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Volume 15

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FOREWORD

Advances in Enzyme Regulation is now in its fifteenth volume. The appreciative reception of this series reflected the need for such a source of information, inspiration, laboratory and teaching companion.

Volume 15 concentrates on subjects which have reached the state of productive summarization and critical evaluation in the light of extensive new results. This book also lives up to its goal of advancing a few steps ahead of the general front of mammalian enzyme regulation studies.

It has been my editorial policy to impose as few restrictions as possible, emphasizing, however, the objectives of excellence of contribution, perfection in presentation, and penetration and scope in interpretation. This principle gives a wide range of freedom to the participants to express their concepts. Thus, the responsibility for detail – accuracy of reporting, preciseness of references, allocations of priority, expressions of judgment and evaluation – lies with the individual authors.

The Editor, who enjoyed the advice of leaders in the field, has been organizing the Symposia and selecting new topics and speakers on the basis of immediate and long-range significance of the scientific contributions. It is hoped that the comments and suggestions of investigators and teachers in this field will continue to come to the Editor's office and contribute to shaping the course of forthcoming conferences and volumes.

Indiana University 1976 GEORGE WEBER, *Editor*

ACKNOWLEDGEMENTS

This is the fifteenth in a series of Symposia dedicated to problems and advances in the regulation of enzyme activity and synthesis in mammalian systems.

I take great pleasure in expressing appreciation for the support and assistance I received in organizing and conducting this Conference. I wish gratefully to acknowledge that Indiana University School of Medicine, Burroughs Wellcome and Co., Hoffman–LaRoche, Johnson & Johnson Co., Eli Lilly and Co., The Upjohn Co., and U.S.V. Pharmaceutical Corporation provided the financial support for this Meeting.

In the planning of the program, selection of the participants and arrangements for the Symposium the advice of the following was invaluable: Ronald W. Estabrook (U.S.A.), Benno Hess (Germany), Frank M. Huennekens (U.S.A.), Nobuhiko Katunuma (Japan), Alan C. Sartorelli (U.S.A.), Sergei E. Severin (U.S.S.R.), Sidney Weinhouse (U.S.A.), and Arnold D. Welch (U.S.A.).

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At Indiana University School of Medicine in the local organization of the Symposium I had the kind assistance of Dean Steven C. Beering. The efficient and competent help of David M. Paul in accommodation arrangements and the expert assistance of Carol Eitzen in the preparation of illustrations are very much appreciated.

Thanks are due to members of my secretarial staff, Agnes Csejtey and Cheryl Klave, who assisted in local arrangements and in typing of the manuscripts.

My highest appreciation is due to my wife, Catherine E. Forrest Weber, whose contribution to the format and English style has been most valuable in the assembling of this volume.

George Weber
Symposium Chairman

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α-KETO ACID DEHYDROGENASES AND ACYL-CoA SYNTHETASES FROM PIGEON BREAST MUSCLE

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INTRODUCTION

Oxidative metabolism of α-keto acids – pyruvic and α-ketoglutaric acids – is in many ways similar. In both cases the same coenzymes function and the end products are CO_2, acyls of CoA, and reduced NAD. Both processes are catalyzed by multienzyme complexes of intricate structure – pyruvate and α-ketoglutarate dehydrogenases, which are in close interaction with other enzymes, acyl-CoA synthetases in particular. Both α-keto acid dehydrogenases, as well as acetyl- and succinyl-CoA synthetases, not only possess similar properties, but also have features that distinguish these enzymes from one another.

The aim of this communication is to present some data on the similarities and differences between these enzymes isolated from pigeon breast muscle.

MATERIALS AND METHODS

α-Keto acid dehydrogenases and succinyl-CoA synthetase were isolated from breast muscles of pigeons. Acetyl-CoA synthetase was isolated from beef and rabbit heart muscles.

Isolation and purification of the pyruvate dehydrogenase complex were carried out essentially as described earlier by Glemzha *et al.*(1). However, the enzyme chromatography on cellulose-suspended calcium phosphate gel and subsequent $(NH_4)_2SO_4$ salting out were replaced by Sepharose 4B gel filtration and ultracentrifugation at 140,000 × *g*. Separation of the pyruvate dehydrogenase component from the complex was carried out by incubating the enzyme with 0.5 M KBr, as described by Khailova *et al.* (2). The activity of the pyruvate dehydrogenase complex was measured from the increase in NADH concentration; the activity of the pyruvate dehydrogenase, i.e. the activity of the decarboxylating component, was determined at 600 nm by dichlorophenol indophenol reduction (3).

Isolation and purification of the α-ketoglutarate dehydrogenase complex was performed according to the method of Sanadi (4) modified by including a Sepharose 6B chromatography step (5). Isolation of the α-ketoglutarate dehydrogenase component was carried out as described earlier by Severin and Gomazkova (5). The activity of the whole complex was measured by observing NADH production at 340 nm on a Hitachi-356 spectrophotometer. The activity

of the α-ketoglutarate dehydrogenase component was determined by ferrocyanide reduction at 420 nm (5).

Acetyl-CoA synthetase was isolated as a homogeneous preparation from acetone powder from rabbit (6) and beef (7) heart by the method of Campagnari and Webster (8), with some modifications.

The activity of the enzyme was measured by several techniques: from the decrease in CoA in the reaction with sodium nitroprusside; by isotope exchange using C^{14}-acetate, H^3-ATP and P^{32}-inorganic pyrophosphate.

The individual reaction stages were also studied by adding synthetic acetyl adenylate to a test tube, and the reaction subsequently was directed towards the formation of either acetyl-CoA or ATP.

Succinyl-CoA synthetase was isolated from pigeon breast muscle according to Meshkova and Matveeva (9). The activity of the enzyme was measured spectrophotometrically from succinyl-CoA formation (absorbance at 235 nm). A unit of activity is determined as the amount of enzyme catalyzing the formation of 1.0 μmol of succinyl-CoA per min at 25°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein molecular weight determination were performed according to Weber and Osborn (10). Molecular weight of multiple forms of the α-ketoglutarate dehydrogenase component was determined electrophoretically in a polyacrylamide gel gradient according to Kopperschläger *et al.* (11).

Paper electrophoresis (horizontal) was carried out at 1–2°C in 0.05 M tris-acetate buffer, pH 7.5, containing 1×10^{-3} M EDTA; the voltage gradient was 40 V/cm and duration 90 min.

Radioactivity was measured on a Nuclear Chicago Mark II automatic scintillation counter.

Electron microscopy studies of samples negatively contrasted with 0.25% solution of sodium phosphotungstate were performed on a Hitachi HU-125 electron microscope at 100 kV and magnification of × 100,000.

γ-P^{32}-ATP with specific activity of 15.6 Ci/mmol was obtained from Amersham; 8-C^{14}-ATP with a specific activity of 477 μCi/mmol was supplied by the Institute for Utilization, Production and Investigation of Radioisotopes (UVVVR, CSSR); $N(CH_3)$-thiamine, $N(CH_3)_2$-thiamine and hydroxythiamine were obtained from A. Schellenberger's laboratory (M. Luther University, Halle, G.D.R.).

RESULTS AND DISCUSSION

*Pyruvate Dehydrogenase (Complex)**

With the exception of the preliminary data obtained by Jagannathan and Schweet in 1952 (18), pyruvate dehydrogenase of skeletal muscles, including pigeon breast muscles, has not been studied so far.

*Data from Glemzha *et al.* (1), Khailova *et al.* (2, 3, 12–15) and Severin *et al.* (16, 17) are used.

The pyruvate dehydrogenase complex consists of pyruvate-lipoate oxidoreductase (EC 1.2.4.1), lipoate acetyltransferase (EC 2.3.1.12) and lipoamide dehydrogenase (EC 1.6.4.3), and catalyzes at least three successive reactions (Fig. 1):

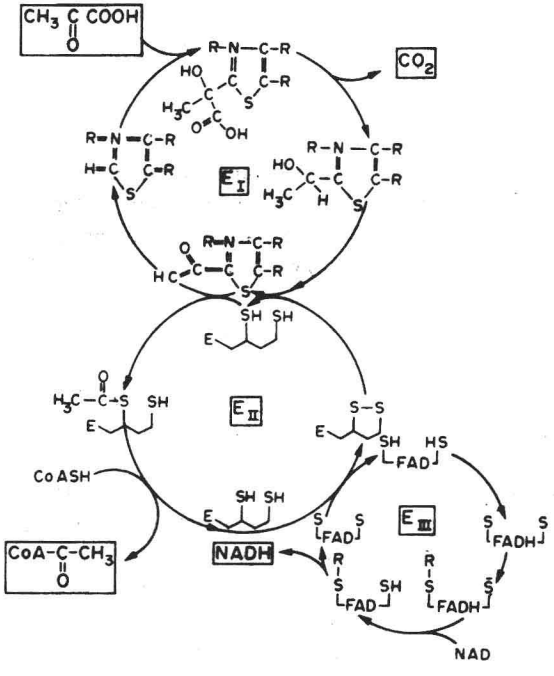

FIG. 1. The reaction sequence catalyzed by the pyruvate dehydrogenase complex. The upper circle represents the reactions catalyzed by pyruvate dehydrogenase (E_I), leading to pyruvate decarboxylation and acetyl lipoate formation. The middle circle represents the lipoate acetyltransferase-catalyzed reactions (E_{II}), which lead to formation of acetyl-CoA and reduced lipoic acid. The lower circle illustrates the lipoamide dehydrogenase (E_{III})-catalyzed reactions, which lead to NAD reduction and formation of the oxidized form of lipoic acid.

1. Decarboxylation of pyruvic acid and oxidation of the reaction product (E_1);

2. Transfer of the acetyl residue to CoA, accompanied by reduction of the disulfide group of lipoic acid (E_2);

3. Oxidation of lipoic acid and production of reduced NAD (E_3).

It has recently been found that there are two more enzymes in the complex, kinase and phosphatase (9), which possess regulatory functions.

The multienzyme pyruvate dehydrogenase complex, isolated from pigeon breast muscle and purified to homogeneity in this laboratory, has a sedimenta-

tion coefficient $S_{20,w}$ = 70S and appears under an electron microscope as a spherical structure. 400 to 450 Å in diameter. The central part of the complex is occupied by the transacetylase molecule, which under an electron microscope exhibits a clear structure at three different projections corresponding to a pentagonal dodecahedron (Fig. 2). This component is 210 to 250 Å in diameter and its $S_{20,w}$ = 27.4S. Transacetylase is surrounded by lipoyl dehydrogenase and pyruvate dehydrogenase molecules with $S_{20,w}$ of 6.1 and 7.8S, respectively. The latter component is about 70 Å in diameter and has a marked tendency to aggregate and to form long strands following negative contrasting (Fig. 3).

The following results were obtained after complete deaggregation of the complex by sodium dodecyl sulfate and subsequent polyacrylamide gel electrophoresis. The lipoyl dehydrogenase component yielded identical subunits with a molecular weight of 57,000. The transacetylase component also produced one kind of subunit, only with a molecular weight of 68,000 (3). A particular feature of the dehydrogenase component was that it consisted of two subunits, α- and β- with molecular weights of 41,000 and 37,000, respectively. The molecular weight of the dehydrogenase component is 156,000 (12), hence its structure apparently corresponds to a formula of $\alpha_2\beta_2$. Different combinations of the subunits may produce oligomers with lower molecular weights (70,000 and 104,000), which differ in specific activity (12).

The primary structure of pyruvate decarboxylating component subunits, in particular the structure of the active site of the enzyme, has not been determined. It is, however, clear that a very important role in the enzymatic decarboxylation of pyruvate is played by the histidine residue of the enzyme. Photoinactivation or diethylpyrocarbonate acylation of this residue deprives the enzyme of its activity. The pH dependence of photoinactivation is characteristic of that for histidine residue inactivation. According to the evidence obtained in this laboratory, histidine serves an anchoring function in binding the thiamine-PP coenzyme (14, 17). The SH-groups of the protein were also shown to be essential for enzymatic activity, since their fixation with sulfhydryl reagents, *p*-chloromercuribenzoate, for example, resulted in the inactivation of the enzyme (13). Using thiamine-PP analogs, we have found that for the first step in the pyruvate dehydrogenase reaction the participation of not only the second carbon atom of the thiamine-PP thiazole ring, but also of the amino group at the fourth carbon of the pyrimidine ring is necessary. Substitution of the amino group by its N-methyl- or N-dimethyl-derivatives or by a hydroxyl results in a loss of enzymatic activity (16) (bicentral mechanism of Schellenberger). The derivatives have no coenzyme activity and are competitive inhibitors:

$$K_{i\ 4'\text{-}N(CH_3)\text{-}TPP} = 4 \times 10^{-5}\ M;\ K_{i\ 4'\text{-}N(CH_3)_2\text{-}TPP} = 8.5 \times 10^{-5}\ M;$$

$$K_{i\ 4'\text{-}N\text{-}OH\text{-}TPP} = 2.9 \times 10^{-6}\ M\ (16).$$

The pyruvate dehydrogenase complex from pigeon breast muscle, like similar enzymes from mammalian heart and kidney (19–21), can be phosphorylated and dephosphorylated, and this markedly affects its enzymatic activity (15). Phosphorylation is catalyzed by a kinase which transfers the terminal phosphoryl moiety of ATP to the subunit of the enzyme, and this subunit may be regarded as a regulatory one (Fig. 4). The transfer results in a decrease or in complete suppression of the decarboxylase activity of the complex or isolated decarboxylating component. Restoration of the activity involves the splitting off

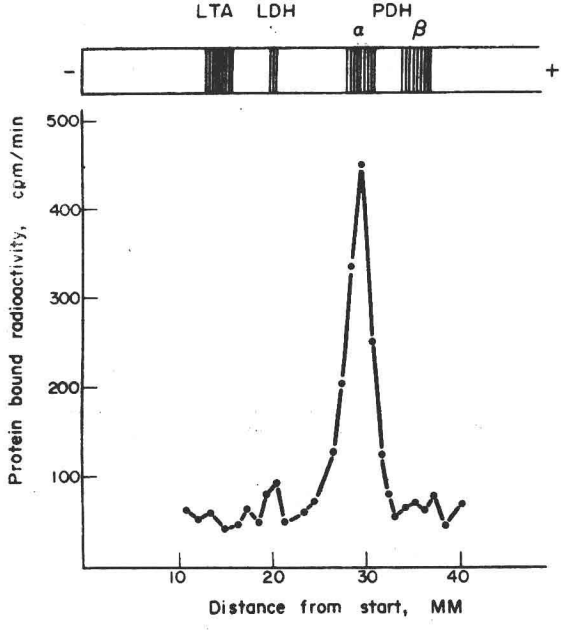

FIG. 4. Gel distribution of protein-bound P^{32} following electrophoretic separation of the pyruvate dehydrogenase complex in the presence of sodium dodecyl sulfate.

of the phosphoryl group by the corresponding phosphatase. Both enzymes, kinase and phosphatase, are parts of the pyruvate dehydrogenase complex. Their activity is regulated by Mg^{2+} concentration: at low Mg^{2+} concentration (and in the presence of ATP) the enzymatic activity is inhibited, since phosphorylation takes place; at higher Mg^{2+} concentrations phosphatase is activated, resulting in the splitting off of the phosphoryl group and the restoration of activity. It should be noted that activation of the phosphatase activity of the pyruvate dehydrogenase complex from pigeon breast muscle requires a much lower Mg^{2+}

concentration (K_m = 0.25 mM) as compared to the mammalian tissue enzymes (K_m = 2 mM).

After the phosphoryl group had been split off, the enzymatic activity of the pyruvate dehydrogenase complex was often higher than the initial one (Fig. 5). This fact indicates that part of the isolated complex was phosphorylated and that the phosphorylation and dephosphorylation processes are a means of an *in vivo* regulation of pyruvate dehydrogenase activity in muscle tissue.

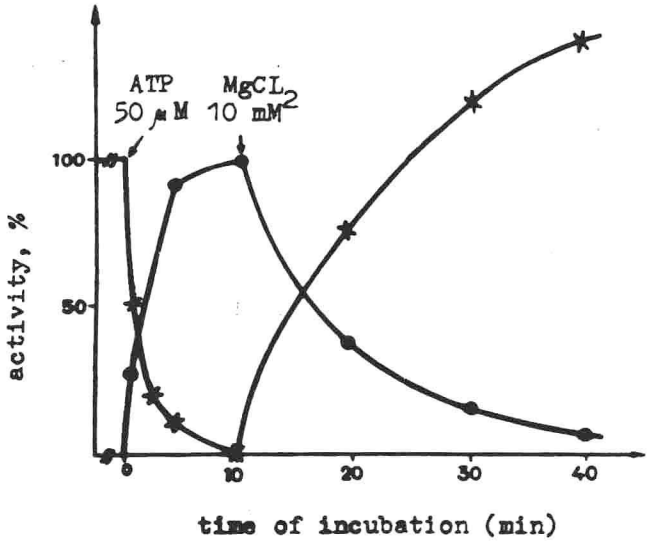

FIG. 5. Changes in pyruvate dehydrogenase activity (– x – x –x –) and protein-bound radioactivity (– • – • – • –) during incubation of the pyruvate dehydrogenase complex with ATP (0.1 mM) and $MgCl_2$ (10 mM).

Thus, several successive reactions resulted in the formation of acetyl-CoA from pyruvate (Fig. 1). The enzymes participating in this process are regulated systems: the first of them, which decarboxylates pyruvate, changes its activity during phosphorylation and dephosphorylation; the second one, which catalyzes the transfer of the acetyl residue to CoA, is regulated by the ratio of free HS-CoA (activator) to its acetyl derivatives (inhibitor).

Many of the described properties of the pyruvate dehydrogenase complex from pigeon breast muscle are similar to those of the enzyme isolated from various mammalian tissues. Table 1 provides a comparative [illegible]