ISOLATION AND PROPERTIES OF PYRUVATE AND α-KETOGLUTARATE DEHYDROGENATION COMPLEXES FROM PIG HEART MUSCLE

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Enzyme systems which catalyze a CoA- and DPN-linked oxidative decarboxylation of α-keto acids (Reaction 1) have been obtained from various sources (Korkes, et al., 1951; Jagannathan, et al., 1952; Schweet, et al., 1952; Gunsalus, 1954 and Goldman, 1959).

$$RCOCO_2H + CoA\text{-}SH + DPN \longrightarrow RCO\text{-}S\text{-}CoA + CO_2 + DPNH + H^+ \qquad (1)$$

Mammalian α-ketoglutarate dehydrogenase complex was isolated in highly purified form from pig heart (Sanadi, et al., 1952; Kaufman, et al., 1953 and Massey, 1960). In their studies of mammalian pyruvate dehydrogenation complex Korkes and co-workers (1952) found that a crude fraction precipitated from pig heart extract with 0.4 saturated ammonium sulfate catalyzed Reaction 1 in the presence of pyruvate; further purification has not been reported. In previous papers, one of the authors reported a series of studies on the pyruvate and α-ketoglutarate dehydrogenation complexes (PDC and KGDC) in Escherichia coli (Crookes strain) (Koike, et al., 1960a, b and c). The PDC was separated into three essential components: (a) pyruvic carboxylase, (b) a component containing the protein-bound lipoic acid and exhibiting dihydrolipoic transacetylase activity, and (c) a flavoprotein, dihydrolipoic dehydrogenase. These components were also reassociated to produce a large unit resembling the original complex in composition and enzymatic activities (Koike, et al., 1963).

In the present communication isolation and study of both multienzyme complexes from pig heart as structural units with molecular weights of 10 (PDC) and 3.3 (KGDC) million are discussed. Both contained the protein-bound lipoic acid, FAD and thiamine-PP as essential coenzymes for catalysis of Reaction 1. For purification our original procedure was followed. Pig heart particles were prepared according to the method of Sanadi, et al., (1952). The resultant amber-colored extract, 6 mg protein per ml, was adjusted pH to 6 with 1 N acetic acid; and KGDC and PDC respectively precipitated by addition of 0 to 0.015 volume and 0.015 to 0.03 volume of 2% protamine sulfate solution (pH 5). Each precipitate was eluted with 0.1 M phosphate buffer, pH 7, dialyzed against 0.05 M phosphate buffer, pH 7 overnight and centrifuged. The supernatant fluids, designated protamine precipitate eluates, which were almost free from each other, were collected by centrifugation for 2.5 hours at 144,000 X g. The resultant yellow pellets were dissolved in 0.05 M phosphate buffer, pH 7, and purified by chromatography on a calcium phosphate gel-cellulose column (Price, et al., 1954) previously washed with 0.05 M phosphate buffer, pH 7.5. The complexes were adsorbed on columns, washed with 0.05 M then 0.1 M phosphate buffer, pH 7.5 and 1% ammonium sulfate in 0.1 M phosphate buffer, pH 7.5, leaving a bright yellow band at the top of each column. When PDC was applied to the column colorless fractions which exhibited both pyruvic carboxylase and dihydrolipoic transacetylase activities were removed with the 0.05 M and 0.1 M phosphate buffer, pH 7.5. The yellow band on each column was then eluted with a solution of 4% ammonium sulfate in 0.1 M phosphate buffer, pH 7.5. Both the yellow and colorless eluates were fractionated with solid ammonium sulfate. PDC and the colorless fractions were precipitated between 0.29 and 0.36 saturation and KGDC was precipitated between 0.24 and 0.29 saturation. A summary of typical purification and recovery data of enzymatic activities is presented in Tables I and II.

Electrophoretic analysis the purified PDC and KGDC gave homogenous bands

Table I

Purification of Pyruvate Dehydrogenation Complex

Fraction	Total protein g	Specific activities (μmoles/hr./mg protein) Dismutation	Yield total mmoles	Carboxylase	Lipoic transac.	Lipoic DeH.
Homogenate	5,800	0.23	1,330	0.03	0.26	-
Particle	330	1.3	429	0.13	1.3	-
Amber-color extract	37	3.6	133	0.4	3.1	66
Protamine ppt. eluate	2.44	13	32	0.82	15	36
Pellet	0.7	41	28	3.0	62	112
AmSO ppt. (0.29-0.36)	0.15	64	16	4.3	81	210

All assays were carried out as described previously (Koike, et al., 1960a), except incubation at 37°C in the presence of crystalline bovine serum albumin. Lipoic transacetylase assay was carried out with 10 μmoles of dihydrolipoamide in 0.05 ml of 95% ethanol. Lipoic dehydrogenase assay was carried out as described by Massey (1960) with some modification in the presence of lipoamide at room temperature.

Table II

Purification of α-Ketoglutarate Dehydrogenation Complex

Fraction	Total protein g	Specific activities (μmoles/hr./mg protein) Carboxylase	Yield total mmoles	DPN Reduction	Lipoic DeH.	CO_2 Evolution
Homogenate	5,800	0.19	1,100	-	-	0.48
Particle	330	1.0	330	-	-	1.7
Amber-color extract	37	2.7	100	25	66	4.2
Protamine ppt. eluate	7.7	6.0	46	64	119	10.4
Pellet	3.1	8.6	27	82	216	16.7
AmSO ppt. (0.24-0.29)	0.7	16.3	12	178	373	24.5

α-Ketoglutarate carboxylase assay was carried out as described by Koike, et al., (1960a). DPN reduction and lipoic dehydrogenase assay were carried out as described by Massey (1960) with some modification. CO_2 evolution was carried out as described by Sanadi, et al., (1952).

which migrated toward the anode as a single boundary in veronal buffer (μ=0.06, pH 8.6) at 4°C. The PDC and KGDC were also essentially homogenous upon ultracentrifugation in Spinco model E analytical Ultracentrifuge with the sedimentation coefficients ($S^0_{20,w}$) of 67.5 S and 36.5 S. The patterns, with highly purified preparations of both complexes, are shown in Figures 1 and 2. Diffusion studies using schlieren optics in a Spinco model H Electrophoresis and Diffusion apparatus gave corrected diffusion coefficients ($D_{20,w}$) of 0.6 X $10^{-7}cm^2sec^{-1}$ and 1.0 X $10^{-7}cm^2sec^{-1}$. The molecular weights were calculated to be approximately 10 X 10^6 (PDC) and 3.3 X 10^6 (KGDC), assuming a partial specific volume of 0.73 ml per g. Further hydrodynamic and light-scattering studies are now under way to confirm the exact molecular weights and shapes.

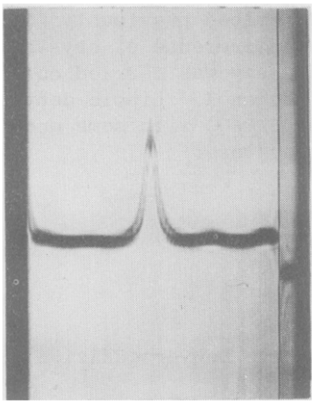

Fig.1. Ultracentrifuge schlieren pattern obtained with PDC, 6.6 mg per ml, after 24 min. at 34,450 r.p.m.; temperature, 6.7°C.

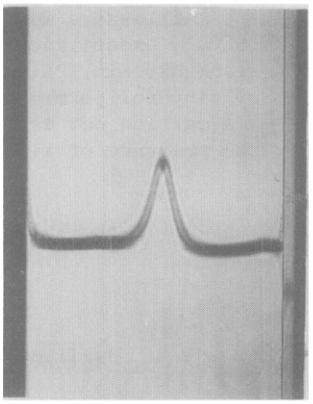

Fig.2. Ultracentrifuge schlieren pattern obtained with KGDC, 10 mg per ml, after 25 min. at 43,700 r.p.m.; temperature, 8.5°C.

The lipoic acid content of the complexes was determined manometrically with lipoic acid-deficient Streptococcus faecalis 10C1 cells (Gunsalus, et al., 1952) after autoclaving in 0.1 N sodium hydroxide in an open test-tube at 15 lb for 3 hours under nitrogen (Wagner, et al., 1956). PDC and KGDC contained respectively 5 and 2 X 10^{-9} moles of (+)-lipoic acid per mg protein. The absorption spectrum of PDC showed a broad shoulder from 400 to 480 mμ; the absorption between 400 to 500 mμ was significantly decreased after addition of sodium dithionite. The absorption spectrum of KGDC showed maxima at 350, 415

and 455 mμ, and a shoulder at 480 mμ and difference spectrum after addition of sodium dithionite similar to that for PDC.

The flavin component has been tentatively identified as FAD by paper chromatography (Huennekens, et al., 1957) and by full activation of recrystallized D-amino acid apo-oxidase system (Negelein, et al., 1939). The FAD contents of the complexes were 1.5 (PDC) and 2.5 (KGDC) X 10^{-9} moles per mg protein (Beinert, et al., 1957). Thiamine-PP was determined by the modified procedure of Kajiro (1957) and Green, et al., (1941). Highly purified PDC was free of thiamine-PP and the activity in the dismutation assay was restored by thiamine-PP. Thus thiamine-PP was dissociated from PDC during purification. The dissociation constant of thiamine-PP in the dismutation assay was 4.2 X 10^{-6} M. KGDC showed a little response to added thiamine-PP in the α-ketoglutarate carboxylase assay; its thiamine-PP content was 1.5 X 10^{-9} moles per mg protein. All preparations of examined PDC showed absolute dependence on added CoA and DPN in the dismutation assay. Neither complex showed a detectable bound DPN as determined fluorometrically by the method of Levitas, et al., (1947). Preparations of the purified complexes showed absorbancy ratios at 280 and 260 mμ of 1.24 and 1.40, suggesting the presence of approximately 1.55 and 0.75% 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 the preparations. PDC contains approximately 50 moles of protein-bound lipoic acid and 15 moles of bound FAD. KGDC contains approximately 6 moles of protein-bound lipoic acid, 8 moles of FAD and 6 moles of thiamine-PP.

Electron microscope studies of the purified PDC and KGDC are now in progress by the negative and positive staining procedures. Electron micrographs of PDC and KGDC negatively stained with buffered phosphotungstate and cytochrome C showed, so far, various aspects of regular globular structure about 600 Å and 400 Å in diameter, respectively. The details of electron microscope studies of both complexes should shed further light on its structural organization.

Reference

Beinert, H., and Page, E., J. Biol. Chem., 225, 479 (1957)

Goldman, D. S., Biochim. Biophys. Acta, 32, 80 (1959)

Green, D. E., Herbert, D., and Subrahmanyan, V., J. Biol. Chem., 138, 327 (1941)

Gunsalus, I. C., Dolin, M. I., and Struglia, L., J. Biol. Chem., 194, 849 (1952)

Gunsalus, I. C., in W. D. McElroy and B. Glass (Editors), The mechamism of enzyme action, Johns Hopkins Press, Baltimore, 1954, p. 545.

Huennekens, F. M., and Felton, S. P., in S. P. Colowick, and N. O. Kaplan (Editors), Methods in enzymology, Vol. III, Academic Press, Inc., New York, 1957, p. 950.

Jagannathan, V., and Schweet, R. S., J. Biol. Chem., 196, 551 (1952)

Kaufman, S., Gilvarg, C., Cori, O., and Ochoa, S., J. Biol. Chem., 203, 869 (1953)

Kaziro, Y., J. Biochem., 44, 827 (1957)

Koike, M., Reed, L. J., and Carroll, W. R., J. Biol. Chem., 235, 1924 (1960a)

Koike, M., and Reed, L. J., J. Biol. Chem., 235, 1931 (1960b)

Koike, M., Shah, P. C., and Reed, L. J., J. Biol. Chem., 235, 1939 (1960c)

Koike, M., Reed, L. J., and Carroll, W. R., J. Biol. Chem., 238, 30 (1963)

Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., J. Biol. Chem., 193, 721 (1951)

Korkes, S., del Campillo, A., and Ochoa, S., J. Biol. Chem., 195, 541 (1952)

Levitas, N., Robinson, J., Rosen, F., Huff, J. W., and Perlzweig, W. A., J. Biol. Chem., 167, 169 (1947)

Massey, V., Biochim. Biophys. Acta, 38, 447 (1960)

Negelein, E., and Bromel, H., Biochem. Z., 300, 225 (1939)

Price, V. E., and Greenfield, R. E., J. Biol. Chem., 209, 363 (1954)

Sanadi, D. R., Littlefield, J. W., and Bock, R. M., J. Biol. Chem., 197, 851 (1952)

Schweet, R. S., Katchman, B., Bock, R. M., and Jagannathan, V., J. Biol. Chem., 196, 563 (1952)

Wagner, A. F., Walton, E., Boxer, G. E., Pruss, M. P., Holly, F. W., and Folkers, K. J. Am Chem. Soc., 78, 5079 (1956)