MECHANISM OF ACTION AND CRYSTALLIZATION OF LACTIC OXIDATIVE DECARBOXYLASE FROM MYCOBACTERIUM PHLEI

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Earlier phases of an investigation concerning the lactic oxidative decarboxylase from Mycobacterium phlei (1, 2) extended the initial observations of Edson (3) and Edson and Cousins (4). A similar type of enzyme, obtained from Mycobacterium avium, has been described by Yamamura et al. (5, 6). The flavoprotein character of the enzyme, as well as the absence of associated coenzymes, has been established. The exact nature of the prosthetic group, riboflavin 5'-phosphate, was determined through the use of paper chromatography, light absorption ratios, and apoenzyme reactivation experiments (2). Certain atypical aspects of the enzyme activity, namely the failure of added catalase to influence the reaction (1, 3, 5) and the oxidation and decarboxylation of lactate as associated with a single protein unit (1), have been noted.

The current report presents additional data supporting a mechanism of action designed to explain the activity of the lactic oxidative decarboxylase and evidence as to the extent of purification achieved in the isolation of the enzyme from M. phlei.

Methods and Materials

The initial steps for the isolation and purification of the enzyme have been described previously (1, 2). Crystalline beef liver catalase and horseradish peroxidase were obtained from the Worthington Biochemical Corporation and were further purified before use. DL-Glycidate, potassium salt, was prepared by the method of Freudenberg (7) from epichlorohydrin. Catalase activity was assayed according to the method described by Beers and Sizer (8) and peroxidase by the method described by Polis and Shmukler (9). Paper electrophoretic data were obtained with a Spinco model R apparatus used in conjunction with an Analytrol scanner. The Durrum type cell was generally operated with a current of 15 ma. for 6 hours and barbiturate buffer of pH 8.0 with an ionic strength of 0.075. Crystalline...
Results

Nature of Metabolic Intermediates

Action of Carbonyl Fixatives—It has been established by chemical balance studies that for each mole of lactate metabolized by the enzyme, 1 mole of oxygen is utilized and 1 mole each of acetate and CO₂ is formed. Experimental values resulting from a typical group of determinations are presented in Table I. Edson (3) and Yamamura et al. (6) have presented similar analytical data supporting the stoichiometry indicated.

The flavoprotein character of the enzyme suggested that an oxidation product such as pyruvate might be intermediate in the metabolic pathway from lactate to acetate and CO₂. It was expected that the fixation of such an intermediate during the course of the reaction would interfere with CO₂ production but not with oxygen utilization. Anaerobic experiments have established the fact that the oxidative reaction step occurs prior to the decarboxylation.

Edson (3) had demonstrated earlier the presence of pyruvate as a metabolic intermediate with use of acetone powders prepared from M. phlei. The experiments were conducted under anaerobic conditions with methylene blue as the hydrogen acceptor. The pyruvate formed was isolated as the

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TABLE I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>L-Lactic acid, initial concentration*</th>
<th>Oxygen</th>
<th>CO₂</th>
<th>Acetic acid†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>9.7</td>
<td>9.4</td>
<td>8.1</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>12.5</td>
<td>12.4</td>
<td>11.5</td>
</tr>
</tbody>
</table>

In Experiment 1, each Warburg vessel contained 467 γ of partially purified enzyme, specific activity 75. The substrate used was L-lactic acid (zinc salt). In Experiment 2, each vessel contained 70 γ of purified enzyme, specific activity 196. In addition, each vessel contained 0.5 ml. of 0.1 M phosphate buffer, pH 6.0; gas, oxygen; final volume, 2.0 ml.; temperature, 37°. The reactions were permitted to go to completion. No residual lactic acid was found.

* Determined according to the method of Barker and Summerson (10).
† After steam distillation in the presence of MgSO₄, as described by Friedemann (11), and microtitration, the nature of the volatile acid was established by the paper chromatography method of Isherwood and Hanes (12). In addition, the acid gave a positive lanthanum-iodine reaction (13).
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2,4-dinitrophenylhydrazone. More recently Sutton (1, 2), using a purified preparation of the enzyme, established the fact that the reaction will not proceed when either methylene blue or neotetrazolium is used as hydrogen acceptors. This result, as well as those reported by Yamamura et al. (5, 6), who utilized a similar enzyme from *M. avium*, invalidates the earlier findings of Edson (3). Clarification as to the exact nature of the metabolic intermediate resulting from the activity of the purified lactic oxidative decarboxylase seemed to be in order.

**Table II**

Effect of Carbonyl Fixatives in Oxidative Decarboxylation of Lactate

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Oxygen utilization</th>
<th>CO₂ utilization</th>
<th>R. Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µl.</td>
<td>µl.</td>
<td></td>
</tr>
<tr>
<td>1†</td>
<td>None</td>
<td>169</td>
<td>167</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Hydroxylamine† (0.1 M)</td>
<td>76</td>
<td>85</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>&quot; (0.01 M)</td>
<td>159</td>
<td>158</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>&quot; (0.001 M)</td>
<td>170</td>
<td>168</td>
<td>0.99</td>
</tr>
<tr>
<td>2§</td>
<td>None</td>
<td>168</td>
<td>166</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Methone (0.009 M) (in 10% ethanol)</td>
<td>136</td>
<td>133</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>10% ethanol</td>
<td>147</td>
<td>142</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Enzyme preparation (specific activity 63), 137 γ of protein per Warburg vessel; 0.5 ml. of 0.4 M lithium lactate solution; 0.5 ml. of 0.1 M phosphate buffer, pH 5.7; gas, oxygen; final volume, 2.0 ml; temperature, 37°. All concentrations are given as final molarities.

* Oxygen utilization and CO₂ production for the second and third 10 minute periods of the reaction.
† Hydrazine sulfate gave similar results.
‡ Efficiency of hydroxylamine-pyruvate fixation demonstrated in the following manner (14): (a) 10 µ moles of pyruvate + 10 µ moles of H₂O₂ → 227 µl. of CO₂ (theory 224); (b) same as above + 0.04 M hydroxylamine (added at the same time as H₂O₂) → 9 µl. of CO₂. Reaction time, 5 minutes.
§ Methone, 5,5-dimethyl-1,3-cyclohexanedione.

A series of experiments in which carbonyl fixatives were added to the reaction mixture established the fact that no reactive carbonyl compound is formed (Table II). It will be seen that the fixatives do not influence the R. Q. values. A general poisoning of the enzyme is apparently responsible for the reduction in the manometric values. The failure to observe fixation strengthens the view that the enzyme in question has a dual function. It is also apparent that the expected oxidation product, pyruvate, if formed, does not dissociate from the enzyme surface and the decarboxylation of this intermediate probably occurs at or near the initial reaction site.

2 2,2-(p-Diphenylene)bis(3,5-diphenyl) tetrazolium ion.
Competitive Inhibition—The fact that carbonyl fixatives fail to inhibit the production of CO₂ strongly suggests that either the carbonyl compound formed is not dissociated from the enzyme surface or the dissociated intermediate does not contain a carbonyl group. The latter possibility suggested that glycidic acid (2,3-epoxypropionic acid) might be the intermediate product resulting from the activity of the lactic oxidative decarboxylase. Initially it was observed that the D,L-glycidate, as previously shown in the case of pyruvate (1), does not serve as a substrate, nor does it react with carbonyl reagents. The fact that the products of enzymatic reactions may serve as inhibitors (15) suggested that glycidate might act in a similar manner. When glycidate and lactate were incubated together in

![Graph showing competitive relationship of potassium glycidate and sodium pyruvate to lactate utilization. Standard assay conditions were used (1, 2). Curves are plotted according to the method of least squares. For additional data see Table II.](image)
the presence of the enzyme, a definite lowering of the rate of reduction of 2,6-dichlorophenol-indophenol was observed in the Beckman spectrophotometer at 620 mp.

More direct evidence of the competitive nature of glycidate was obtained with manometric experiments. The results of a typical series of experiments are presented in the form of a Lineweaver-Burk plot (16) (Fig. 1). Of even more interest are the results obtained with pyruvate under similar experimental conditions (Fig. 1 and Table III). As with glycidate, the

### Table III

**Michaelis Constants and Associated Values Based on Lineweaver-Burk Treatment of Competitive Inhibition Data**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Equation</th>
<th>$K_s$ or $K_i$ (mole per l.)</th>
<th>$V_{max}^i$ or $V_{max}^i$ (mole per min.)</th>
<th>$\frac{1}{K_s}$</th>
<th>$\frac{1}{K_i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$y = 0.043 \times 10^6 \times +2.6 \times 10^6$</td>
<td>$1.7 \times 10^{-2}$</td>
<td>$3.9 \times 10^{-7}$</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Glycidate, 0.025 M</td>
<td>$y = 0.17 \times 10^6 \times +2.0 \times 10^6$</td>
<td>$6.4 \times 10^{-3}$</td>
<td>$4.0 \times 10^{-7}$</td>
<td>157*</td>
<td></td>
</tr>
<tr>
<td>Glycidate, 0.0025 M</td>
<td>$y = 0.06 \times 10^6 \times +2.5 \times 10^6$</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$3.7 \times 10^{-7}$</td>
<td>900</td>
<td></td>
</tr>
</tbody>
</table>

Standard assay conditions were used (1, 2). All concentrations are expressed as final molarities. The enzyme preparation used had a specific activity of 149, and each Warburg vessel contained 48.8 mg of protein.

* If only L-glycidate is utilized, $K_i = 3.2 \times 10^{-7}$ and $1/K_i = 314$.

added pyruvate functions as a competitive inhibitor. The reciprocal of the calculated $K_s$ and $K_i$ values may be considered to be a measure of the relative affinity of the enzyme for the substrate and for the inhibitor (Table III) (17). The data indicate that the enzyme has the greatest affinity for pyruvate and the least for the substrate. It has also been established that β-chlorolactate can function as an inhibitor.

**Metabolic Intermediate**—The fact that pyruvate can act as a competitive inhibitor suggested that, if the enzyme were used in substrate quantities under anaerobic conditions, it might be possible to demonstrate the presence of this intermediate on the enzyme surface through the use of a
carbonyl fixative. 10 ml. of an enzyme preparation having a specific activity of 63 (2) were incubated with 2 ml. of 0.4 m lithium lactate solution under anaerobic conditions in a large Warburg vessel. On addition of the substrate from the side arm of the vessel, the yellow color associated with the enzyme immediately disappeared. A saturated solution of 2,4-dinitrophenylhydrazine in dilute HCl was added to the main chamber from a second side arm. The reaction mixture was extracted with 3 volumes of ether. The dried ether extract was dissolved in a chloroform-ethanol mixture (4:1) and extracted with 1 N Na₂CO₃ solution. The carbonate layer was adjusted to pH 4.5 with 1 N HCl solution and extracted with the chloroform-ethanol mixture. The extract was reduced to dryness under a vacuum and the residue dissolved in 0.1 M phosphate buffer, pH 7.2. The resulting solution was examined by descending paper strip chromatography according to the procedures described by Altmann et al. (18) and Cavallini et al. (19). The unknown 2,4-dinitrophenylhydrazone had the same mobility as a control sample of the 2,4-dinitrophenylhydrazone of pyruvate applied to the same strip.

Relation of Peroxide to Enzyme Activity

Evidence for Formation of Peroxide—The extensive researches of Keilin and Hartree (20, 21) and others (22) have established the fact that the oxidations catalyzed by flavin enzymes result in the formation of H₂O₂. The addition of catalase to these systems reduces the oxygen requirement to one-half as a result of the catalytic decomposition of H₂O₂. In certain of these systems, e.g. d-amino acid oxidase and glycolic acid oxidase, the H₂O₂ formed is utilized in a spontaneous non-enzymatic reaction. If catalase is added to either of these systems, the oxygen utilization is reduced to one-half and no CO₂ is produced. Moreover, the addition of ethanol plus catalase to a flavin enzyme results in a coupled oxidation and the formation of acetaldehyde (20, 23, 24).

In contrast to these flavin systems, the addition of catalase to the lactic oxidative decarboxylase reaction system does not diminish the amount of oxygen utilized or CO₂ produced (1, 3, 5). In addition no coupled oxidation of alcohol has been observed (6). It is of interest to note that the PABA red² reaction of Lipmann (26, 27) has been reported to be positive by Edson and Cousins (4). These investigators limited their observations to the measurement of oxygen utilization and a qualitative determination of peroxide formation based on the PABA red reaction. It seemed of interest to confirm this finding and to extend the experiments to include the production of CO₂. A reduction in the amount of CO₂ produced by the enzy-

² PABA, p-aminobenzoic acid. The exact chemical nature of the PABA red complex is not known. A suggestion as to the nature of the complex can be found in the investigations of Saunders and Mann (25).
matic reaction would be a quantitative measure of the extent of the peroxide reaction with PABA. It will be seen (Table IV) that no significant departure of the R. Q. values from the theoretical value of one was found. The amount of peroxide diverted by the peroxidase-PABA reaction is not sufficient to yield a manometrically measurable reduction in CO₂ production.

It is of interest that the addition of an excess of catalase to the peroxidase-lactate oxidative decarboxylase system blocks the formation of both PABA red and purpurogallin. Stern and Bird (28) have reported the suppression of catalase activity by peroxidase and its substrates.

### Table IV

<table>
<thead>
<tr>
<th>Test Group No.</th>
<th>Incubation</th>
<th>Oxygen utilization µl.</th>
<th>CO₂ utilization µl.</th>
<th>R. Q.</th>
<th>H₂O₂ µmoles</th>
<th>Found µmole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>91</td>
<td>89</td>
<td>0.98</td>
<td>4.1</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>190</td>
<td>187</td>
<td>1.02</td>
<td>8.5</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>291</td>
<td>287</td>
<td>0.99</td>
<td>13.0</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>388</td>
<td>385</td>
<td>0.99</td>
<td>17.3</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Each Warburg vessel contained 70 µg of enzyme preparation, specific activity 196, 0.5 ml. of 0.4 m lithium lactate solution, 0.5 ml. of 0.1 m phosphate buffer, pH 6.0, 0.5 ml. of 0.1 m PABA solution, 0.5 ml. of peroxidase solution (6850 units per ml.); gas, oxygen; final volume, 2.0 ml.; temperature, 37°.

* Micromoles of H₂O₂ possible based on oxygen utilization.
† Micromoles of H₂O₂ corresponding to the amount of PABA red formed. These values correspond to optical density values of the range 0.050 to 0.2 at 475 µm in the Beckman spectrophotometer.

### Evidence of Enzyme Purity

**Crystallization**—A recent modification in the method used for the purification of the crude lactic oxidative decarboxylase involves a change in the ammonium sulfate concentration used to precipitate the enzyme. The fraction containing the enzyme is at present collected at 0.45 saturation rather than at 0.5 saturation. This change results in a preparation with slightly greater activity. The specific activity (2) at this point in the purification varies between 50 and 65 units of activity per mg. of protein. The further purification of the enzyme by precipitation at essentially the isoelectric point of pH 5.1 (2) effectively separates the lactic oxidative decarboxylase from the principal contaminant, a hydroperoxidase. This procedure can be accomplished with less loss of activity by reducing the

Details for the isolation of this enzyme will be published elsewhere.
dialysis time to a few hours. At this point in the purification the specific activity varies between 150 and 180 units of activity per mg. of protein. In a single instance a preparation with a specific activity of 240 was obtained.

The bright yellow amorphous sediment resulting from the precipitation at pH 5.1 is suspended in a minimal volume of 0.1 M phosphate buffer, pH 6.0. It has been observed that the addition of a small amount of a saturated solution of ammonium sulfate (approximately 0.8 ml. for each ml. of buffer solution) aids in rendering the enzyme soluble. The process can be speeded by warming to 37°. The resulting supernatant fluid, after centrifugation, contains the enzyme. The initial crystallization of the enzyme can be accomplished by adjusting the solution to an ammonium sulfate concentration sufficient to produce an incipient turbidity. If slightly less salt is added than that amount required to produce visible turbidity, there will be less chance of an amorphous precipitate forming. The solution is chilled at 4-6° for 1 hour at which time a silky sheen characteristic of crystalline plates begins to appear. Complete crystallization, with a water-clear supernatant fluid, is apparent after 8 to 10 hours. Recrystallization is easily accomplished by removing the crystals by centrifugation and dissolving them in a minimal volume of buffer. The ammonium sulfate treatment is then repeated. Fig. 2 shows the yellow transparent plates obtained after three recrystallizations by the method described. The crystals are essentially non-birefringent and may be rendered soluble by warming to 37° and crystallized again by chilling to 4°.

Physical Characterization—Partially purified preparations of the enzyme have been examined in the Tiselius electrophoresis apparatus and the results reported (2). The preparations so examined were found to contain a single major component accompanied by a small minor component. The minor component was also observed on paper electrophoresis examination of these preparations. In barbiturate buffer at pH 8.6 and an ionic strength of 0.075, the minor component has the same mobility as a purified sample of the hydroperoxidase mentioned above. Paper electrophoretic and ultracentrifugal examinations indicate that the first crystallization of the enzyme removes essentially all of the contaminant.

The crystals, from which the photograph shown in Fig. 2 was made, were removed from the suspending medium by centrifugation and dissolved in pH 6.9 phosphate buffer having an ionic strength of 0.1. This solution was examined in the Spinco analytical centrifuge. The sedimentation pattern obtained is given in Fig. 3. This result demonstrates that the crystalline lactic oxidative decarboxylase produces but a single boundary on ultracentrifugation. A second examination of the same sample in the presence of substrate demonstrated that the yellow color of the pros-
Fig. 2. Crystalline lactic oxidative decarboxylase from *M. phlei*. Three times recrystallized material was suspended in 0.1 M phosphate buffer, pH 6.0. The images of certain crystals are blurred due to their movement through the suspending medium and the slow speed of the film used. The distinct rectangular crystals are actually end views of the crystalline plates. Photograph taken with a high dry objective (λ = 0.65, 50:1) and a 10X eyepiece of an "MEF" Reichert Universal camera microscope; 2150X.

Fig. 3. Sedimentation pattern obtained in a Spinco analytical ultracentrifuge. Three times recrystallized material was dissolved in phosphate buffer, pH 6.9, and ionic strength of 0.1. Frame 1 was taken immediately after the rotor attained speed of 59,780 r.p.m.
thetic group can be reduced to the colorless form without changing the sedimentation characteristics of the enzyme.

*Molecular Weight*—By using data obtained by measuring the absorption spectrum of the crystalline enzyme in its oxidized and reduced form, it has been possible to calculate an equivalent molecular weight. Assuming a molecular extinction coefficient for flavoproteins at 455 nm of $1.04 \times 10^7$ cm$^2$ X mole$^{-1}$ (29), the average equivalent molecular weight for several crystalline enzyme samples has been calculated to be 125,700.

The ultracentrifugal experiments provide values in the range between $12.7 \times 10^{-13}$ for the sedimentation constant. The diffusion constant was found to be $4.36 \times 10^{-7}$ cm$^2$ sec.$^{-1}$. Assuming a partial specific volume of 0.73, as recorded for flavin enzymes and similar proteins (30, 31, 32), it is possible to calculate a molecular weight of about 260,000. These molecular weight data suggest that the lactic oxidative decarboxylase has two prosthetic groups. It should be noted that a similar situation in regard to the equivalent molecular weight and that determined by ultracentrifugation has been reported by Keilin and Hartree for glucose oxidase (33). Determination of additional physicochemical properties of the crystalline enzyme is in progress.

**SUMMARY**

1. The lactic oxidative decarboxylase from *Mycobacterium phlei*, a riboflavin 5'-phosphate-containing enzyme, has been crystallized, and the homogeneity of the material established by two physicochemical methods, e.g. ultracentrifugation and electrophoresis.

2. A tentative molecular weight of 260,000 has been assigned the enzyme. The equivalent molecular weight, based on spectrophotometric data, has been calculated to be 125,700. These data suggest that the enzyme contains two prosthetic groups.

3. The enzyme has a dual function, *i.e.* oxidation and decarboxylation of lactate to acetate and CO$_2$. A single protein is responsible for both of these activities. The failure of the intermediate reaction products, *i.e.* pyruvate and H$_2$O$_2$, to dissociate from the enzyme surface and the further reaction of these substances under the influence of the enzyme to yield acetate and CO$_2$ differentiate this enzyme from the known flavin enzymes.

The author is particularly indebted to members of the Physicochemical Division for the ultracentrifugal determinations. The author wishes to thank Mr. William R. Cherry for taking the photomicrographs.

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