ENZYME SYSTEMS IN THE MYCOBACTERIA

V. THE PYRUVIC DEHYDROGENASE SYSTEM

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In the preceding communication[1] the purification and some of the properties of a soluble pyruvic oxidase isolated from cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* were described. This enzyme, like its counterpart in animal tissues and microorganisms[2–5] reacts with ferricyanide, PIP**, oxygen and DPN. This report deals with the stoichiometry of the oxidative reaction and with some of the coenzyme requirements of the H37Ra pyruvic oxidase***.

MATERIALS AND METHODS

Unless otherwise specified all enzymes and coenzymes used in this investigation were of the highest purity available from commercial sources.

The pyruvic dehydrogenase of H37Ra was prepared as described in the preceding paper[1]. Phosphotransacetylase was prepared from dried cells of *Clostridium kluyveri* by the procedure of STADTMAN, NOVELLI AND LIPMANN[8]. Lipoic acid (oxidized and reduced) was generously provided by Dr. DAVID GIBSON. Acetyl CoA and acetyl phosphate were analyzed colorimetrically after conversion to acethydroxamic acid[9]. The preparation of the malic dehydrogenase and the condensing enzyme of H37Ra has been described[10,11].

The DPN assay system for the pyruvic dehydrogenase contains, in μmoles, Tris or phosphate buffer of pH 7.0 (40), cysteine (3), CoA (0.15), DPT (0.025), $MgCl_2$ (5), Li-pyruvate (10), DPN (1.0) and enzyme (0.1 to 1.0 mg). The final volume is 1.0 ml. The reaction is started by the addition of DPN. The change in optical density at 340 mμ is measured at min intervals for 5 min. Optical densities are measured with a Beckman DU spectrophotometer. The extinction coefficient of DPNH[12] is taken as $6.22 \cdot 10^6$ cm $\times$ mole^{-1}. Specific activity is defined in terms of PIP reduction[1].

RESULTS

Characteristics of the assay system

The optimum pH for the pyruvic dehydrogenase assay system is 7.0. Under the assay conditions described above the reduction of DPN proceeds linearly with time for about 5 minutes, providing the overall reduction of DPN in this period of time is less than

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** The following abbreviations will be used: PIP, 2,6-dichlorophenolindophenol, H37Ra, the H37Ra strain of *M. tuberculosis* var. *hominis*; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; DPT, thiamine pyrophosphate; tris, trishydroxymethyl-aminomethane buffer; CoA, acetylation coenzyme.

*** The oxidative reaction catalyzed by this enzyme, in its purified state, appears to be a standard DPN-requiring reaction. The ability of the enzyme to utilize oxygen may be due to an associated enzymic activity quite separate from the oxidizing enzyme[6]. The term "pyruvic dehydrogenase" rather than "pyruvic oxidase" will, accordingly, be used.

A preliminary account of this work has appeared[7].

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0.03 μmoles/ml. Under these conditions the rate of DPN reduction is proportional to enzyme concentration. At higher enzyme levels, the rate of DPN reduction falls off with time; this is probably due to the reoxidation of DPNH by the lactic dehydrogenase which is present in the enzyme preparation. In the above assay system, the pyruvic dehydrogenase shows an absolute requirement for CoA and DPT. The dependence of the assay system on several of the components is shown in Fig. 1. An occasional preparation of pyruvic dehydrogenase will show only a partial requirement for DPT. Magnesium ions often stimulate the rate of DPN reduction. In some preparations little or no stimulation is noted, in others the rate may be doubled. Accordingly, magnesium ions are always included in the assay mixture. Addition of manganese, lipoic acid, hog liver extract or yeast extract to the assay system had no effect on the rate of oxidation.

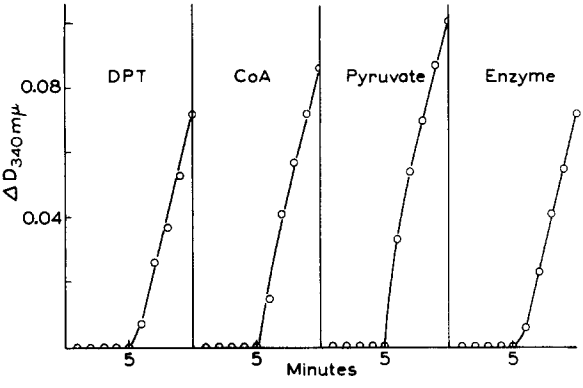

Fig. 1. Dependence of pyruvic dehydrogenase assay system on various components. See the text for assay conditions. In each instance all components of the assay system, except the component indicated, were mixed. Five minutes later the remaining component was added.

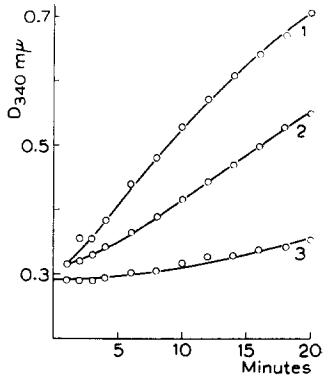

Fig. 2. Catalytic activity of CoA in the pyruvic dehydrogenase activity system. The standard assay system using phosphate buffer was set up. CoA was used as follows: Curve 1, 0.005 μmoles; Curve 2, 0.0025 μmoles; Curve 3, no CoA. Each cuvette contained 0.35 mg of pyruvic dehydrogenase of specific activity 597.

Formation of acetyl phosphate and acetyl CoA from pyruvate

The oxidation of pyruvate by pyruvic dehydrogenase, in the presence of stoichiometric amounts of DPN and CoA, should yield acetyl CoA, CO_2 and DPNH. Experiments designed to show acetyl CoA formation were negative (Fig. 2). CoA acted catalytically, not as a substrate. The presence of an acetyl CoA deacylase[13] was a possible explanation for this lack of formation of acetyl CoA. To overcome this difficulty of deacylation it was planned to trap acetyl CoA as acetyl phosphate by adding phosphotransacetylase to the reaction mixture[14]. DPN can be used in catalytic amounts by adding lactic dehydrogenase to the reaction mixture; the DPNH formed on the oxidation of pyruvate is oxidized by the excess pyruvate in the system. As shown in Table II, acetyl phosphate accumulates in such a system. The data of Table I demonstrate that a majority of the activity of the whole system is retained despite removal of any one of several of the reactants. In the system described in Table II, it is impossible to stop acetyl phosphate formation unless either pyruvate or DPT is omitted.

Since phosphotransacetylase cannot operate in the absence of phosphate, a simple method was at hand to demonstrate acetyl CoA accumulation. Fig. 3 shows the

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TABLE I

BALANCE FOR PYRUVATE-ACETYL PHOSPHATE DISMUTATION REACTION

Each tube contained, in μmoles, phosphate buffer of pH 7.0 (70), Li-pyruvate (11.2), $MgCl_2$ (5), DPT (0.40), CoA (0.40), cysteine (8), DPN (0.10), crystalline lactic dehydrogenase (20 μgm), phosphotransacetylase (50 μgm) and pyruvic dehydrogenase (specific activity of 380) as shown. The final volume was 1.0 ml. The reaction mixtures were incubated for 2.0 hours at 38° and then analyzed for acetyl phosphate and residual pyruvate.

Expt. no.	*Enzyme concentration (mg/ml)*	*Final pyruvate*	*Δ Pyruvate*	*Final acetyl phosphate*	$\frac{\Delta \text{ pyruvate}}{\Delta \text{ acetyl P}}$
1	0	11.2	0	0	
2	1.19	5.54	5.7	2.06	2.8
3	2.38	4.68	6.5	2.32	2.8

TABLE II

FORMATION OF ACETYL PHOSPHATE FROM PYRUVATE

The complete system contained, in μmoles: phosphate buffer of pH 7.0 (100), Li-pyruvate (100), $MgCl_2$ (10), DPT (0.50), CoA (0.50), cysteine (10), DPN (0.20), crystalline lactic dehydrogenase (20 μg), phosphotransacetylase (50 μg) and pyruvic dehydrogenase (0.864 mg of specific activity 715). The final volume was 2.0 ml. The reaction mixtures were incubated at 38° for 20 min and then analyzed for acetyl phosphate.

Mixture	*μMoles acethydroxamic formed in 20 min**
Complete system	0.65
no lactic dehydrogenase	0.38
no phosphotransacetylase	0.56
no CoA	0.34
no DPN	0.49

* Corrected for the blank in the control without pyruvic dehydrogenase.

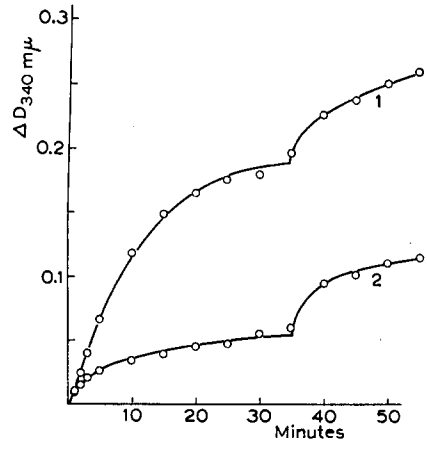

Fig. 3. CoA dependence of pyruvic dehydrogenase. Each micro-cuvette contained, in μmoles: tris buffer of pH 7.0 (40), L-cysteine (8), DPT (0.020), Li-pyruvate (10), DPN (1.0), CoA (Curve 1, 0.05; Curve 2, 0.025) and pyruvic dehydrogenase (0.94 mg; specific activity of 380). The final volume was 1.0 ml. The reaction proceeded for 35 min and additional CoA, equal to the amount present at the start of the reaction was added. Values have been corrected for a no-CoA blank.

results of an experiment in which acetyl CoA was formed from pyruvate and stoichiometric quantities of CoA in a phosphate-free system. The reduction of DPN proceeds until the added CoA is exhausted; additional CoA allows the formation of more DPNH.

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Identity of pyruvate-oxidation product

Two methods were used to prove that acetyl CoA is formed from pyruvate:

1. *Chromatography.* The products of the reaction described as the "complete system" in Table II, as well as those of a control system without pyruvate were reacted with hydroxylamine and the resulting mixtures were isolated and chromatographed on paper. Ascending chromatography was carried out for 20 hours in *n*-butanol/3*N* NH_4OH[15]; the papers were dried and then sprayed to develop the iron-hydroxamate color[16]. An hydroxamic acid having an R_F (0.17) identical with that of known acethydroxamic acid was isolated from the "complete system" tube; the control tube contents showed no hydroxamate spot.

2. *Coupling of the pyruvic dehydrogenase and condensing enzyme systems.* Additional proof for the formation of acetyl CoA by the pyruvic dehydrogenase was obtained by linking reactions catalyzed by the pyruvic and malic dehydrogenases with that of the condensing enzyme reactions. Oxalacetate was generated by the oxidation of L-malate by the DPN-specific malic dehydrogenase. Fig. 4 shows the successful coupling of these reactions. In the absence of any one component of the condensing

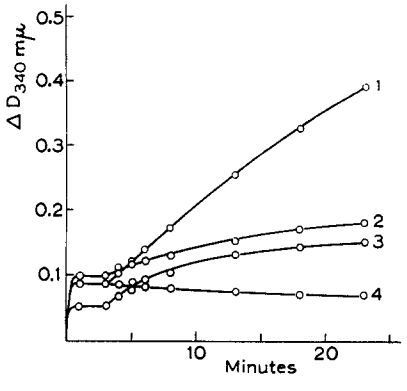

Fig. 4. Coupling of the pyruvic dehydrogenase, malic dehydrogenase and condensing enzyme. Each microcuvette contained, in micromoles: tris buffer of pH 7.0 (50), $MgCl_2$ (5), L-cysteine (8), DPT (0.025), L-malate (5), Li-pyruvate (10), DPN (1.0), CoA (0.05), pyruvic dehydrogenase (0.118 mg; specific activity of 380), malic dehydrogenase (0.026 mg; specific activity of 16) and condensing enzyme (0.013 mg; specific activity of 3.5). Total volume was 1.0 ml. Omissions from reaction mixtures were as follows: Curve 1, none; Curve 2, condensing enzyme; Curve 3, L-malate; Curve 4, CoA. The reactions were started by the addition of DPN; 3 minutes later CoA, as indicated, was added.

enzyme system, the rate of DPN reduction is governed by the pyruvic dehydrogenase. In the absence of any component of the pyruvic dehydrogenase system, there is no DPN reduction after the malic dehydrogenase reaction reaches equilibrium. When the two systems are coupled, a rapid and continuing reduction of DPN results.

Action of inhibitors

Versene is a powerful inhibitor of the pyruvic dehydrogenase of H37Ra. This is a general inhibition and is not dependent on the electron acceptor used[1]. In the absence of magnesium ions, the pyruvic dehydrogenase is inhibited 84% by $2 \cdot 10^{-4} M$ and 14% by $5 \cdot 10^{-5} M$ versene. This inhibition can, in part, be reversed by magnesium ions. Fluoride, arsenate and arsenite do not affect the rate of oxidation of pyruvate by this enzyme.

K_s values for CoA and DPN

Michealis constants for DPN and CoA in the pyruvic dehydrogenase assay system are $1.3 \cdot 10^{-5}$ and $5.5 \cdot 10^{-5} M \cdot l^{-1}$, respectively.

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Ultracentrifugation

The pyruvic dehydrogenase (specific activity of 470) is not sedimented by centrifugation at 105,000 × *g* for 2 hours.

Enzymes associated with the pyruvic dehydrogenase

Preparations of the purified H37Ra pyruvic dehydrogenase consistently show the presence of a phosphotransacetylase, lactic dehydrogenase and lipoic dehydrogenase. The presence of the first two enzymes was discussed above. Lipoic dehydrogenase was demonstrated by direct assay[17]. This combination of associated activities is either fortuitous or is possibly due to the presence of a pyruvic dehydrogenase complex which contains all these activities.

DISCUSSION

Pyruvic dehydrogenases have been purified from several animal tissues and microorganisms. JAGANNATHAN AND SCHWEET[18] purified a pyruvic oxidase from pigeon breast muscle. The highly purified enzyme is a large molecule and no resolution of activities associated with the several reactions involved in pyruvate oxidation has been reported. LITTLEFIELD AND SANADI[19] were able to show that, under the proper conditions, both DPN and CoA are required by this enzyme. A pyruvic dehydrogenase was isolated from pig heart muscle by KORKES *et al.*[3]. This enzyme was partially separated into two fractions whose combined activity for the oxidation of pyruvate was greater than the sum of the activities of the two fractions acting separately. A completely resolved and highly purified pyruvic dehydrogenase was isolated from *Escherichia coli* and *Streptococcus faecalis* by KORKES *et al.*[4] and by GUNSALUS[20]. One of the two fractions (Fraction A) oxidizes pyruvate in the presence of DPT and an electron acceptor (dye). Fraction B contains the enzymic complement necessary to form acetyl CoA and DPNH[17, 20]. Lipoic acid is a necessary coenzyme for this reaction[20].

The pyruvic dehydrogenase of H37Ra is a soluble enzyme of low molecular weight. DPN, DPT, CoA and Mg^{++} are required for the oxidation of pyruvate; acetyl CoA is the product of the reaction. The enzyme has not yet been resolved into two or more fractions as have the dehydrogenases of *E. coli* and *S. faecalis*.

The pyruvic dehydrogenase of H37Ra is associated with several other enzymes related to pyruvate metabolism. These include a phosphotransacetylase, a lactic and a lipoic dehydrogenase. The fact that these four activities remain associated with each other through several purification steps for the pyruvic dehydrogenase suggests that we are dealing here not exclusively with a pyruvic dehydrogenase but with a pyruvic dehydrogenase complex. This infers that these enzymes are in some manner associated with each other in the intact cell, are solubilized together during the sonic disruption of the cells, and are purified together. The ratio of enzymic activities at each stage in the pyruvic dehydrogenase purification procedure cannot be used as proof of the existence of a complex and, consequently, the lack of correspondence of purification factors for the several enzymes cannot be used as proof for the non-existence of a complex. That this is true may be inferred from the theory of protein purification. In the purification of an enzyme, conditions are selected to give minimum removal and/or destruction of contaminating proteins. Both the phosphotransacetylase and the lactic dehydrogenase associated with the pyruvic dehydrogenase of H37Ra are inactivated

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during dialysis of pyruvic dehydrogenase fractions. Thus, while the pyruvic dehydrogenase has been purified 80- to 100-fold over the initial crude extract, the phosphotransacetylase and lactic dehydrogenase have been purified only slightly. It is of importance to note, however, that in every case where all activities have been followed through various purification steps the activities remain associated, one with the others; no fraction other than that containing the pyruvic dehydrogenase contains significant phosphotransacetylase, lactic dehydrogenase or lipoic dehydrogenase activity. This concept of enzymic complexes is currently under investigation in this laboratory.

It must be borne in mind, however, that this apparent association of activities may, in fact, be an artifact. A crude cell-free extract undoubtedly contains many enzymes that tend to fractionate together. By chance this group of enzymes could include the several enzymes described above which act on pyruvate or acetyl CoA.

SUMMARY

A soluble pyruvic dehydrogenase has been isolated from cell-free extracts of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. Pyruvate is oxidatively decarboxylated to acetyl CoA and CO_2. Magnesium, DPN, CoA and DPT are required for this oxidation. The pyruvic dehydrogenase is inhibited by versene; magnesium can, in part, reverse this inhibition. K_s values for DPN and CoA are $1.3 \cdot 10^{-5}$ and $5.5 \cdot 10^{-5}$ moles/liter, respectively. The pyruvic dehydrogenase appears to be part of an enzyme complex. Associated with the pyruvic dehydrogenase are a phosphotransacetylase, a lactic dehydrogenase and a lipoic dehydrogenase.

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