Induction of $D(-)$- and $L(+)$-Lactic Cytochrome c Reductase in Yeast*

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Aerobically grown bakers’ yeast has been shown to contain two different lactic cytochrome $c$ reductases, one of which is specific for $L(+) -$lactate (1) and the other of which is specific for $D(-)$-lactate (2). Neither of these two enzymes is present in anaerobically grown yeast, which contains instead a $D(-)$-lactic dehydrogenase that does not reduce cytochrome $c$. No $L(+-)$-lactic dehydrogenase is present in anaerobically grown bakers’ yeast. Slonimski et al. (3) have shown that amino acid analogues inhibit the development of several respiratory enzymes during the aerobic induction of anaerobically grown yeast, but that the development of $L(+-)$-lactic cytochrome $c$ reductase is not inhibited. They have suggested that anaerobic $D(-)$-lactic dehydrogenase is converted on induction into $L(+-)$-lactic cytochrome $c$ reductase (4).

The studies described in the present paper were designed to obtain more information about the interrelationship of the various enzymes during the induction process. The results confirm the observation of Slonimski et al., and show also that the development of $D(-)$-lactic cytochrome $c$ reductase is not inhibited by amino acid analogues.

Short communications on parts of this work have appeared elsewhere (5–7).

EXPERIMENTAL PROCEDURES

Materials

DPN and cytochrome $c$ (grade II from horse heart) were obtained from Sigma Chemical Company. Ergosterol, DL-norleucine, and DL-para-fluorophenylalanine were purchased from Nutritional Biochemicals Corporation. L-Malate, D-lactate, and L-lactate were obtained from the California Corporation for Biochemical Research. Nitrogen, containing less than 6 parts of oxygen per million, was kindly provided by Norsk Hydro-Elektrisk Kvælstoffaktieselskap, Oslo.

The calcium salts of $D$- and $L$-lactate (lots 440092 and 301142, respectively) were dissolved in water, and calcium was precipitated with phosphate at pH 7. The sample has been shown to contain 0.25% of the $D$ isomer (2).

Methods

$D$- and $L$-lactic cytochrome $c$ reductase as well as anaerobic $D$-lactic dehydrogenase were assayed in phosphate buffer, pH 7.1, $\mu$ 0.008, containing 0.001 M Versene (ethylenediaminetetraacetate) (2). Anaerobic $D$-lactic dehydrogenase was determined with ferricyanide ($5 \times 10^{-5} M$) and $2,6$-dichlorophenolindophenol ($5 \times 10^{-2} M$) as acceptors. The unit of enzymatic activity is defined as $\mu$eq of acceptor reduced per hour.

Cytochrome oxidase was assayed spectrophotometrically in 0.03 M phosphate buffer, pH 6.8. The concentration of reduced cytochrome $c$ was $4 \times 10^{-5} M$. Cytochrome $c$ was reduced with ascorbic acid. The amount of reducing agent employed was slightly less than that needed for complete reduction of cytochrome $c$. The unit of enzymatic activity is defined as $\mu$eq of ferrocytochrome $c$ oxidized per hour.

Malic dehydrogenase was determined spectrophotometrically in 0.1 M glycine buffer, pH 9.7. The concentration of DPN and L-malate were $2.5 \times 10^{-4}$ and $2 \times 10^{-4} M$, respectively.

For each of the enzymes mentioned above specific activity is defined as units per mg of protein.

Protein was measured by the biuret method (8). When lipoprotein complexes were present, 20 $\mu$l of 10% deoxycholate per 3 ml of reaction mixture were added, and the mixture was centrifuged before reading.

The cells were disrupted, as described previously, for the determination of $D$- and $L$-lactic cytochrome $c$ reductase in yeast grown aerobically (2).

Culture Techniques and Aeration of Resting Cells—Cultures of bakers’ yeast were kindly provided by De Norske Gjær & Spiritfabriker A/S, Oslo (2). Single cell colonies were selected, and cultures for inoculations were grown in standing Erlenmeyer flasks containing 50 ml of wet (10$^6$B). The various media were autoclaved for 20 minutes at 15 pounds pressure and left to cool under nitrogen. The inoculum size was 1 ml per liter, and the incubation time was 3 days at 28$^\circ$. Carbon dioxide escaped through a water trap. At the end of the incubation period carbon dioxide evolution was very slow.

The yeast was washed twice with distilled water at 2$^\circ$, and then suspended in 0.067 M phosphate buffer, pH 6.7, containing glucose, or glucose plus $D$-lactate (9, 10). Ten grams of centrifuged yeast were suspended in 100 ml of buffer. The aeration was carried out at room temperature in gas washing bottles provided with a cotton filter.

Medium—Five different media were used. They contained per liter: Medium I. Beet molasses, to give 15$B$; MgSO$_4$, 0.3 g; ammonium sulfate, 1.7 g; ergosterol, 20 mg; Tween 80, 5 ml; ethyl alcohol, 5 ml; at pH 4.5. Yield, 25 g (centrifuged yeast). pH after growth, 4.5.

Medium II. Glucose, 30 g; vitamins and salts as in medium 5.
of Slonimski (11), ergosterol, Tween 80, and alcohol as in Medium I; at pH 4.5. Yield, 4 g per liter. pH after growth, 2.6.

Medium IIb. Medium II + 3 g of D-lactate; at pH 4.8. Yield, 4 g. pH after growth, 4.4.

Medium III. Glucose, 54 g; Difco yeast extract, 10 g; ammonium sulfate, 1.2 g; KH₂PO₄, 1.0 g; ergosterol, 10 mg; Tween 80, 1 ml; ethanol, 2 g; at pH 5.4. Yield, 8 g. pH after growth, 4.4.

Medium IV. Wort to give 10°B; at pH 5.3. Heat-coagulative proteins were removed by centrifugation. pH after growth, 4.3. Yield, 6 g.

Evaluation of Methods. Lactic Dehydrogenase in Anaerobically Grown Yeast—The assay methods of D- and L-lactic cytochrome c reductase were examined in the following manner. Particular D-lactic cytochrome c reductase and soluble L-lactic cytochrome c reductase were isolated from bakers' yeast homogenate as described previously (2) and tested with 5 × 10⁻³ M L- and D-lactate, respectively. The rates of the reactions were too low to be measured. Since all D-lactic cytochrome c reductase was present in particulate form in yeast homogenates (2), and since practically all L-lactic cytochrome c reductase was obtained in the soluble fraction, it was concluded that L-lactic cytochrome c reductase did not affect the determination of D-lactic cytochrome c reductase, or vice versa.

Since D-lactic cytochrome c reductase does not reduce ferriyanide or 2,6-dichlorophenolindophenol (2), the reaction of these acceptors with D-lactate as substrate is a measure of anaerobic D-lactic dehydrogenase alone.

D- and L-lactic cytochrome c reductase were not present in detectable concentration in any of the five homogenates from yeast grown anaerobically. Anaerobic D-lactic dehydrogenase was located in the soluble fraction of the homogenates. Half-maximal velocity was obtained with 3 × 10⁻³ M D-lactate; the enzyme was practically saturated with 15 × 10⁻³ M of the substrate which was used in the assays.

The relative rates with ferriyanide and 2,6-dichlorophenolindophenol were 3:1. At high concentrations of the homogenate, the rate was not strictly proportional to the concentration. This is illustrated in Fig. 1. The deviation was too small to have any practical significance for the assay. Another factor, however, influenced the assay of anaerobic D-lactic dehydrogenase; in several instances in Culture I, anaerobic D-lactic dehydrogenase increased with time after homogenization. The results obtained with a homogenate after various periods of aeration are shown in Fig. 2; the increase with time was larger in the preparation from nonaerated cells than in the preparation from cells which had been aerated for 4 hours, and no increase was observed after 8 and 24 hours of aeration. In most homogenates the activity decreased significantly in the course of 24 hours at 3°, and no increase of the activity was observed. The supernatant of the heat-denatured homogenate did not inhibit the enzyme.

Yield and specific activity of anaerobic D-lactic dehydrogenase after various periods of stirring are shown in Table I. Maximal yield was obtained after 15 minutes. Thus, the enzyme was extracted from anaerobic yeast at the same rate as D- and L-lactic cytochrome c reductase from aerobically grown yeast (2).

The specific activity of anaerobic D-lactic dehydrogenase in homogenates of Cultures I, II, III, and IV were 32, 9, 8, 29, and 25, respectively. The activity was measured with ferriyanide as acceptor and 24 hours after homogenization. Occasionally, increase of activity with time was observed, but only in Culture I.

Lactic Dehydrogenase during Aeration of Resting Cells—Qualitatively, the effect of aeration on the development of D- and L-lactic cytochrome c reductase was the same in the five cultures. Initially, the D enzyme developed as rapidly, or more rapidly than the L enzyme. After a lag period, however, the concentration of the L enzyme was twice that of the D enzyme. This is illustrated in Figs. 3 and 4 for Cultures I and II, respectively. The apparent concentration of the anaerobic enzyme was less in Culture II than in Culture I, but D- and L-lactic cytochrome c reductase developed to a greater extent in Culture II than in Culture I. The concentrations of D- and L-lactic cytochrome c reductase in commercial bakers' yeast were 4 and 10, respectively (2). This is twice as much as was produced in the best adaptation experiments.

In Cultures I, III, and IV, the anaerobic D-lactic dehydrogenase decreased rapidly during aerobic induction. In Culture II,
however, no decrease of the concentration of anaerobic \( \text{n-lactic dehydrogenase} \) was observed.

A number of respiratory enzymes are induced during aeration of yeast grown anaerobically (11). The development of cyto-

**TABLE II**

Effect of \( p \)-fluorophenylalanine on induction of \( \text{D- and L-lactic cytochrome c reductase, cytochrome oxidase, and malic dehydrogenase} \)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>0 hr Control</th>
<th>+ ( p )-Fluorophenylalanine</th>
<th>7 hrs</th>
<th>+ ( p )-Fluorophenylalanine</th>
<th>22 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{n-Lactic cytochrome c reductase} )</td>
<td>0.0</td>
<td>0.39</td>
<td>0.91</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>( \text{L-Lactic cytochrome c reductase} )</td>
<td>0.0</td>
<td>0.43</td>
<td>0.71</td>
<td>1.78</td>
<td>3.12</td>
</tr>
<tr>
<td>( \text{Cytochrome oxidase} )</td>
<td>0.1</td>
<td>1.7</td>
<td>1.7</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>( \text{Malic dehydrogenase} )</td>
<td>31</td>
<td>36</td>
<td>35</td>
<td>103</td>
<td>78</td>
</tr>
</tbody>
</table>

The main pathway of induced enzyme synthesis in nondividing cells of yeast appears to involve the utilization of the internal free amino acids (13). Amino acid analogues will, when added to the cell suspension, enter the pool and suppress the synthesis of active enzymes. Thus, the inhibition of cytochrome oxidase and malic dehydrogenase by \( p \)-fluorophenylalanine and norleucine indicates that these enzymes are synthesized from the amino acid pool. Conversely, the fact that \( \text{D- and L-lactic cytochrome c reductase} \) escapes the inhibition suggests that they may be formed from complex precursors. In this connection it is in-
interesting that the lactic cytochrome c reductases are formed more rapidly than cytochrome oxidase and malic dehydrogenase in several of the cultures.

It is possible that anaerobic D(-)-lactic dehydrogenase is the precursor of D(-)-lactic cytochrome c reductase. The conversion would involve incorporation of a soluble or readily solubilized protein into the lipoprotein complex of respiratory particles. The fact that no apparent decrease of the anaerobic enzyme takes place during the formation of D(-)-lactic cytochrome c reductase in Culture II would seem to contradict this possibility. However, the determination of D(-)-lactic dehydrogenase is too imprecise to support this objection. The experiment illustrated in Fig. 2 indicates that the enzyme obtained from nonaerated cells may require time for full development. The lability of the enzyme may explain why this process is not observed in all cultures.

The relatively rapid induction of D(-)-lactic cytochrome c reductase compared with L(-)-lactic cytochrome c reductase seems to be a general phenomenon in stationary phase cells, independent of the medium used for growth. Enzymes oxidizing D-lactate have no obvious physiological function, and the possibility should be considered that lactate is not the natural substrate for this enzyme. In some respects the precursor-product relationship suggested by Slonimski would appear to be more reasonable for the conversion of one D-lactate-specific enzyme to another D-lactate-specific enzyme, rather than for the conversion of a L-specific enzyme to an L-specific one. The situation is too complex to arrive at definite conclusions.

SUMMARY

Bakers' yeast was grown anaerobically in five differing media, and suspensions of stationary phase cells were aerated in the absence of an added source of nitrogen. Anaerobic D(-)-lactic dehydrogenase, D(-)- and L(+)-lactic cytochrome c reductase, malic dehydrogenase, and cytochrome oxidase were determined in cell-free extracts.

Anaerobic D(-)-lactic dehydrogenase increased with time in some of the homogenates.

The enzymatic changes observed during aeration are summarized as follows. Anaerobic D(-)-lactic dehydrogenase decreases or remains constant, whereas D(-)- and L(+)-lactic cytochrome c reductase develop. A lag period is obtained in the induction of L(+)-lactic cytochrome c reductase. In several of the cultures D(-)- and L(+)-lactic cytochrome c reductase develop more rapidly than cytochrome oxidase and malic dehydrogenase. The induction of D(-)-lactic cytochrome c reductase is not inhibited by p-fluorophenylalanine or norleucine, L(+)-lactic cytochrome c reductase is stimulated, and malic dehydrogenase and cytochrome oxidase are inhibited by the amino acid analogues.

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REFERENCES
Induction of d(-)- and l(+)-Lactic Cytochrome c Reductase in Yeast
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