadiastase by the thiochrome method of Fujisawa and Matus (14). Ultra centrifugal analyses were performed at 3–7° in a Beckman model E analytical ultracentrifuge equipped with schlieren and Rayleigh optical systems. Sedimentation coefficients were calculated and corrected as described by Schachman (15). Molecular weights were determined by the meniscus depletion sedimentation equilibrium method described by Yphantis (16). For the equilibrium runs, the An-D rotor was used at higher speeds with a 12-mm six-channel Kel-F or filled Epon centerpiece and sapphire windows, while at speeds below 12,000 rpm the heavy An-J rotor was used. Weight-averaged molecular weights were represented as the average of those obtained from each of three black fringes. Errors were indicated as standard deviations from the mean molecular weight. In the calculations of sedimentation coefficients and molecular weights, the partial specific volume (3) was assumed to be 0.73 ml per g. Electron microscopy of the specimen negatively stained with 0.25% sodium phosphotungstate (pH 7.2) essentially as described by Hayakawa et al. (4) was performed in a JEM model 7A electron microscope operating at 80 kv.

Enzyme Assays—Ferricyanide-linked 2-oxoglutarate dehydrogenase activity was measured spectrophotometrically as described by Massey (17). Lipoate succinyltransferase (succinyl-CoA-dihydrolipoate 2-succinyltransferase) assay was a modification (3) of the procedure described by Smith (18). The reaction mixture (1.0 ml) contained 100 μmoles of Tris-HCl buffer (pH 7.2), 180 μmoles of potassium succinate (pH 7.2), 10 μmoles of magnesium chloride, 6.5 μmoles of potassium ATP (pH 7.0), 0.1 μmoles of CoA, 10 μmoles of DL-dihydrolipoamide, 5 units of Escherichia coli succinyl-CoA synthetase (succinate:CoA ligase (ADP), EC 6.2.1.5), 0.13 μmoles of diethiothreitol, and 2 mg of bovine serum albumin. The mixture was incubated for 30 min at 37° and then assayed for succinylthioester (19). All operations at 80 kv. Thiamine-PP was also assayed spectrofluorometrically as described by Massey (17). Lipoate succinyltransferase (succinate:CoA ligase (ADP), EC 6.2.1.5), 0.13 μmoles of diethiothreitol, and 2 mg of bovine serum albumin. The mixture was incubated for 30 min at 37° and then assayed for succinylthioester (19). All operations at 80 kv.

Errors were indicated as standard deviations from the mean.

Results

Purification of 2-Oxoglutarate Dehydrogenase Complex

Preparation of the amber extract of the pig heart Keilin-Hartree preparation (20) and subsequent protamine sulfate fractionation were carried out essentially as described in previous papers (2, 3).

Step 1: Protamine Precipitate Eluate—The first protamine precipitate resulting from the addition of 0.01 volume of 2% protamine sulfate solution (pH 5.0) to the amber extract was suspended in 0.1 M potassium phosphate buffer (pH 7.0) equal to 0.05 volume of the amber extract and homogenized with a Teflon pestle homogenizer. The homogenate was dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) overnight at 0° and then centrifuged for 20 min at 27,000 × g. The supernatant solution was kept at −20° for 3 days.

Step 2: Triton X-100 Extraction—To 190 ml of the protamine precipitate eluate containing 4.7 g of protein, 21 ml of 20% Triton X-100 in 0.05 M potassium phosphate buffer (pH 7.0) was added, to give a final concentration of 3%. The mixture was homogenized with a Teflon pestle homogenizer for 5 min, stirred for 1 hour, and then centrifuged for 30 min at 27,000 × g. The supernatant solution, denoted as Triton X-100 extract, was diluted to give a protein concentration of 30 to 50 mg per ml with 0.1 M potassium phosphate buffer (pH 7.5).

Step 3: First Calcium Phosphate Gel-Cellulose Column Chromatography—Approximately 1 g of protein of Triton X-100 extract was applied to a column (4 × 8 cm) of calcium phosphate gel-cellulose (less than 1 month old) which had been washed previously with about 200 ml of 0.1 M potassium phosphate buffer (pH 7.5). The column was then washed with 5 to 7 column volumes of the same buffer, leaving a bright yellow band in the upper part of the column. The yellow fluorescent band was eluted with a solution of 1% ammonium sulfate in 0.1 M potassium phosphate buffer (pH 7.5). The yellow eluates from five such columns were combined, and fractionated with ammonium sulfate between 0.24 and 0.29 saturation. The precipitate was collected by centrifugation for 15 min at 27,000 × g, and dissolved in a small volume of 0.05 M potassium phosphate buffer (pH 7.0). After overnight dialysis against the same buffer the solution was centrifuged for 20 min at 27,000 × g to remove insoluble materials.

Step 4: Second Calcium Phosphate Gel-Cellulose Column Chromatography—To 15 ml of the yellow supernatant solution from the previous step containing 545 mg of protein, 2.65 ml of 20% Triton X-100 solution were again added, and the mixture was frozen for several days at −20°. After thawing the mixture was homogenized and centrifuged. The supernatant solution was applied to a calcium phosphate gel-cellulose column (4 × 8 cm) and fractionated as described at Step 3. The yellow eluate was concentrated by ammonium sulfate precipitation between 0.24 and 0.29 saturation, and the yellow precipitate was dissolved in a small volume of 0.05 M potassium phosphate buffer (pH 7.0), followed by dialysis overnight against the same buffer. Any insoluble material was removed by centrifugation. A summary of the purification is given in Table I. The purified preparation was bright yellow and highly fluorescent, and was nearly homogeneous as revealed by ultracentrifugation with s20,w = 31.5 S (cf. Fig. 2A). The weight-average molecular weight of the complex was 2,706 ± 0.015 million and this value was very close to that reported in a previous paper (3). The preparation was stored at −20° and lost less than 10% of its activity in 1 month.

Resolution and Reconstitution

Resolution of 2-Oxoglutarate Dehydrogenase Complex with 2.5 M Urea—The complex was dissociated into two components, the colorless fraction and lipoamide dehydrogenase, by a modification of the procedure of Massey (17). A solution containing 100 mg of the complex in 3.2 ml of 0.05 M potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA was mixed with an equal volume of a solution of 5 M urea and 2% ammonium sulfate in 0.1 M potassium phosphate buffer (pH 7.0). The mixture was applied to a calcium phosphate gel-cellulose column (4 × 8 cm),
which had been previously washed successively with 200 ml of 0.1 m potassium phosphate buffer (pH 7.5) and 10 ml of a solution of 1% ammonium sulfate in 0.1 m potassium phosphate buffer (pH 7.5). After adsorption of the protein, the column was washed with 17 ml of a solution of 2.5 m urea and 1% ammonium sulfate in 0.1 m potassium phosphate buffer (pH 7.5), and then with a solution of 1% ammonium sulfate in 0.1 m potassium phosphate buffer (pH 7.5). A colorless protein was eluted, leaving a broad yellow, fluorescent band on the column. Immediately after elution, the colorless fraction, comprising about 60 mg of protein in a volume of 60 ml, was collected by ammonium sulfate fractionation between 0.23 and 0.50 saturation. The precipitate was dissolved in a minimum volume of 0.05 m potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer. Any insoluble material was removed by centrifugation. The yellow fluorescent band was then eluted with a solution of 4% ammonium sulfate in 0.1 m potassium phosphate buffer (pH 7.5). The eluate, comprising 15.7 mg of protein in a volume of 60 ml, was concentrated by ammonium sulfate fractionation between 0.4 and 0.8 saturation. The precipitate was dissolved in a small volume of 0.05 m potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer.

Recovery of protein, coenzyme composition, and enzymatic activities of the component enzymes from the complex are summarized in Table II. The colorless fraction contained most of the protein-bound lipoic acid and thiamine-PP found in the complex and exhibited both 2-oxoglutarate dehydrogenase and lipoate succinyltransferase activities. Lipase dehydrogenase contained most of the FAD in the complex and exhibited lipoate succinyltransferase activities. Lipoamide dehydrogenase—The colorless fraction from the previous step was separated into two components, 2-oxoglutarate dehydrogenase and lipoate succinyltransferase, by Sepharose 6B gel chromatography in the presence of 0.7 m guanidine hydrochloride, 0.5% Triton X-100, and 2 m dithiothreitol. First, about 80 ml of Sepharose 6B gel suspended in 0.05 m potassium phosphate buffer (pH 7.0) was added to a jacketed glass chromatographic tube (2.2 × 46 cm). The column was allowed to pack and then washed with about 120 ml of a solution of 2 m dithiothreitol in 0.05 m potassium phosphate buffer (pH 7.0). On this column, another 80 ml of Sepharose 6B gel, which had been previously washed four times with 80 ml of a solution of 0.7 m guanidine hydrochloride in 0.05 m potassium phosphate buffer (final pH 7.0) containing 0.5% Triton X-100 and 2 m dithiothreitol, were poured in layers. The column was allowed to pack overnight by gravity and cooled to 7°C. The mixture of 2.2 ml of the colorless fraction containing 50 mg of protein, 1 mg of dithiothreitol, 1.13 ml of 2.1 m guanidine hydrochloride in 0.05 m potassium phosphate buffer (pH 7.0) and 0.085 ml of 20% Triton X-100 in 0.05 m potassium phosphate buffer (pH 7.0) was applied carefully on the column. After this mixture had passed over the column, the column was developed successively with 20 ml of a solution of 0.7 m guanidine hydrochloride in 0.05 m potassium phosphate buffer (final pH 7.0) containing 0.5% Triton X-100 and 2 m dithiothreitol and then with 110 ml of a solution of 2 m dithiothreitol in 0.05 m potassium phosphate buffer (pH 7.0). Immediately after elution, each 5-ml fraction was exhaustively dialyzed overnight against three changes of 1 liter of 2 m mercaptoethanol in 0.05 m potassium phosphate buffer (pH 7.0). The protein, enzymatic activity, and elution profiles of a Sepharose 6B chromatographic separation of the colorless fraction into two components are given in Fig. 1. The protein emerged as two peaks. The peak eluted first exhibited lipoate succinyltransferase activity and the second 2-oxoglutarate dehydrogenase activity. It is essential for retaining 2-oxoglutarate dehydrogenase activity to maintain the flow rate over 30 ml per hour.

Fractions 19 to 25 exhibiting high 2-oxoglutarate dehydrogenase activity were combined and applied to a calcium phosphate gel-cellulose column (3.0 × 2.6 cm) which had been previously equilibrated with 0.02 m potassium phosphate buffer (pH 7.0). After adsorption of the protein the column was washed with 34 ml of 0.05 m potassium phosphate buffer (pH 7.0) to remove Triton X-100 and then the active protein was eluted with 0.25 m potassium phosphate buffer (pH 7.0). The enzyme protein was collected by ammonium sulfate fractionation between 0 and 0.40 saturation. The precipitate was collected by cen-

TABLE I

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<th>Enzyme</th>
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TABLE II

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<tr>
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<td>8.32 0 0</td>
</tr>
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TABLE III

Weight-average molecular weights and coenzyme contents of native complex, its isolated components, and reconstituted complex

| Enzyme                        | Molecular weight | Nearest integer |
|-------------------------------|------------------|-----------------
|                               | Lipic acid | Thiamine-PP | FAD  |
| 2-Oxoglutarate dehydrogenase  | 2.706 ± 0.015 x 10⁶ | 8     | 6    | 12   |
| Colorless fraction            | 2.029 ± 0.016 x 10⁶ | 8     | 6    | 0    |
| Lipoamide dehydrogenase       | 1.080 ± 0.007 x 10⁶ | 0     | 0    | 2    |
| Lipoate succinyltransferase   | 9.580 ± 0.018 x 10⁶ | 8     | 0    | 0    |
| 2-Oxoglutarate dehydrogenase  | 2.302 ± 0.015 x 10⁶ | 0     | 1    | 0    |
| Reconstituted complex         | 2.405 ± 0.011 x 10⁶ | 8     | 6    | 12   |

*Experiments were carried out in 0.05 M potassium phosphate buffer (pH 7.0) for 88 hours at 4,059 rpm, 72 hours at 4,609 rpm, 23 hours at 21,740 rpm, 87 hours at 6,569 rpm, 40 hours at 16,200 rpm, and 87 hours at 4,059 rpm, respectively, according to the order of the enzyme specimens in this column.

Fig. 1. Gel chromatography on Sepharose 6B of the colorless fraction in the presence of 0.7 M guanidine hydrochloride. Fractions of 5 ml were collected at a flow rate of 30 ml per hour. The void volume of the column was 50 ml. Immediately after elution, each fraction was dialyzed against a solution of 2 mM mercaptoethanol in 0.05 M potassium phosphate buffer (pH 7.0). Then, protein concentration (●—●), and 2-oxoglutarate dehydrogenase (●—●) activities in the effluent were examined as described under "Experimental Procedure." The details of the gel chromatography are described in the text.

Fig. 2. Sedimentation patterns obtained with the 2-oxogluta-
rate dehydrogenase complex, its component enzymes, and the re-
constituted complex. A, the native complex after 24 min at
47,660 rpm; B, the reconstituted complex after 24 min at 47,660
rpm; C, the colorless fraction after 25 min at 47,660 rpm; D, lipo-
amide dehydrogenase after 72 min at 20,780 rpm; E, lipoate succi-
nyltransferase after 36 min at 52,640 rpm; and F, 2-oxoglutarate
dehydrogenase after 42 min at 50,780 rpm. Protein concentrations
were, respectively, 0.09, 0.09, 0.62, 0.55, 0.8, and 0.25 g/100 ml of
0.05 M potassium phosphate buffer (pH 7.0).
of the protein-bound coenzyme content per mole of the enzyme is
given in the same table. From these data it is apparent that
each molecule of lipoate succinyltransferase, 2-oxoglutarate
dehydrogenase, and lipoamide dehydrogenase contains 8 mole-
cules of lipoic acid, 1 molecule of thiamine-PP, and 2 molecules
of FAD, respectively. The complex appeared to be a non-
covalently bound aggregate of 1 molecule of lipoate succinyl-
transferase, 6 molecules of 2-oxoglutarate dehydrogenase, and 6
molecules of lipoamide dehydrogenase.

Reconstitution of Complex from Its Component Enzymes—The
2-oxoglutarate dehydrogenase complex has been reassembled in
two ways; one from the three isolated component enzymes,
lipoate succinyltransferase, 2-oxoglutarate dehydrogenase, and
lipoamide dehydrogenase, the other from the colorless fraction
and lipoamide dehydrogenase.

Reassociation of Three Component Enzymes—All three com-
ponent enzymes were required to exhibit CoA- and NAD-linked
oxidative decarboxylation of 2-oxoglutarate (Reaction 1). Asso-
ciation of three component enzymes to reconstitute the complex
has been attempted and confirmed by the following experiment.
A mixture containing 3.0 mg (35.7%) of lipoate succinyltrans-
ferase and 3.4 mg (40.5%) of 2-oxoglutarate dehydrogenase was
incubated for 15 min at 0°, followed by addition of 2.0 mg (23.8%) of
lipoamide dehydrogenase. The mixture was then diluted to a
final volume of 1.8 ml with 0.05 m potassium phosphate buffer
(pH 7.0), and centrifuged for 150 min at 100,000 × g in a No. 50
rotor of a Beckman model L-2 ultracentrifuge. The yellow
pellet was dissolved in 1.8 ml of 0.05 m potassium phosphate
buffer (pH 7.0) and recentrifuged under the same conditions to
remove any noncombined excess of 2-oxoglutarate dehydro-
genase and lipoamide dehydrogenase. The second pellet was
redissolved in an appropriate volume of 0.05 m potassium phos-
phate buffer (pH 7.0). As given in Table IV, the coenzyme
compositions and enzymatic activities of the reconstituted
complex were in good agreement with the values of the native
complex (cf. Table II). About 90% of the over-all reaction ac-
tivity of the native complex could be restored by the recon-
stituted complex. The sedimentation pattern of the reconsti-
tuted complex showed a single boundary with s20w value of
32.1 S, which was very close to that of the native complex (Fig.
2B). The weight-average molecular weight of the reconstituted
complex was 2.495 ± 0.011 million and the value was very close
to that of the native complex. From the nearest integer of the
protein-bound coenzyme content per mole of the complex and
various enzymatic activities, the reconstituted complex ap-
peared to be an enzyme mosaic which was reconstituted by
noncovalent binding of 1 molecule of lipoate succinyltransferase,
6 molecules of 2-oxoglutarate dehydrogenase, and 6 molecules
of lipoamide dehydrogenase.

It must be emphasized that 2-oxoglutarate dehydrogenase and
lipoamide dehydrogenase did not combine with each other
directly, but did combine with lipoate succinyltransferase. The
direct evidence for the latter has been confirmed by the following
experiments. First, the colorless fraction, which is a lipoate
succinyltransferase-2-oxoglutarate dehydrogenase subcomplex,
was prepared by mixing of 2.5 mg (47%) of lipoate succinyltrans-
ferase and 2.8 mg (53%) of 2-oxoglutarate dehydrogenase,
followed by ultracentrifugation, as described above. The
coenzyme compositions, enzymatic activities, and sedimentation
coefficient of the reconstituted colorless fraction were in very
good agreement with those of the native colorless fraction as
given in Table IV and Fig. 3A (cf. Table II and Fig. 2C). The
lipoate succinyltransferase-lipoamide dehydrogenase subcomplex
denoted as the yellow fraction was prepared by mixing of 3.0 mg
(60%) of lipoate succinyltransferase and 2.0 mg (40%) of
lipoamide dehydrogenase, followed by ultracentrifugation as
above. The resultant yellow pellet was homogeneous upon an-
alytical ultracentrifugation with s20w value of 23.9 S (Fig. 3B),
and exhibited lipoate succinyltransferase and lipoamide dehy-
drogenase activities (Table IV), suggesting the binding of these two
enzymes. As reported in a previous paper (6), the lipoamide
dehydrogenase was interchangeable with that of the pyruvate
dehydrogenase complex; however, the other two component
enzymes were not.

Reassociation of Colorless Fraction and Lipoamide Dehydro-
genase—The complex could be reconstituted from the colorless
fraction, lipoate succinyltransferase-2-oxoglutarate dehydro-
genase subcomplex, and lipoamide dehydrogenase. A mixture of
3.0 mg (67%) of the colorless fraction and 1.5 mg (33%) of
lipoamide dehydrogenase was centrifuged as described above.
The properties of the yellow pellet were very similar to those of
the native complex (Table IV).

Morphology of Lipoate Succinyltransferase and
2-Oxoglutarate Dehydrogenase Complex

The electron microscopic images of the isolated lipoate suc-
cinyltransferase molecule negatively stained with 0.25% sodium

| TABLE IV | Enzymatic activities and coenzyme contents of reconstituted complexes and subcomplexes |
|----------|-------------------------------------------------|---------------------------------|-------------------------|
|          | Reconstituted enzyme complex | Protein-bound coenzyme | Specific activities |
|          | | Lipic acid | Thiamine-PP | FAD | Over-all reaction | 2-Oxoglutarate dehydrogenase | Lipoate succinyltransferase | Lipoamide dehydrogenase |
| The complex | | nmoles/mg protein | nmoles/hr/mg protein | | | | | |
| Colorless fraction | 3.3 | 2.3 | 4.3 | 335 | 125 | 71 | 1012 |
| Yellow fraction | 5.5 | 0 | 6.3 | 0 | 165 | 104 | 0 |
| The complex | 3.3 | 2.2 | 4.4 | 330 | 115 | 69 | 1059 |

* Reconstituted from three component enzymes.
* Reconstituted from the colorless fraction and lipoamide dehydrogenase.
however, the appearance of the isolated 2-oxoglutarate dehydrogenase is not yet known because of aggregation of this enzyme molecule during staining. It was also difficult to obtain satisfactory electron micrograph of the reconstituted complex and its subcomplexes as the two component enzymes tended dissociate from the core enzyme during staining. These observations suggest that the molecules of the 2-oxoglutarate dehydrogenase and lipoamide dehydrogenase are distributed in a regular manner on the surface of the molecule of lipoate succinyltransferase and are bound noncovalently to it.

**DISCUSSION**

It was essential to repeat freezing and thawing at least seven times to solubilize the 2-oxoglutarate dehydrogenase complex from the pig liver Keilin-Hartree preparation. By contrast the pyruvate dehydrogenase complex was released by freezing and thawing only two or three times as reported in a previous paper (3). The concomitant loss of 2-oxoglutarate dehydrogenase activity was always increased in proportion to the number of times of freezing and thawing. This difficulty was overcome by employing the nonionic detergent Triton X-100 instead of freezing and thawing and ultracentrifugation. This improved procedure the recovery of the complex from the protamine precipitate eluate was more than doubled with appreciable loss of the 2-oxoglutarate dehydrogenase activity.

The lipoic acid-mediated over-all oxidation of 2-oxoglutarate (Reaction 1) is apparently analogous to pyruvate oxidation (20) and catalyzed by three component enzymes, 2-oxoglutarate dehydrogenase, lipoate succinyltransferase, and lipoamide dehydrogenase, which act sequentially. This paper reports the mammalian 2-oxoglutarate dehydrogenase complex was separated into three active component enzymes and reconstituted from these isolated enzymes. Also the stoichiometry of the reconstituted complex from the mammalian multienzyme complex has been shown. Binding studies indicate that the 2-oxoglutarate dehydrogenase complex contains a core consisting of 1 molecule of lipoate succinyltransferase, to which 6 molecules of each of the other two component enzymes, 2-oxoglutarate dehydrogenase and lipoamide dehydrogenase, are noncovalently bound. The core enzyme, lipoate succinyltransferase plays a catalytic structural role, as shown by reconstituting both the core fraction, lipoate succinyltransferase-2-oxoglutarate dehydrogenase subcomplex, and the yellow fractions, lipoate succinyltransferase-lipoamide dehydrogenase subcomplex, from these isolated enzymes. We have not yet investigated the binding sites of the two component enzymes on lipoate succinyltransferase. These results confirm and extend previous studies by Massey (17) and by Mukherjee et al. (27) on the mammalian and bacterial 2-oxoglutarate dehydrogenase complex.

As mentioned in this paper, the biochemical studies indicate that the mammalian 2-oxoglutarate dehydrogenase complex like the mammalian and bacterial pyruvate dehydrogenase complex (4, 20), is an organized mosaic of enzymes in which the component enzymes are arranged to allow efficient coupling the individual reactions catalyzed by three component enzyme.

This concept was confirmed and extended by electron microscopy which was employed for a study of the macromolecular structure of the E. coli pyruvate and 2-oxoglutarate dehydrogenase complexes (21-23). The gross appearances of the pig heart 2-oxoglutarate dehydrogenase complex and isolated lipoate succinyltransferase closely resemble those of the E. coli a-ketoglutarate dehydrogenase complex negatively stained with 0.25% sodium phosphotungstate. A mixture of 0.4 ml of an enzyme solution containing about 40-50 μg of protein in a solution of 0.0015M sucrose in 1.25 mM potassium phosphate buffer (pH 7.0) and 0.4 ml of 0.5% sodium phosphotungstate solution (pH 7.2) was cooled to 0° and sprayed on a moistened Butvar 98 film, supported on a carbon-stabilized net of Butvar 72A mounted on a copper grid by a multiple spraying apparatus (4). After 2 to 5 min the specimen was examined in an electron microscope. The micrographs were taken at an electron optical magnification of 100,000 times. A, lipoate succinyltransferase (× 300,000); B, its selected individual image (circled in A) showing a tetramer structure (× 600,000); and C and D, selected individual images of the 2-oxoglutarate dehydrogenase complex which has the core (arrow) (× 600,000).
dehydrogenase complexes and the beef kidney 2-oxoglutarate dehydrogenase complex (24). Further structural analyses of the complex and its component enzymes are in progress. Investigation of the characteristics of these component enzymes in respect to enzymatic, physical, chemical, and optical properties and their chemical subunits is in progress and these results will appear elsewhere.

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