α-KETO ACID DEHYDROGENASE COMPLEXES, VIII. COMPARISON OF DIHYDROLIPOYL DEHYDROGENASES FROM PYRUVATE AND α-KETOGLUTARATE DEHYDROGENASE COMPLEXES OF ESCHERICHIA COLI*

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Communicated by Roger J. Williams, July 21, 1967

Enzyme systems which catalyze a coenzyme A- and diphosphopyridine nucleotide-linked oxidative decarboxylation of pyruvate and α-ketoglutarate (reaction (1))

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

have been isolated from *Escherichia coli* as multienzyme complexes with molecular weights of several million.[1] The pyruvate dehydrogenase complex (PDC) has been separated into three enzymes—pyruvate dehydrogenase,[2] dihydrolipoyl transacetylase, and a flavoprotein, dihydrolipoyl dehydrogenase.[3] The complex has been reconstituted from the isolated enzymes. The α-ketoglutarate dehydrogenase complex (KGDC) also has been separated into three enzymes, and it too has been reassembled from the isolated enzymes.[4] The three enzymes are α-ketoglutarate dehydrogenase, dihydrolipoyl transsuccinylase, and dihydrolipoyl dehydrogenase.

Combinations of the enzymes comprising PDC with those of KGDC have been examined. The two α-keto acid dehydrogenases and the two transacylases are not functionally interchangeable, nor do these components form "hybrid" complexes; although functionally similar these enzymes apparently are chemically different. This paper reports physical, enzymic, and immunochemical data indicating the identity of the two dihydrolipoyl dehydrogenases. Genetic implications of these findings are discussed with respect to the results of Henning *et al.*[5, 6] on the biosynthesis of PDC and KGDC and their genetic control in *E. coli* K12.

Materials and Methods.—Enzyme preparations: Pyruvate and α-ketoglutarate dehydrogenase complexes were isolated from sonic extracts of *E. coli* (Crookes strain) and were resolved on columns of calcium phosphate gel suspended on cellulose as described in previous publications.[1, 3, 4, 7] The flavoprotein fraction from the gel-cellulose columns was subjected to fractionation with solid ammonium sulfate, and the uncomplexed flavoprotein was collected between 0.50 and 0.80 saturation. The preparations were stored in the frozen state (−20°) and retained full activity for at least several months. The subcomplex consisting of pyruvate dehydrogenase and dihydrolipoyl transacetylase was obtained by fractionation of PDC on gel-cellulose in the presence of 4 *M* urea as described previously.[3, 7]

Enzyme assays: Dihydrolipoyl dehydrogenase activity was determined spectrophotometrically by measuring the rate of DPNH oxidation in the presence of the dehydrogenase and lipoamide.[7] The DPN-reduction assay for the intact PDC, which is based on reaction (1), was performed as described previously.[4]

Flavin determination: The flavin content of protein fractions was determined according to the method of Beinert and Page[8] by measuring the absorbance of neutralized trichloroacetic acid extracts at 450 mμ before and after reduction with

dithionite. Protein was determined by the biuret method[9] and, in certain cases, by the method of Lowry *et al.*[10]

Disc electrophoresis: Polyacrylamide gel electrophoresis was performed with 7.5 per cent "standard gel" (running at pH 9.5) as described in the manual supplied by the Canal Industrial Corporation (Bethesda, Md.). A more critical comparison of the two flavoproteins was obtained by use of a split spacer gel[11] according to directions furnished by Dr. J. G. Flaks. The spacer gel was split by means of a plastic insert which extended from the top of the column through one half of the spacer gel. Each flavoprotein (50 μg of protein) was added to a separate portion of upper gel and the two mixtures were placed on opposite sides of the divider.

Immunological procedures: Rabbit sera directed against each purified flavoprotein were prepared as outlined by Kaplan *et al.*[12] Double diffusion utilizing agar plates (Ouchterlony technique) was performed as described by Campbell *et al.*[13] Immunoelectrophoresis was carried out as described by Grabar and Williams.[14] Quantitative microcomplement fixation was performed as described by Wasserman and Levine.[15]

Results.—Some physical and chemical properties of the two flavoproteins: The two dihydrolipoyl dehydrogenases isolated from PDC and KGDC, respectively, appeared to be homogeneous by polyacrylamide gel electrophoresis and yielded single boundaries in the analytical ultracentrifuge ($s_{20,w}$ = 6.2 to 6.3S).[3, 4] The polyacrylamide gel electrophoresis pattern obtained with the two flavoproteins utilizing a split spacer gel showed a single component (Fig. 1), as revealed by fluorescence under

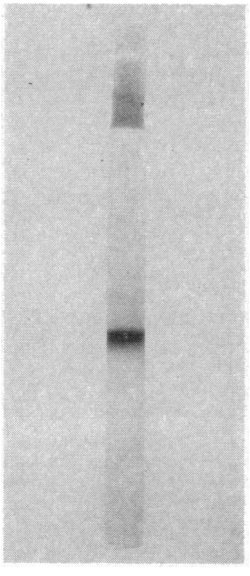

FIG. 1.—Polyacrylamide gel electrophoresis pattern obtained with the two flavoproteins using a split spacer gel.

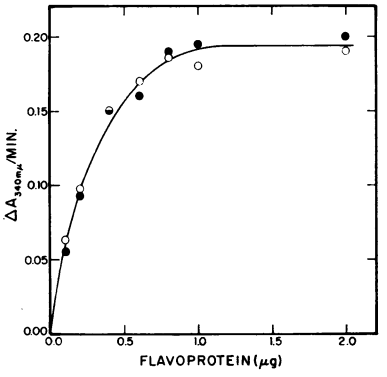

FIG. 2.—Titration of the subcomplex consisting of pyruvate dehydrogenase and dihydrolipoyl transacetylase with the PDC flavoprotein (○) and with the KGDC flavoprotein (●). Six micrograms of the subcomplex and the indicated amounts of each flavoprotein were mixed in a total volume of 0.25 ml of 0.02 M potassium phosphate buffer, pH 7.0, and the solutions were allowed to stand at 0° for 15 min. The other components required for the DPN-linked oxidation of pyruvate were then added to give a final volume of 3.0 ml, and the assay was carried out as described previously.[4]

ultraviolet light and by staining with amidoschwarz. The flavin (FAD) contents (18 mμmoles per mg of protein) of the two enzymes were equal within the precision of the measurements. Some preparations of the two enzymes contained a faster-sedimenting component ($s_{20,w}$ = 9.0*S*). This latter component was separated from the 6*S* enzyme by filtration through Sephadex G-200. Its flavin content and specific activity were identical with those of the 6*S* enzyme. The 9*S* component is believed to be a dimer of the 6*S* enzyme. Except for this presumed dimer, no other electrophoretically distinguishable forms of the *E. coli* dihydrolipoyl dehydrogenase have been detected.

Functional identity of the two flavoproteins: The specific activities (140 units per mg) of the two dihydrolipoyl dehydrogenases were equal within the precision of the measurements. Fractionation of PDC on gel-cellulose columns in the presence of 4 *M* urea yields free flavoprotein and a subcomplex consisting of pyruvate dehydrogenase and dihydrolipoyl transacetylase.[3] It was shown previously that these two fractions reassociate spontaneously at neutral pH to reconstitute PDC.[3] The flavoprotein is bound to the dihydrolipoyl transacetylase component of PDC. The binding sites must be specific since the over-all reaction (1) involves a coordinated sequence of reactions between protein-bound intermediates. Therefore, reconstitution of the DPN-linked oxidation of pyruvate (reaction (1)) provides a sensitive test of the functional identity of the two dihydrolipoyl dehydrogenases. Titration of the subcomplex consisting of pyruvate dehydrogenase and dihydrolipoyl transacetylase with each of the flavoproteins gave virtually identical results (Fig. 2), as measured by reconstitution of the DPN-linked oxidation of pyruvate.

Immunochemical comparison: Rabbit antisera were prepared against the two

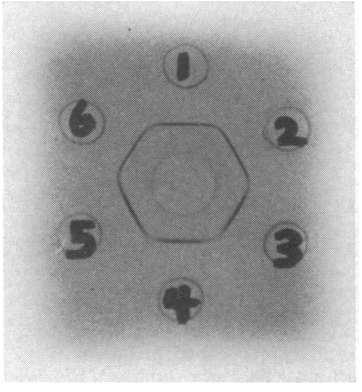

FIG. 3.—Ouchterlony double-diffusion patterns obtained with the two flavoproteins. The center well contained a 1:5 dilution of antiserum to the PDC flavoprotein. Outer wells 1, 3, and 5 were filled with the PDC flavoprotein (300 μg per ml), and wells 2, 4, and 6 contained KGDC flavoprotein (300 μg per ml).

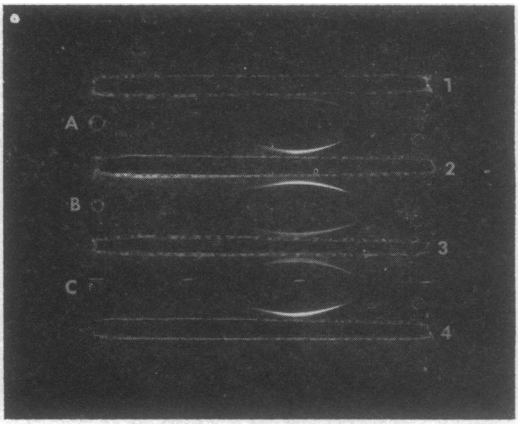

FIG. 4.—Immunoelectrophoresis patterns obtained with the two flavoproteins. Antigen wells *A*, *B*, and *C* were filled, respectively, with PDC flavoprotein (3.8 mg per ml), KGDC flavoprotein (3.7 mg per ml), and an equal mixture of the two flavoproteins. The samples were subjected to electrophoresis for 6 hr at 4° and 20 ma. Antibody troughs *1* and *3* were filled with antiserum to KGDC flavoprotein (1:4 dilution) and troughs *2* and *4* with antiserum to PDC flavoprotein (1:2 dilution). Diffusion was allowed to occur for 24 hr at 4°. The precipitin arcs were stained with 0.1% amidoschwarz.

purified dihydrolipoyl dehydrogenases as described in the *Materials and Methods* section. The two enzyme preparations showed complete identity in Ouchterlony double-diffusion experiments (Fig. 3). Similar results were obtained when the two flavoproteins were tested by immunoelectrophoresis (Fig. 4). A third immunological procedure which can provide a quantitative and highly sensitive comparison of proteins is the microcomplement fixation technique.[15] Utilizing this technique Reichlin, Hay, and Levine[16] have been able to distinguish between hemoglobins differing by only one amino acid residue. Figure 5*A* shows the results obtained with antiserum directed against the KGDC flavoprotein, and Figure 5*B* presents the results obtained with antiserum directed against the PDC flavoprotein. No significant antigenic differences were observed between the two flavoproteins with this sensitive technique.

Discussion.—Genetic and biochemical analyses of acetateless and succinateless mutants of *E. coli* K12 by Henning *et al.*[5, 6] indicate that the structural genes for the pyruvate dehydrogenase and the transacetylase are closely linked (acetate (*ace*) locus), and are located between the leucine and T1,5^R loci. The structural genes for the corresponding enzymes of KGDC (succinate (*suc*) locus) appear to be located far from the *ace* region.[5] The location of the structural gene(s) for the dihydrolipoyl dehydrogenase(s) is not yet known. The present studies, involving physical, enzymic, and immunological examination of the two dihydrolipoyl dehydrogenases isolated from PDC and KGDC, respectively, are consistent with the complete identity of the two flavoproteins. This finding suggests that there is only one genetic locus which specifies the synthesis of the flavoprotein, and that this structural gene is not closely linked to the *ace* or *suc* loci. Yet, as shown by Henning *et al.*,[6] mutations in the *ace* locus can result in a considerable decrease in dihydrolipoyl dehydrogenase activity, indicating that the flavoprotein concentration (or activity) can be regulated by the *ace* locus. Other examples of such coordinate regulation of linked and unlinked genes have been reported, and models have been proposed to explain this phenomenon.[6, 17]

Multiple enzymically active forms of dihydrolipoyl dehydrogenase from pig heart[18, 19] and beef liver[20] have been detected by electrophoresis, in contrast to the results obtained with the *E. coli* enzyme. The immunochemical identity of the multiple forms of the pig heart enzyme is indicated by the results of Ouchterlony

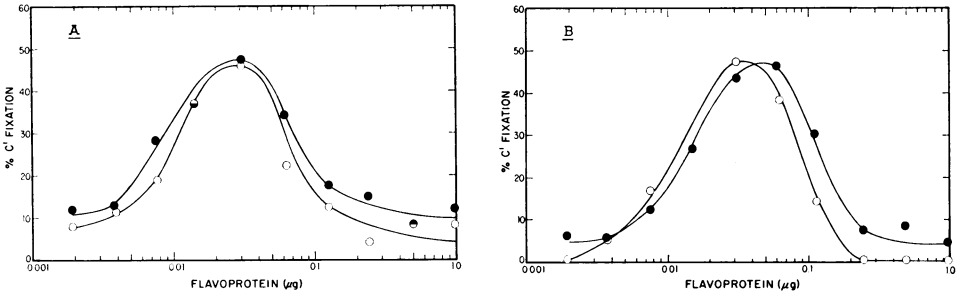

FIG. 5.—Complement fixation experiments showing identical cross reaction between antiserum to KGDC flavoprotein and the two flavoproteins (*A*), and between antiserum to PDC flavoprotein and the two flavoproteins (*B*). The antibody dilutions were 1:10,000 and 1:5,000, respectively. ●, KGDC flavoprotein; ○, PDC flavoprotein.

double-diffusion experiments.[21, 22] Massey, Hofmann, and Palmer[23] have presented evidence indicating that pig heart dihydrolipoyl dehydrogenase consists of two identical polypeptide chains. In view of these results it would appear that the multiple electrophoretic forms of the pig heart dihydrolipoyl dehydrogenase, and, presumably, of the beef liver enzyme, are not due to differences in the primary structure. The multiple forms may result from conformational differences, as proposed by Kitto, Wassarman, and Kaplan[24] to explain the occurrence of multiple electrophoretic forms of chicken mitochondrial malic dehydrogenase.

Summary.—The properties of purified dihydrolipoyl dehydrogenases isolated from the *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes have been compared using physical, enzymic, and immunological methods. No detectable differences were found between the two enzyme preparations.

The authors are indebted to Miss Lynn Hamilton and Mr. Jack Smith for expert technical assistance and to Mr. Donald Horney and Drs. Barid Mukherjee, Henry Henney, Jr., and G. Barrie Kitto for their advice and assistance in various aspects of this work.

* Supported in part by a grant (GM-06590) from the U.S. Public Health Service.

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[2] It has been observed that this enzyme catalyzes a reductive acetylation of lipoate or lipoamide by pyruvate. As a component enzyme of PDC, it apparently catalyzes both the decarboxylation of pyruvate and the reductive acetylation of the lipoyl moiety which is bound covalently to the transacetylase. Therefore, it seems appropriate to designate this enzyme pyruvate dehydrogenase rather than pyruvate decarboxylase.[3]

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