ENZYMES AND COENZYMES OF THE PYRUVATE OXIDASE OF PROTEUS*

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The oxidative decarboxylation of pyruvate has been shown to occur by several distinct pathways. The first cell-free system studied was that from Lactobacillus delbrueckii, which produces acetyl phosphate (1) without the intermediate formation of acetyl coenzyme A (2). The dismutase systems from Streptococcus faecalis and from Escherichia coli produce acetyl coenzyme A (3), but the pyruvate oxidase system from Proteus vulgaris produces free acetate without the intermediate formation of acetyl phosphate (4) or acetyl coenzyme A (5).

The pyruvate oxidase of P. vulgaris consists of two separable protein components (6) which have been identified as a particulate autoxidizable cytochrome system (Component 1) and a soluble enzyme (Component 2), which catalyzes the following reaction in the presence of diphosphothi-amine (DPT) and an appropriate electron acceptor: pyruvate + H₂O → acetate + CO₂ + 2H⁺ + 2e. Either 2,6-dichlorophenolindophenol or ferricyanide may serve directly as an electron acceptor. Oxygen is consumed only when Component 1 is present to serve as an intermediate electron carrier. No other coenzyme in addition to DPT has been demonstrated to function in this system. A brief report of some of these results has appeared (7).

EXPERIMENTAL

Test Systems

A. Oxygen Consumption—Conventional manometric techniques were used at a temperature of 37⁰. Each flask contained 0.02 M potassium phosphate buffer, pH 6.0, 100 γ of DPT, 200 γ of MnSO₄·H₂O, 100 μmoles of potassium pyruvate, enzyme fractions, and water to 3.0 ml. The center well contained 0.2 ml of 20 per cent KOH. The rate of oxygen consumption was calculated from the second 10 minute period after the addition of pyruvate.

B. Reduction of Ferricyanide—The reaction mixture contained 0.03 M

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potassium phosphate buffer, pH 6.0, 50 μ of DPT, 100 μ of MnSO₄·H₂O, 50 μmoles of potassium pyruvate, 25 μmoles of potassium ferricyanide, enzyme fractions, and water to 1.0 ml.; temperature, 25°. The rate of ferricyanide reduction was calculated from the amount of ferrocyanide produced in the first 10 minutes after the addition of ferricyanide.

C. Reduction of 2,6-Dichlorophenolindophenol (2,6-DCP)—The reaction mixture was the same as that of Test System A, except that 200 μ of 2,6-DCP were added; temperature, 25°. The rate of 2,6-DCP reduction was calculated from the second 1 minute interval after the addition of 2,6-DCP by observing the decrease in optical density at 600 μm in a 1 cm. cuvette.

1 unit of Component 2 is equivalent to the utilization of 0.5 μl. of oxygen per hour in the presence of an excess of Component 1 with Test System A. 1 unit of Component 1 is that amount of Component 1 which permits half maximal velocity of 1 unit of Component 2 in Test System A.

Enzyme Fractionation and Purification

Cells were grown, collected, and disrupted by sonic oscillation as previously described (5). Unless otherwise indicated, all operations were carried out at 4°; enzyme solutions or suspensions were brought to the indicated decimal fraction of saturation with ammonium sulfate by the addition of a saturated solution of ammonium sulfate; centrifugation was at 25,000 × g in the SS-1 head of the Servall refrigerated centrifuge.

Purification of Component 1. Step I—Cell fragments and intact cells were removed from the suspension by centrifuging for 30 minutes. Fraction 1, yield (from 65 gm. of wet cell paste): 3920 mg. of protein, 693,000 units, per cent recovery 100, specific activity 176.

Step 2—Fraction 1 was brought to 0.62 of saturation with ammonium sulfate, held for 2 hours, and centrifuged for 25 minutes. The supernatant fluid was discarded and the precipitate was suspended in a minimal amount of distilled water and dialyzed against 10 liters of distilled water for 20 hours. Fraction 2, yield: 3180 mg. of protein, 565,000 units, per cent recovery 82, specific activity 178.

Step 3—Fraction 2 was brought to 0.38 of saturation with ammonium sulfate, held for 1 hour, and then centrifuged for 20 minutes. The precipitate was washed once in 0.38 saturated ammonium sulfate, the washed precipitate containing Component 1; its treatment is described below. The supernatant fluid and wash liquid were combined, and solid ammonium sulfate was added to saturation. After 1 hour the protein suspension was decanted from the excess ammonium sulfate and centrifuged for 20 minutes. The supernatant fluid was discarded. The precipitate was dissolved in distilled water and dialyzed against 2 liters of distilled water for 16 hours. After dialysis, one-ninth volume of 0.20 M potassium phosphate
buffer, pH 6.0, was added to the enzyme solution, and it was heated at 58-60°C for 10 minutes. The denatured protein was removed by centrifuging for 30 minutes. The supernatant fluid contained the active material. Fraction 3, yield: 421 mg. of protein, 440,000 units, per cent recovery 64, specific activity 1040.

Step 4—Fraction 3 was brought to 0.5 times of saturation with ammonium sulfate, held for 1 hour, and centrifuged for 20 minutes. The precipitate was discarded, and the supernatant fluid was brought to 0.7 times saturation by adding solid ammonium sulfate. After 1 hour the suspension was centrifuged for 20 minutes. The supernatant fluid was discarded; the precipitate was dissolved in a small volume of distilled water and dialyzed against 2 liters of distilled water for 16 hours. The precipitate which was formed in the dialysate contained 80 per cent of the remaining activity. It was collected by centrifuging for 20 minutes and washed twice, each time with 10 ml. of distilled water. Fraction 4, yield: 40 mg. of protein, 238,000 units, per cent recovery 34, specific activity 5950.

Step 5—Fraction 4 was suspended in 11 ml. of 1 per cent KCl. The suspension was shaken every 20 minutes for 4 hours, then centrifuged for 20 minutes, and the precipitate discarded. Phosphate buffer at pH 6.5 was added to the supernatant fluid to a final concentration of 0.033 M. Fraction 5, yield: 7.4 mg. of protein, 186,000 units, per cent recovery 27, specific activity 25,170. The purification of Component 2 is summarized in Table I.

Component 2 activity determined in Test System A in the presence of excess Component 1 (5800 units, 5 mg. of protein).

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Protein</th>
<th>Units</th>
<th>Recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coll-free extract</td>
<td>3920 mg.</td>
<td>603,000 units</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.62 saturated ammonium sulfate ppt.</td>
<td>3180 mg.</td>
<td>565,000 units</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>0.38 &quot; &quot; &quot; supernatant fluid, heated</td>
<td>421 mg.</td>
<td>440,000 units</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>Dialyzed and washed 0.51-0.70 saturated ammonium sulfate ppt.</td>
<td>40 mg.</td>
<td>238,000 units</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Ppt. from dialyzed salt solution of Fraction 4</td>
<td>7.4 mg.</td>
<td>186,000 units</td>
<td>27</td>
</tr>
</tbody>
</table>

Component 2 activity determined in Test System A in the presence of excess Component 1 (5800 units, 5 mg. of protein).
Proteus Pyruvate Oxidase

which can be removed by adsorption on calcium phosphate gel. Component 1 can also be freed of Component 2 by precipitation of Component 1, which occurs after alternate freezing and thawing. The latter treatment is preferred since more than 80 per cent of Component 1 activity is recoverable. The dialyzed suspension of the 0.38 saturated ammonium sulfate precipitate was kept in a deep freeze at -17° for 5 days and thawed once every 24 hours. Component 1 was then precipitated by centrifuging for an hour. It was suspended in distilled water and evenly dispersed by sonic oscillation at 9 kc. per second for 8 minutes.

![Graph of the difference in absorption between oxidized and reduced Component 1. Test System A. The inset shows the Soret bands.](image)

**Functions and Properties of Protein Components**

Component 1 is reddish brown and shows a marked Tyndall effect. The active material is associated with particles sedimentable in a centrifugal field of 60,000 X g. Component 1 is comparatively resistant to physical agents (5). The cytochrome system of Component 1 is autoxidizable when reduced by pyruvate in the presence of Component 2 in Test System A. Fig. 1 shows the spectrum of the difference in absorption of Component 1 between its oxidized state and enzymatically reduced state, measured in the recording spectrophotometer of Chance (8) by Dr. Lucile Smith. The same spectrum is obtained when hydrosulfitre is the reducing agent. Distinct peaks occur at 627 and 560 mμ. There is a lower peak at 595 mμ. The fused β-bands have a single peak at 530 mμ. The Soret band has a peak at 429 mμ with a shoulder at 442 mμ. The peaks due to the difference
in absorption between the oxidized and reduced states appear only when the oxygen tension in one cuvette is zero. These peaks indicate the presence and functioning in Component 1 of cytochromes $b_1$, $a_2$, and possibly $a_1$.

The most highly purified Component 2 preparations are water-clear. This component is soluble in dilute salt solution but insoluble in distilled water, suggesting that it is a globulin. In the purified form it loses 50 per cent of its activity in 48 hours at 4° and is completely inactivated by freezing. However, in 0.033 M phosphate buffer, pH 6.5, it may be stored at 4° for a week without losing more than 10 per cent of its activity.

Component 2 reduces 2,6-DCP and ferricyanide in Test Systems C and B respectively. It does not permit oxygen uptake in Test System A unless

<table>
<thead>
<tr>
<th>Table II</th>
</tr>
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<tbody>
<tr>
<td>Inhibition of Action of Component 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>2,6-Dichlorophenolindophenol reduced per hr., μmoles per mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>11.6</td>
</tr>
<tr>
<td>&quot; - Component 2</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot; - pyruvate</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot; - diphosphothiamine</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot; + 0.00001 M HgCl₂</td>
<td>0.0</td>
</tr>
<tr>
<td>&quot; + 0.03 M NaAsO₂</td>
<td>11.1</td>
</tr>
</tbody>
</table>

The complete system consisted of Test System C with 55 γ of 2,6-dichlorophenolindophenol and 29 γ of Component 2 protein.

Component 2 is added (Table III). The DPT concentration required for half maximal rate in the reduction of 2,6-DCP by Component 2 is the same as that required for the aerobic oxidation of pyruvate by the *P. vulgaris* pyruvate oxidase preparation of Stumpf (4). Component 2 activity is strongly inhibited by mercuric chloride and unaffected by sodium arsenite (Table II). A product recovery showed that 30 μmoles of 2,6-DCP were reduced by Component 2 with the formation of 27.8 μmoles of acetate and 24 μmoles of carbon dioxide.

Component 2, in the presence of an excess of Component 1, oxidizes pyruvate at similar rates with either oxygen, ferricyanide, or 2,6-DCP as the final oxidant; however, the concentration of Component 1 necessary for half maximal rate of pyruvate oxidation varies with the electron acceptor (Table III). This indicates that the rate at which Component 2 oxidizes pyruvate and reduces the cytochromes of Component 1 is independent of the manner of reoxidation of Component 1, although the rate
of reoxidation of Component 1 is affected by the choice of electron acceptors. Ferricyanide is the most efficient oxidant.

The presently available data suggest that there are few, if any, similarities between the acetate-generating pyruvate oxidase of *P. vulgaris* and acetyl phosphate- or acetyl coenzyme A-generating pyruvate oxidases of other bacteria. The acetyl phosphate-generating system of *L. delbrueckii* apparently consists of a single protein requiring flavin adenine dinucleotide (FAD) and DPT (9). Coenzyme A (CoA) does not function (2). The acetyl CoA-generating oxidases are multienzyme systems requiring, in addition to CoA, diphosphopyridine nucleotide (DPN), DPT, and lipoic acid.

**Table III**

Comparison of Electron Acceptors

<table>
<thead>
<tr>
<th>System</th>
<th>Pyruvate oxidized per hr. in presence of</th>
<th>( O_2 )</th>
<th>( K_2Fe(CN)_6 )</th>
<th>2,6-Dichlorophenolindophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component 1*</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>2*</td>
<td>0</td>
<td>22.2</td>
<td>719</td>
</tr>
<tr>
<td>&quot;</td>
<td>2 + excess Component 1*</td>
<td>1092</td>
<td>994</td>
<td>719</td>
</tr>
<tr>
<td>&quot;</td>
<td>1 required for half maximal velocity of 1</td>
<td>66.6</td>
<td>43.7</td>
<td>206.0</td>
</tr>
</tbody>
</table>

| mg. of Component 2             |

Oxygen uptake was determined in Test System A with 28.4 \( \gamma \) of Component 2 and 0 to 6.23 mg. of Component 1. Ferricyanide reduction was determined in Test System B with 5.7 \( \gamma \) of Component 2 and 0 to 1.87 mg. of Component 1. 2,6-Dichlorophenolindophenol reduction was determined in Test System C with 2.84 \( \gamma \) of Component 2 and 0 to 0.62 mg. of Component 1.

* The values are given in micromoles per mg. of component 2 protein.

(10). It has been shown that neither acetyl phosphate (4) nor acetyl CoA is an intermediate of the acetate-generating system and that CoA is not required and does not function (5).

The pyridine nucleotide coenzymes do not appear to function in the *P. vulgaris* system. The addition of DPN or triphosphopyridine nucleotide (TPN) does not affect the rate of pyruvate oxidation, nor is there an increase in the optical density of the system at 340 m\( \mu \) after zero oxygen tension has been reached. Additional evidence for the non-participation of DPN is the failure of the system to dismute pyruvate in the presence of added lactic dehydrogenase and DPN and the ability of crude extracts to oxidize pyruvate at an undiminished rate after the DPNH oxidase in the extract was completely inactivated by heating at 55° for 20 minutes. The non-function of lipoic acid is suggested by the fact that this coenzyme
is present in the *P. vulgaris* system at one-hundreth of the level found in acetyl CoA-generating systems. The failure of 0.03 M arsenite to inhibit the reduction of 2,6-DCP by Component 2 and oxygen uptake by both components suggests also the non-function of lipoic acid, as arsenite at lower concentrations is a potent inhibitor of lipoic acid-requiring enzymes (3). The amount of the complete *P. vulgaris* system which permits an oxygen uptake of 1200 μl per hour contains 3.66 γ of FAD. The d-amino acid oxidase system requires 33.3 γ of FAD to achieve a similar rate. The failure of this system to reduce nitrofurans and tetrazolium salts, compounds readily reduced by flavin-linked oxidases, also suggests that FAD is not functional in this system.

**Table IV**

<table>
<thead>
<tr>
<th></th>
<th>Diphosphothiamine Requirements for Pyruvate Oxidation or Dismutation by Proteus vulgaris</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The pyruvate was metabolized in micromoles per ml. of cell-free extract.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell-free extract prepared from</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Dismutation</td>
</tr>
<tr>
<td>16 hr. cells</td>
<td>+ DPT</td>
<td>238.0</td>
</tr>
<tr>
<td></td>
<td>- “ “</td>
<td>64.4</td>
</tr>
<tr>
<td>6 hr. cells</td>
<td>+ “ “</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Pyruvate oxidation was determined in Test System A; dismutation was determined in a system similar to that of Korkes et al. (16) by estimating acetyl phosphate production.

The only coenzyme common to both the *P. vulgaris* acetate-generating system and the acetyl CoA-generating system is DPT. The former system is obtained resolved for DPT in the preparation of cell-free extracts by ultrasonic oscillation (4) or by sonic oscillation (6), while the latter systems are obtained from *E. coli* and *S. faecalis* (3) containing sufficient bound DPT for maximal activity. Cell-free extracts of *P. vulgaris* contain both the acetyl CoA-generating dismutase system and the acetate-generating oxidase, making it possible to determine whether the variation in the DPT-enzyme associations is a species difference or a difference between enzyme systems. The dismutation rate in extracts prepared from 16 hour cells is less than 5 per cent of the oxidation rate. The addition of DPT causes a 40-fold stimulation of pyruvate oxidation, but it has no effect on the dismutation rate. The dismutation rate in an extract from 6 hour cells is 50 per cent of the oxidation rate. The addition of DPT to this extract causes a 3-fold stimulation of the oxidation rate and has only a small effect on the...
The difference in the nature of the DPT requirement is not due to widely varying DPT-enzyme association constants since these constants are similar when both systems are resolved for DPT. If one DPT-requiring enzyme functioned in both acetate and acetyl CoA generation, it would be necessary that bound DPT be available to this enzyme for one kind of reaction and unavailable to that same enzyme for another kind of reaction. A more plausible explanation of these results is the existence of two separate enzymes, one functioning in acetate generation and the other in acetyl CoA generation. Supporting the latter interpretation are the separation of both types of enzyme systems from E. coli and P. vulgaris and the demonstration that the fractions of each type system would not replace each other (11).

**DISCUSSION**

The oxidation of pyruvate to free acetate by the enzyme system from P. vulgaris had been thought to occur through pathways differing only in minor details from the mechanism by which acetyl CoA is formed from pyruvate (6). However, partial purification and further study of the acetate-generating system have led to the conclusion that differences between this system and the acetyl CoA-generating systems are extensive.

The reactions responsible for disposing of the 2 carbon units produced by the primary decarboxylation of pyruvate in the acetyl CoA-generating systems require, in addition to CoA, lipoic acid and DPN. It is doubtful whether these reactions occur also in the acetate-generating pyruvate oxidase of P. vulgaris, as CoA, lipoic acid, and DPN do not function in this system. The primary cleavage of pyruvate in acetyl CoA-generating systems is apparently catalyzed by an enzyme, “carboxylase,” which in the presence of 2,6-DCP or ferricyanide decarboxylates pyruvate to free acetate. Component 2 of the acetate-generating system catalyzes the same over-all reaction. The observation that, in an extract of P. vulgaris, acetyl CoA generation is dependent on an enzyme with bound DPT, that acetate generation is dependent on an enzyme resolved for DPT, and that “carboxylase”- and Component 2-containing fractions are not mutually replaceable suggests that Component 2 and “carboxylase” are not the same enzyme.

The oxidation of pyruvate to free acetate by the enzyme system of P. vulgaris may be seen to occur in the following manner: (1) pyruvate + Component 2 + DPT → “activated complex;” (2) “activated complex” + Component 1 → acetate + CO₂ + DPT + reduced Component 1 + Component 2; or (3) “activated complex” + 2,6-DCP or ferricyanide → acetate + CO₂ + DPT + Component 2 + 2,6-DCPH₂ or ferrocyanide.

Pyruvate is oxidized by Component 2, which may be considered to be a
pyruvic dehydrogenase, to acetate and carbon dioxide. The electrons are slowly accepted by artificial oxidants or more rapidly by the cytochrome system of Component 1, which is in turn reoxidized by oxygen or by the artificial electron acceptors. The rapid reaction with the cytochrome system and the slower reaction with artificial oxidants have in common the failure to preserve the high energy moiety of the pyruvate molecule.

Methods and Materials

Ferrocyanide was determined by forming ferriferrocyanide (Prussian blue) on the addition of a ferric salt (10). Protein and nucleic acid were estimated from the ratio of optical densities at 280 and 260 mμ according to the calculations of Warburg and Christian (12). Metabolites were estimated by the following methods: CoA by the stimulation of bacterial transacetylase, lipoic acid by the method of Gunsalus et al. (13), FAD by the stimulation of d-amino acid oxidase (this assay was made by Dr. Arnold Brodie), acetate by the lanthanum nitrate method of Hutcheson and Kass (14), and acetyl phosphate by the hydroxamic acid method of Lipmann and Tuttle (15). Crystalline lactic dehydrogenase was prepared according to the directions of Korkes et al. (16), and transacetylase was prepared from water extracts of Clostridium butyricum by precipitation with acetic acid at pH 4, followed by ammonium sulfate fractionation between 0.60 and 0.70 saturation.

Other chemicals used were obtained from the following sources: CoA, 75 per cent pure, from the Pabst Laboratories, DPT from Merck and Company, Inc., DPN, 80 per cent pure, from the Schwarz Laboratories, Inc., TPN, 80 per cent pure, and DPNH from the Sigma Chemical Company, and 2,6-DCP from The Matheson Company, Inc.

SUMMARY

The pyruvate oxidase system of Proteus vulgaris produces free acetate without the intermediate formation of acetyl coenzyme A. It consists of a particulate autoxidizable cytochrome system and of soluble pyruvic dehydrogenase. The pigments of the cytochrome system have been identified as cytochromes a₁, a₂, and b₁. The dehydrogenase has been extensively purified.

The dehydrogenase can reduce artificial electron acceptors; the cytochrome system greatly increases their rate of reduction and also enables oxygen to serve as the terminal oxidant.

Evidence is presented which indicates that coenzymes required by acetyl coenzyme A-generating pyruvate oxidases, diphosphopyridine nucleotide, coenzyme A, and lipoic acid do not function in the acetate generating pyruvate oxidase of P. vulgaris. Diphosphothiamine, a coenzyme common
to all pyruvate oxidases, is required by the pyruvic dehydrogenase of *P. vulgaris*; however, this enzyme does not function in the acetyl coenzyme A-generating system.

It has been concluded that the existence of common intermediates and enzymes in both the *P. vulgaris* pyruvate oxidase system and the acetyl coenzyme A-generating systems is doubtful.

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