MAMMALIAN α -KETO ACID DEHYDROGENASE COMPLEXES

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In recent years the main pathway of α -keto acid oxidation in animal tissues has been studied in this laboratory by the joint efforts of Dr. Hayakawa, Dr. Hirashima, Dr. Hamada and Dr. Aikawa. Electron microscopy was performed by the collaboration of electron microscopists, Mr. Suematsu from our medical school, and Mr. Yotsumoto and his staff members of Japan Electron Microscope Laboratory Company.

As already mentioned by Dr. Reed, there are available evidences that several enzymes participate in the CoA- and NAD-linked multistage oxidative decarboxylation of α -keto acids (Reaction A), that is, pyruvate and 2-oxoglutarate. Reaction

A proceeds successively via decarboxylation, acyl generation, acyl transfer and electron transfer reaction sequences, as shown in Scheme 1.

SCHEME 1 *Proposed Reaction Sequence*

E_1 =pyruvate dehydrogenase; E_2 =lipoate acyltransferase; E_3 =lipoamide dehydrogenase; $\text{R}=\text{CH}_3$ and $\text{HO}_2\text{C}\cdot(\text{CH}_2)_2$.

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The results, that will be mentioned later, strongly suggest that these enzymes are uniquely located to permit efficient implementation of the above consecutive reaction on the organized mosaic of enzymes, namely, multienzyme complex. These multienzymes have been isolated from pigeon breast muscle (1, 2), *Escherichia coli* (3), pig heart muscle (4-8) and beef kidney mitochondria (9) as a soluble and well organized complex with molecular weights of several million.

Since early studies of Ochoa and his co-workers in 1951 (10), no additional studies of pyruvate dehydrogenase complex (PDC) from mammalian tissue have been reported. Mammalian 2-oxoglutarate dehydrogenase complex (OGDC) was isolated by Sanadi and his co-workers in 1952 (4) from pig heart muscle and further studies of this complex was achieved by Massey in 1960 (5). Recently we have succeeded in isolating both complexes from pig heart muscle in highly purified states as soluble multienzyme units with high molecular weights by a convenient procedure devised in our laboratory (6-8).

ISOLATION AND PROPERTIES

1. Isolation and Purification Procedure from Pig Heart Muscle Particles

Isolation and purification procedures from Keilin-Hartree preparation are summarized in Scheme 2. Our original purification procedure was performed as follows: Amber-color extract was obtained by freezing and thawing three times the acid-precipitated particle which was prepared by the same method described by Sanadi *et al.* (4). Both complexes were isolated from the amber-color extract by a combination of fractionations with protamine precipitation, ultracentrifugation, calcium phosphate gel-cellulose column chromatography and finally ammonium sulfate.

SCHEME 2 Purification Procedure of α -Keto Acid Dehydrogenase Complexes

2. Enzymatic and Physicochemical Properties of PDC and OGDC

Solutions of purified preparation were opalescent, bright yellow and highly fluorescent. Both complexes lost less than 20 % of their activities during storage

at 0° in the presence of $4 \times 10^{-3} M$ EDTA for as long as 40 days (PDC) and at -20° for 1 month (OGDC), respectively. The pyruvate dismutation (over-all reaction), pyruvate dehydrogenase, lipoate acetyltransferase and lipoamide dehydrogenase activities were associated with the highly purified PDC over the 280-fold range of purification (Table 1). The over-all oxidation of 2-oxoglutarate, 2-oxoglutarate dehydrogenase, lipoate succinyltransferase and lipoamide dehydrogenase activities were associated with the highly purified OGDC over the 74 fold range of purification (Table 1). These results strongly suggest the presence of, at least, three constituent

TABLE 1
Enzymatic Activities of PDC and OGDC

Complex	Over-all reaction	Dehydrogenase	Lipoate acyltransferase	Lipoamide dehydrogenase	Protein-bound lipoate
		<i>μmoles/hr/mg protein</i>			<i>μg/mg protein</i>
PDC	99.6	5.7	96.1	370	0.66
OGDC	210	28	17	510	0.49

enzymes in each single complex. Both complexes were also essentially homogeneous upon ultracentrifugation and electrophoresis. Hydrodynamic parameters are summarized in Table 2. The molecular weights of both complexes were calculated to be 9 to 9.7 million (PDC) and 2.7 to 2.8 million (OGDC), respectively.

The absorption spectra of the complexes showed a broad shoulder from 400 to 480 mμ (PDC), and maxima at 455 and 350 mμ and a shoulder at 480 and 415 mμ (OGDC). Difference spectra before and after dithionite reduction suggested the presence of FAD in both complexes. Acid extractable flavin moiety in both complexes was identified as FAD by the full activation of apo-D-amino acid oxidase and by paper chromatography. PDC contained protein-bound lipoic acid and FAD in the ratio of 2:1, but it was free from thiamine-PP. OGDC contained protein-bound thiamine-PP, lipoic acid and FAD in the ratio of 1:1:1.5. Co-enzyme contents of both complexes are summarized in Table 3.

TABLE 2
Comparison of Sedimentation and Diffusion Coefficients and Molecular Weights of PDC and OGDC

Enzyme sources	S° _{20,W}	D _{20,W}	Molecular weight		References
			From S and D	From the Archibald method	
× 10 ⁶					
PDC					
Pig heart	67.5	0.62	9.7	9.0	Hayakawa <i>et al.</i> (7)
Pigeon breast muscle	58 ^a	1.35	4.0	—	Schweet <i>et al.</i> (2)
<i>E. coli</i>	64.5	1.2	4.8	4.9	Koike <i>et al.</i> (3)
OGDC					
Pig heart	31-36 ^a	1.5-1.76	2.0	—	Sanadi <i>et al.</i> (4)
Pig heart	35.7	1.18	2.8	2.7	Hirashima <i>et al.</i> (8)
<i>E. coli</i>	40.0	1.51	2.4	—	Koike <i>et al.</i> (3)

^a Represented S_{20,w} values.

TABLE 3
Comparison of Contents of Bound Cofactors in PDC and OGDC

Complex	Thiamine-PP	Lipoic acid	FAD
	<i>moles per mole of complex</i>		
PDC	0 ($K_m = 4.2 \times 10^{-6} M$)	32	14
OGDC	6	6	9

Both complexes showed absolute dependence on added CoA and NAD in the over-all reaction. Neither complex showed a detectable bound-NAD. Preparation of the purified complexes showed absorbancy ratios at 280 and 260 $m\mu$ of 1.24 and 1.27, suggesting the presence of approximately 1.55 % (PDC) and 1.40 % (OGDC) nucleic acid. Lipid, extracted into hot methanol-chloroform (2:1) and then into petroleum ether, amounted to approximately 0.8 % of the dry weight of each complex preparation.

RESOLUTION AND RECONSTITUTION OF PYRUVATE DEHYDROGENASE COMPLEX

SCHEME 3 *Resolution Procedure of PDC*

PDC has been successively resolved into three constituent enzymes, (a) thiamine-PP-dependent pyruvate dehydrogenase, (b) lipoate acetyltransferase containing protein-bound lipoic acid, and (c) flavoprotein, lipoamide dehydrogenase by fractionations with calcium phosphate gel-cellulose column chromatography in the presence of 4 M urea, ammonium sulfate, and 0.3 M potassium iodide, and gel-cellulose column chromatography in the presence of 0.02 M ethanolamine (11). The resolution procedure is summarized in Scheme 3. All three components were required to reconstitute the complex to be essential for CoA- and NAD-linked oxidative decarboxylation of pyruvate. A mixture of the three enzymes (ratio of 4.9:3.7:1.1 by weight in above order) was centrifuged at $198,000 \times g$ for 2 hours. The hydrodynamic parameter and enzymatic activities of the resultant yellow pellet were in good agreement with the values of the native complex, that is, the three

FIG. 1 Sedimentation Patterns in the Course of the Resolution and Reconstitution Studies of PDC

isolated components combined simultaneously to produce a large unit resembling the native complex. The pyruvate dehydrogenase and lipoamide dehydrogenase did not combine with each other, but each of these components did combine with lipoate acetyltransferase. Sedimentation patterns of the complex and its constituent enzymes in the course of the resolution and reconstitution studies are shown in Figure 1. The lipoamide dehydrogenase was exchangeable not only with lipoamide dehydrogenase specific for OGDC and free lipoamide dehydrogenase from the amber-color extract but also with human liver enzyme. Hydrodynamic parameters and molecular weights of the native complex and its constituent enzymes, and number of molecules of these enzymes per mole of the complex are summarized in Table 4. In summary, these results strongly suggest that mammalian OGDC may also consist of three constituent enzymes containing three coenzymes, thiamine-PP, lipoic acid and FAD, which play a role in the course of the oxidative decarboxylation of 2-oxoglutarate.

TABLE 4
*Comparison of Hydrodynamic Parameters of PDC and Its
Constituent Enzymes*

Enzymes	S [°] _{20,w}	D _{20,w}	Molecular weight		No. of molecules per mole of complex
			From S and D	From Archibald method	
			× 10 ⁶		
PDC	67.5	—	—	900	
Pyruvate dehydrogenase	8.06	4.62	19.4	—	29
Lipoate acetyltransferase	35.9	1.31	246	—	1
Lipoamide dehydrogenase	5.9	4.71	11.2	—	8

ELECTRON MICROSCOPY

In view of the results so far achieved one ought to introduce electron microscopy to determine the structural organization of such giant complex molecules which catalyze a multistage reaction sequences. In 1964 correlative electron microscopic studies of *E. coli* PDC have been established by Dr. Reed with the collaboration of Dr. Fernández-Morán (12). The appearance of the highly purified lipoate acetyltransferase particle negatively stained with a 0.25–0.75 % solution of sodium phosphotungstate (pH 7.2) suggested hexagonal structure with a diameter of approximately 190–200 Å (Figure 2a). These structures were also seen in particles of the yellow fraction after negative staining (Figure 2b). The pig heart PDC and colorless fraction tend to dissociate or to shrink under the negative staining condition used. Electron micrographs showed images very similar to those obtained with purified lipoate acetyltransferase and much smaller particles in the background (Figure 3a, 3b). Other images of lipoate acetyltransferase with a diameter of about 250–310 Å, subunits surrounded by small particles, were observed. These small particles or peripheral are not clearly defined as in the complex Reconstituted PDC showed very similar images as obtained with the native complex (Figure 3c). Experiments to locate each of the constituent enzymes in the complex are in progress.

FIG. 2 *Electron Micrograph of Mammalian Lipote Acetyltransferase (a), and Yellow Fraction (b)*

The pig heart OGDC and colorless fraction obtained from it also tend to dissociate during negative staining with 0.25–0.5 % sodium phosphotungstate (pH 7.2). Two types of images of the complex were observed: (a) polyhedron with a diameter of about 220 Å and (b) tetrad structure with a side of 110 Å (Figure 4) like *E. coli* lipoate succinyltransferase (13).

FIG. 3 *Electron Micrograph of Mammalian PDC (a), Colorless Fraction (b), and Reconstituted PDC (c)*

The appearances in the electron microscope of lipoate acetyltransferase and lipoate succinyltransferase from beef kidney are very similar to those of the highly purified corresponding enzymes from pig heart muscle (9).

FIG. 4 *Electron Micrograph of Mammalian OGDC (a and b)*

IMMUNOCHEMICAL IDENTITY OF PIG HEART LIPOAMIDE DEHYDROGENASES

It is worthwhile to investigate the immunochemical relationship among three lipoamide dehydrogenase from pig heart muscle: (a) free lipoamide dehydrogenase, (b) the lipoamide dehydrogenase specific for OGDC, and (c) the lipoamide

FIG. 5 *Ouchterlony Double Diffusion Studies*

Center well (A) contains 0.1 ml (3.6 mg of protein) of the immune globulin, the outside wells contain 50-60 μ g of protein of each enzyme, 1: free lipoamide dehydrogenase, 2: lipoamide dehydrogenase from 2-oxoglutarate dehydrogenase complex, 3: lipoamide dehydrogenase from pyruvate dehydrogenase complex, 4: human liver lipoamide dehydrogenase, 5: *E. coli* lipoamide dehydrogenase.

FIG. 5 Titration of NADH_2 -Lipoamide Activity with Immune Globulin

●, free lipoamide dehydrogenase; ○, lipoamide dehydrogenase from 2-oxoglutarate dehydrogenase complex; □, lipoamide dehydrogenase from pyruvate dehydrogenase complex; △, human liver lipoamide dehydrogenase.

dehydrogenase specific for PDC. Rabbit antisera have been prepared against free lipoamide dehydrogenase. In the Ouchtelony double diffusion test (Figure 5 a and b) and titration studies of several enzymatic activities with partially purified immune globulin (Figure 6), immunochemical identity of these three lipoamide dehydrogenase was confirmed (14). Human liver and *E. coli* lipoamide dehydrogenases gave no immunochemical line of identity and neither were their activities inhibited by immune globulin. These findings strongly suggest that all of the flavoproteins in respiratory chain might be immunochemically identical.

EFFECT OF VARIOUS BIVALENT CATIONS AND CHELATING AGENTS ON THE OXIDATIVE DECARBOXYLATION OF α -KETO ACID

Some of highly purified α -keto acid dehydrogenase complexes are activated by additional Mg^{2+} . Pigeon breast muscle PDC was activated by Mn^{2+} in place of Mg^{2+} , but strongly inhibited by Cu^{2+} (15). Crude pig heart enzyme did not require the addition of Mg^{2+} , Mn^{2+} or thiamine-PP for the oxidation of pyruvate (10). Both purified pig heart complexes were activated by Ca^{2+} to the same extent as Mg^{2+} , which had been considered one of typical activators of the oxidative decarboxylation reaction of α -keto acids (16). *E. coli* PDC, however, was activated only by Mg^{2+} , and not by Ca^{2+} . These activating effects were in good agreement with the metal contents of the complexes obtained by atomic absorption analysis. Both pig heart and *E. coli* PDC were strongly inhibited by EDTA at low concentration. By contrast pig heart OGDC was only slightly inhibited by EDTA and rather strongly inhibited by 8-hydroxyquinoline. Further investigation of the mechanism of the oxidative decarboxylation of α -keto acids, for instance, interactions among thiamine-PP, metal ions and enzyme protein is now in progress.

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DISCUSSION

Dr. J. Avigan: In view of the very rapid metabolism of long chain isoprenoid fatty acids by an α -oxidative mechanism in mammalian tissues it would be of interest to determine whether the α -keto dehydrogenase enzymes are specific for the longer chain substrates.

Dr. M. Koike: Pyruvate dehydrogenase complex is specific for the oxidation of pyruvate, and 2-oxoglutarate dehydrogenase complex is specific for 2-oxoglutarate and relatively specific for α -ketoadipate.

Dr. Y. M. Ostrovsky: Is there a possibility to acetylate some amines by acetyl-TPP without lipoic acid?

Dr. M. Koike: I do not know.

Dr. R. E. Olson: I believe Dr. Koike's data suggest that thiamine pyrophosphate is

more loosely bound to pyruvate dehydrogenase complex than to α -ketoglutarate dehydrogenase complex, since only a K_m was given to pyruvate dehydrogenase complex and 6 moles/mole for α -ketoglutarate dehydrogenase complex. This has some physiological implication for beriberi, since pyruvate dehydrogenase activity is greatly reduced by thiamine depletion, whereas α -ketoglutarate dehydrogenase activity remains fairly high as measured by fatty acid oxidation in thiamine-deficient dog heart.

Dr. M. Koike: Thank you for your suggestion.

Dr. L. J. Reed: I agree with your suggestion. In both *E. coli* and animal tissue, thiamine pyrophosphate is apparently bound more

tightly to the α -ketoglutarate dehydrogenase complex than to the pyruvate dehydrogenase complex.

Dr. D. R. Sanadi: (1) Both Dr. Reed and Dr. Koike have been complimented on their excellent presentation and continued fine work in this area. I would like to ask Dr. Koike why some of the cofactor analyses seem to be inconsistent with the number of proposed subunits. (2) Have any attempts been made to see these structures within mitochondria. They seem to be large and characteristic enough to be easily identified?

Dr. M. Koike: (1) Contents of lipoic acid and FAD in mammalian complexes were consistent. However, we do not know yet how many subunits consist of one mole of lipoate acetyltransferase. (2) We have not identified yet. However, I hope some methods will open the way of this problem.

Dr. L. J. Reed: we have not yet attempted to locate these complexes in mitochondria

by electron microscopy. I believe it will be technically very difficult to do so.

Dr. R. E. Olson: I would like to mention that the animal pyruvate dehydrogenase is slightly leaky in the sense that it generates some free acetaldehyde which can be reduced to ethanol by alcohol dehydrogenase in liver. Dr. McManus in my laboratory (Picosburgh, 1963-65) showed that labeled pyruvate could be converted to ethanol by mammalian pyruvate dehydrogenase and alcohol dehydrogenase *in vitro*. This appears to be a minor physiological pathway in mammals, since ethanol appears in the blood of humans abstinent from spirits in amounts of 0.05-0.2 mg %.

Dr. M. Koike: Mammalian pyruvate dehydrogenase oxidized pyruvate in the presence of external dye to produce acetic acid, but not aldehyde. We do not have any data according to your experimental results.