Anaerobic Formation of d-Lactate and Partial Purification and Characterization of a Pyruvate Reductase from *Chlamydomonas reinhardtii*¹

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ABSTRACT

D-Lactate accumulation in *Chlamydomonas reinhardtii* was dependent on anaerobic conditions. As much as 50% of the 14C after 2 minutes of photosynthetic 14CO2 fixation moved into D-lactate from sugar phosphates if the cells became anaerobic for short time periods. No lactate accumulated in the dark until the O2 concentration decreased to less than 0.1%. Lactate was determined to be of the D-configuration using stereo-specific lactate dehydrogenases. D-Lactate produced anaerobically by algae grown on 5% CO2 was only slowly metabolized aerobically in the light or dark, and in the dark, only a trace of the lactate was excreted.

A pyruvate reductase (D-lactate: diphenophosphoryl nucleotide oxidoreductase, EC 1.1.1.28) was partially purified 47-fold from *Chlamydomonas*. Because this enzyme catalyzes an essentially irreversible reaction in the direction of pyruvate reduction, it is considered to be a pyruvate reductase. The reductase activity in extracts of *Chlamydomonas* was 30 micromoles per hour per milligram chlorophyll. For the partially purified enzyme, the apparent Kₘ (pyruvate) was 0.5 millimolar, and the pH optimum was 7.0. Studies with cycloheximide and chloramphenicol indicated that the enzyme was constitutive in aerobic cells. Potassium phosphate stimulated the reductase, and high salt and diithiothreitol were required for stability. The enzyme demonstrated substrate inhibition and was inhibited by ATP. Pyruvate reductase was separated from a hydroxy-pyruvate reductase by gel filtration chromatography, indicating the presence of separate reductases for these two substrates in *Chlamydomonas*.

The anaerobic production of d-lactate by unicellular green algae was first noted in *Chlorella* by Warburg (31). D-Lactate has also been observed in various other algae (11, 14). Tolbert et al. (29) showed that if *Chlamydomonas* were pelleted by rapid centrifugation after 2 minutes of photosynthesis, 51% of the newly fixed 14CO₂ was found in lactate in cells which had been grown with air enriched with 5% CO₂ and 26% in cells which had been grown with air levels of CO₂. Despite the evidence for the formation of D-lactate, the metabolic role of this compound in algae is not known.

Studies of anaerobic metabolism in algae have concentrated on quantitative analyses of starch fermentation products formed after several hours of anaerobiosis. The results indicate that various fermentation pathways exist in algae, but most unicellular algae undergo some type of mixed acid fermentation (7, 16). The long-term (6 h) anaerobic starch fermentation products of *Chlamydomonas reinhardtii* include formate, acetate, ethanol, and smaller amounts of CO₂ and H₂. D-Lactate was observed in these long-term studies only at extreme pH values or when the pathway of formate formation was inhibited by hypophosphite (16). However, little is known as to what metabolic changes occur during the initial minutes of anaerobic conditions. The present report demonstrates the rapid accumulation of D-lactate in *C. reinhardtii* during short-term anaerobic experiments of a few seconds to 30 min. Because D-lactate accumulation has not been noted in 6-h periods of anaerobiosis, the possible involvement of D-lactate metabolism during anaerobiosis was also investigated. A preliminary accounting of the studies of D-lactate metabolism in *Chlamydomonas* has been published (12).

D-Lactate is found in yeast (9) and in bacteria. In *E. coli*, D-lactate serves as an energy source for membrane transport (13). D-Lactate is produced in *E. coli* by a cytosolic D-LDH and is subsequently oxidized by a membrane-bound D-LDH which can donate electrons to a bacterial membrane electron transport chain (13, 26). In unicellular algae, D-lactate can be oxidized slowly by a dehydrogenase, which has generally been named glycolate dehydrogenase (18), and is associated with the mitochondrial membrane (1). The electron acceptor or cofactors for this dehydrogenase are unknown, but there is some evidence that it is coupled to the mitochondrial electron transport system (3, 20). An enzyme activity which reduces pyruvate has been reported for various green algae (10, 12, 14, 24) and in this paper its partial purification and characterization are further reported. This enzyme catalyzes an essentially irreversible reaction in the direction of pyruvate reduction; this irreversibility is also characteristic of the cytosolic D-LDH from *E. coli* (27). To relate the enzyme nomenclature with the in situ activity, the soluble enzyme in *Chlamydomonas* will be called pyruvate reductase and the mitochondrial activity as D-lactate or glycolate dehydrogenase.

**MATERIALS AND METHODS**

**Algae.** *Chlamydomonas reinhardtii* (Dang.) UTEX 90, *Anabaena variabilis* UTEX B 337, and *Synechococcus leopoldiensis* UTEX 625 were from the R.C. Starr collection at the University of Texas, Austin. The F-60 mutant of *C. reinhardtii* was a gift from Dr. R.K. Togasaki, Indiana University.

Wildtype *Chlamydomonas* were grown at 23 to 25°C in a high phosphate medium with (NH₄)NO₃ at pH 6.8 (19). The F-60 mutant was grown at pH 7.3 in Tris-acetate-phosphate medium (8). The blue-green algae were grown at pH 8.5 in the medium

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² Abbreviations: LDH, lactate dehydrogenase; Ches, 2-(N-cyclohexylaminio)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Epps, N-(2-hydroxyethyl)piperazine-N'-2-propane sulfonic acid.

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C of Kratz and Myers (15) with the addition of 10 mM sodium
N-tris(hydroxymethyl)aminoethyl-2-aminoethanesulfonic acid.
The cells were aerated with either air (0.03% CO2) or with air enriched
with 5% CO2 in flat Erbach flasks and were continuously illu-
minated under 125 μE·m−2·s−1 from cool white fluorescent
tubes.

Cells were harvested after 3 to 4 d, near the end of the log
phase of growth by centrifuging at 900g and 4°C for 5 min. The
cells were washed once by resuspension in distilled H2O and
were recentrifuged in tared Corex tubes at 12,000g for 10 min.
The algal wet weight was recorded and the pelleted cells were
resuspended for photosynthetic experiments in 3 mM K-phos-
phate, pH 7.5, or 25 mM Heps at pH 7.5. The algal suspensions
were stored on ice and all photosynthetic experiments were done
within 1 h after harvest. Chl concentration was measured spec-
trophotometrically after acetone extraction.

14C-Labeling of Algae. After harvesting, suspensions of 2% or
5% (w/v) Chlamydomonas cells were prepared in 3 mM phos-
phate buffer, pH 7.5, for photosynthetic experiments. An aliquot
of this suspension was placed in a 2-cm diameter glass vial with
a water jacket so that the light was 20 cm from the vial. This
reaction vial was held in a plastic holder in a circulating
water bath at 25°C and the contents stirred by a magnetic bar.
The samples were preilluminated for 2 min by 1000 μE·m−2·s−1
of light from a projector. After this light adaptation, [14C]Na-
HCO3 was added to the suspension to a final concentration of
1 mM. When illumination times were longer than 5 min, more
[14C]NaHCO3 was added to 1 mM at 5-min intervals to ensure
a near saturating level of inorganic carbon for the cells. Aliquots
were removed at various time points and CO2 fixation stopped
by one of two methods. In one procedure designed to assay the
cellular components and the surrounding medium separately,
the cells were immediately separated from the suspension by
centrifugation for 5 s in an Eppendorf microfuge. The superna-
 tant was removed with a Pasteur pipette and added to an equal
volume of methanol. The pellet was resuspended in 50% meth-
anol to a volume equal to the original volume of the aliquot in
order to terminate enzymic reactions and to solubilize the cellu-
lar contents. In the other procedure, an aliquot of the photosyn-
thesizing cells was removed and directly added to methanol and
mixed, without cell separation from the medium.

To count radioactive content in these samples, 50 μl were added to
0.45 ml of 0.5 N acetic acid and allowed to sit for 3 h to release
unfixed 14C. Scintillation fluid (4.5 ml) was then added and
the nonvolatile radioactivity was determined in a liquid scintillation
spectrometer.

Two-Dimensional Paper Chromatography. Aliquots of the cell
and supernatant samples from 14C labeling experiments were
chromatographed for product identification (2). The radioactive
compounds were located by exposure to Kodak XAR-5 X-ray
film for about 3 weeks. To determine the percentage of label in
each compound, the spots were cut out of the chromatograms
and into approximately 5 mm2 pieces and placed into scintilla-
tion vials. Compounds were eluted off the paper by adding 1.5
ml of water to each vial, followed by gentle mixing for about 2
h. Scintillation fluid was then added and the samples were mixed
and the radioactivity counted.

Lactate was identified by cochromatography with a [14C]lactate
standard and with an unlabeled lactate standard. The unlabeled
carrier was located on the chromatograms by spraying with a
0.04% solution of bromoresol green in ethanol adjusted
to pH 7.0. Lactate was also identified by GC-MS of the trimeth-
lysilyl derivatives of the organic acid fraction from the algal cells.
In this case, identification was based upon both the retention
index and mass spectrum.

Products of 14CO2 Fixation by Chlamydomonas under Decreas-
ing O2 Concentrations. Cells grown with 5% CO2 were harvested
and used to prepare a 5% suspension (w/v) at pH 7.5 in a buffer
of 3 mM K-phosphate or 25 mM Heps (approximately 100–120
μM Chl/ml). An aliquot of this suspension was placed in a Rank
Brothers (Bottingham-Shire) O2 electrode chamber for continu-
ous monitoring of the O2 concentration. The cells were illu-
ninated with 800 μE·m−2·s−1 of light from a projector and [14C]Na-
HCO3 (0.25 mM/mmol) was added to a final concentration of
1 mM. The cells were allowed to photosynthesize for 2 to 4
min and then the light was turned off and the chamber covered
with aluminum foil. In the dark, the system became anaerobic
within 5 to 10 min depending on how high the O2 concentration
had reached during photosynthesis. Throughout the experiment,
200 μl samples were taken and immediately added to 500 μl of
methanol to terminate reactions. After each aliquot was re-
 moved, the chamber cap was adjusted to prevent formation of
air bubbles in the system. The samples were concentrated by
evaporation to about 100 μl and analyzed by paper chromatog-
raphy.

Determination of the Stereospecific Configuration of the Lac-
tate Produced by C. reinhardtii. Cells were grown with 5% CO2,
harvested at late log phase in 3 mM K-phosphate, pH 7.5, for
photosynthesis. [14C]NaHCO3 was added to the suspension
and photosynthesis was terminated 1 h and 15 min. After 30 min,
the cells were pelleted by centrifugation at 7700g for 5 min at room
temperature to produce anaerobic conditions. Reactions were
terminated by resuspending the pelleted cells in 5.8 N HClO4.
The sample was neutralized with KOH and centrifuged. Bacterial
NAD+:LDH (Lactobacillus leichmanii) and porcine heart l-LDH were
determined to determine the presence of either d- or l-lactate in the
resulting supernatant sample according to the method of Gawehn
and Bergmeyer (6). The reaction mixture contained 453 mM glycine,
352 mM hydroxylamine, 2.47 mM NAD. An aliquot of the
supernatant (1.1 ml) was added to 3.3 ml of assay buffer, and
0.5 ml of this was used for each assay. After mixing, the initial
absorbance at 340 nm was measured. The reaction was initiated
with the addition of d-LDH or l-LDH to a concentration of 60
units/ml (units were μmol of NADH reduced/min at 25°C). After
90 min at 25°C, the A at 340 nm was compared with a control
which contained no algal sample. No further increase in absorb-
ance was detected after this 90-min time period.

Formation of d-Lactate by the Pyruvate Reductase. An enzyme
fraction was prepared from 5% CO2-grown Chlamydomonas by
extraction in 50 mM K-phosphate, pH 7.0., containing 3% Tri-
ton, 1 mM DTT, and 5 mM EDTA for 40 min, followed by
centrifugation. The supernatant was used to prepare a 45 to 80%
saturated (NH4)2SO4 fraction. The protein was resuspended in
3 mM K-phosphate at pH 6.2 and used for pyruvate reductase
assays. No enzyme fraction was added to the blank, and the
control assay was run without pyruvate. The assay contained K-
phosphate at pH 7.0, 0.12 mM NADH, and 30 μl enzyme
fraction. The reaction was initiated with 2 mM pyruvate, incu-
bated for 90 min at 25°C, and then terminated by protein
precipitation with 5.8 N HClO4. After neutralization with KOH
and centrifugation to pellet the protein and KClO4, the super-
natant samples were used as substrates in assays with the stereo-
specific lactate dehydrogenases, as described above, to determine
the configuration of the lactate produced in the pyruvate reduc-
tase reaction.

Enzyme Assays. NADH-dependent pyruvate reductase was
measured by following the decrease in A at 340 nm. Units of
activity were μmol of NADH oxidized per min at 25°C. The
1-ml assay mixture contained 50 mM K-phosphate at pH 6.2 or
7.0, 0.12 mM NADH, and 2 mM potassium pyruvate. Enzyme
fraction (20–30 μl) was added to initiate the reaction. Assays
for hydroxyurepivate and glyoxylate reductase activities used 2 mM
hydroxyurepivate or 10 mM glyoxylate as substrates in place of
pyruvate. No NADH was oxidized in a control cuvette contain-
ing all components except substrate. The reverse reaction in the direction of lactate oxidation was run at pH 9.2 in 50 mM sodium pyrophosphate or at pH 9.4 in 25 mM Chex with 2.7 mM NAD, enzyme fraction, and 50 mM D- or L-lactate (Li salt). All assays were performed in duplicate or triplicate and average values are reported.

Partial Purification of the Pyruvate Reductase from *Chlamydomonas*. Protein solutions were maintained at 4°C throughout the purification steps. Protein concentrations were determined according to the Lowry procedure.

To prepare cell free extracts, harvested cells were resuspended in 3 volumes (w/v) of 50 mM K-phosphate or 25 mM Mops, pH 7.0, containing 1 mM DTT and 5 mM EDTA. The cells were lysed by two passes through a Yeda press (1,500 p.s.i. of N2 gas for 5 min). The solution was centrifuged at 15,000g for 15 min to remove unbroken cells and membrane material. All of the pyruvate reductase activity was in the resulting supernatant. In an alternate procedure, the harvested cells were resuspended in 3 volumes (w/v) of 0.3% sodium deoxycholate or in 50 mM K-phosphate at pH 7.0, containing 3% Triton X-100, 1 mM DTT, and 5 mM EDTA. The mixture was stirred for 40 min. Cellular debris was removed by centrifugation at 12,000g for 10 min and the supernatant decanted and used for subsequent assays. No activity was found in the pellet fraction.

Saturated (NH4)2SO4, adjusted to pH 7.0 with KOH, was added to the supernatant from the Yeda press extraction (0.67 ml/ml extract) to provide a final concentration of 40% saturation. After 30 min, the suspension was centrifuged at 17,000g for 15 min and the precipitate discarded. A saturated solution of (NH4)2SO4 was slowly added to the supernatant to 65% saturation (0.71 ml/ml supernatant). After 60 min, the suspension was centrifuged at 17,000g for 15 min and the supernatant was discarded. The pellet was dissolved in 25 mM K-phosphate at pH 7.0, containing 1 mM DTT and 5 mM EDTA. This phosphate buffer was used throughout the purification procedures unless otherwise noted.

The dissolved pellet from the previous step was applied to a Sephadex G-25 column (2.5 × 4.3 cm) which was equilibrated and eluted with the K-phosphate buffer. Fractions containing pyruvate reductase activity eluted in the void volume from the column and were pooled. The pooled fractions were loaded onto a column of Affi-Gel Blue, 100 to 200 mesh (2.0 × 5.0 cm), which had been equilibrated with the K-phosphate buffer. The column was washed with this buffer until the A<sub>280</sub> of the eluate was less than 0.03. The enzyme was eluted with a linear gradient of 0 to 1 M KCl in 50 mM K-phosphate, pH 7.0, containing 1 mM DTT and 5 mM EDTA. Pyruvate and hydroxypyruvate reductase activities eluted in a broad peak with the bulk of the protein at approximately 0.6 to 0.7 M KCl.

The fractions containing the reductase activities were pooled and concentrated to approximately 25% of the original volume with a 50-ml Amicon concentrator using a Diaflo PM-30 ultrafiltration membrane (43 mm) and 50 p.s.i. of N<sub>2</sub> gas. The concentrated fraction was loaded onto a Sephacryl S-300 (superfine) column (2.5 × 88 cm) which was equilibrated and eluted with the K-phosphate buffer containing 0.2 M KCl. The pyruvate reductase activity eluted after the bulk of the protein and before the main peak of hydroxypyruvate or glyoxylate reductase activity. The fractions containing the pyruvate reductase activity were pooled and used for subsequent characterization.

**Materials.** [14C]NaHCO<sub>3</sub> was from New England Nuclear. Enzyme grade (NH4)2SO4 was from the Schwarz/Mann Inc. DEAE-cellulose (DE-52), cellulose phosphate (P-11), and carboxymethyl cellulose (CM-23) were obtained from Whatman Chemical Separation Ltd. Affi-Gel Blue was from BioRad and Sephacryl S-300 (superfine) was from Pharmacia. Matrix Gel Red A and Diaflo PM-30 ultrafiltration membranes (43 mm) were from the Amicon corporation. All other biochemicals, buffers, and enzymes were purchased from Sigma Chemical Company. Common laboratory chemicals were of reagent grade and solutions were prepared in deionized, distilled H<sub>2</sub>O.

**RESULTS**

Anaerobic Formation of D-Lactate. The distribution of [14C] among the methanol-soluble, nonvolatile products of *C. reinhardtii* was examined at decreasing O<sub>2</sub> concentrations as monitored in an O<sub>2</sub> electrode (Fig. 1). The O<sub>2</sub> concentration decreased due to O<sub>2</sub> uptake by the algae from dark respiration. [14C]Lactate did not begin to significantly accumulate in the cells until the O<sub>2</sub> concentration was less than 0.9 μM (approximately 0.1%). The amount of lactate subsequently increased with time under anaerobic conditions for at least 20 min. By then, 15% of the fixed [14C] was in lactate. In confirming experiments, aliquots of photosynthesizing cells were removed from the light and incubated in 1.5-ml microfuge tubes with a large air space and with frequent stirring to maintain aerobic conditions in the dark for time periods of 10 s to 1 h, before adding them directly to methanol to stop reactions. No [14C]Lactate accumulated aerobically in the dark (data not shown).

In other experiments, samples were removed directly from a suspension of photosynthesizing cells after short times (10 s to 2 min) with [14C]HCO<sub>3</sub> and made anaerobic by rapidly centrifuging them into a tightly packed pellet in the dark. The percentage of the [14C] in lactate reached 51% during centrifugation after 10 s of photosynthesis (Table I). The cell suspension that was killed
Table I. The Effect of Killing Techniques on the \( ^{14} \text{C} \) Labeled Products of C. reinhardtii

Cells were allowed to photosynthesize in 3 mm potassium phosphate buffer, pH 7.5, with 1 mm \( ^{14} \text{C} \)NaHCO\(_3\) for 10 s. Reactions were then terminated by either adding the suspension of cells directly to methanol or by rapidly centrifuging the cells to separate them from the media and then resuspending them in methanol. Samples were concentrated and analyzed by paper chromatography.

<table>
<thead>
<tr>
<th>Products</th>
<th>Method Used to Terminate Reaction</th>
<th>% ( ^{14} \text{C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar phosphates</td>
<td>Methanol Centrifugation, directly then methanol</td>
<td>74 11 0.1 51</td>
</tr>
<tr>
<td>D-Lactate</td>
<td></td>
<td>3.6 15 0.5 3.0 0.9 7.0</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle intermediates</td>
<td></td>
<td>0.0 0.0 21 13</td>
</tr>
<tr>
<td>Aspartate and glutamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine, glycine, and serine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

by direct addition to methanol without prior centrifugation contained no lactate. The \( ^{14} \text{C} \)lactate was apparently derived from the newly labeled \( ^{14} \text{C} \)-sugar phosphate pools because the label in this fraction decreased dramatically during the centrifugation period.

Although \( ^{14} \text{C} \)lactate was formed in both of these short-term experiments, the difference in the amount of \( ^{14} \text{C} \)lactate accumulated may be explained in part by the difference in the time period to obtain anaerobic conditions. During the dark period in the experiment using the \( \text{O}_2 \) electrode, much of the label from the sugar phosphate pools immediately flowed into the tricarboxylic acid cycle intermediates (malate, succinate, citrate, and fumarate) and related amino acids (glutamate, aspartate, and glutamine). When the system became anaerobic, the sugar phosphate pool was further depleted as lactate began to accumulate in the cells. However, by this time in the dark period less \( ^{14} \text{C} \)-sugar phosphates were available to be converted to pyruvate and lactate via 3-PGA, as 45% of the original \( ^{14} \text{C} \) fixed was in the tricarboxylic acid cycle intermediates and related amino acids. This carbon could flow back to pyruvate and into lactate from malate by the malic enzyme reaction. Indeed, the percentage of \( ^{14} \text{C} \) in malate dropped slightly from 9% to 3% in the first 12 min of anaerobic conditions. However, this flow of carbon to lactate appears to be slower than that from the sugar phosphates to lactate via 3-P-glycerate and pyruvate, as evidenced by the large percentages of label seen in lactate in the experiment using rapid centrifugation in which the pelleted cells were presumably anaerobic for only 45 s before the reactions were terminated with methanol.

The anaerobic experiments performed in the \( \text{O}_2 \) electrode or by centrifugation were done with wildtype \textit{Chlamydomonas} cells which had been grown photautotrophically with 5% \( \text{CO}_2 \). Similar experiments were done with cells grown on air levels of \( \text{CO}_2 \) or in a Tris-acetate phosphate medium in the light, and with the acetate-requiring F-60 mutant of \textit{Chlamydomonas}. In all cases, the cells produced lactate during short times under anaerobic conditions, which was formed from newly fixed \( ^{14} \text{C} \), and all cells were shown to have levels of pyruvate reductase activity (30 \( \mu \text{mol NADH oxidized/h-mg Chl} \) comparable to that of the wildtype cells grown photautotrophically (see below). The bluegreen algae, \textit{A. variabilis} and \textit{S. lepoleimis}, however, had only 2 to 4 \( \mu \text{mol NADH oxidized/h-mg Chl} \) of the NADH-dependent pyruvate reductase activity and no NADPH-dependent activity. Correspondingly, these blue-green algae accumulated only 1.1% of \( ^{14} \text{C} \) in lactate during anaerobic conditions.

When the \( \text{CO}_2 \)-grown \textit{Chlamydomonas} were returned to the light after lactate had accumulated anaerobically in the cells, very little turnover of the lactate was observed. The D-lactate remained in the cells and was not excreted as is glycolate. A small amount of the \( ^{14} \text{C} \) from lactate moved into the sugar phosphates after 1 h. If unlabeled NaHCO\(_3\) was added as a chase, the specific activity of the label in lactate was not diluted out after several minutes in the light, as if lactate synthesis or turnover was not occurring aerobically. If the cells were returned to aerobic conditions in the dark after lactate accumulation, only a small amount of the label in lactate flowed into the tricarboxylic acid cycle, and only a trace of lactate, tricarboxylic acid cycle intermediates, and sugar phosphates leaked into the media. Preliminary data with air-grown \textit{Chlamydomonas} indicate that the lactate which accumulated anaerobically in these cells turned over more rapidly than in the \( \text{CO}_2 \)-grown algae. This might be expected since air-grown \textit{Chlamydomonas} have higher levels of glycolate dehydrogenase (and hence D-LDH activity) than do cells grown under high levels of \( \text{CO}_2 \) (18).

**Determination of the Configuration of the Lactate Produced by \textit{Chlamydomonas}.** The lactate produced by the algae in vivo was oxidized preferentially by the stereospecific D-LDH (Table IIa). It was also shown that the lactate formed during assays of the pyruvate reductase reacted preferentially with the D-LDH (Table IIb). The reason for the lack of apparent absolute specificity of the stereospecific LDHs for the appropriate isomer of lactate was discussed above.

**Table II. Determination of the Stereospecific Configuration of the Lactate Produced by C. reinhardtii (a) and Formation of D-Lactate by the Pyruvate Reductase from \textit{Chlamydomonas} (b)**

Sample preparation and assays were performed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \text{D-LDH Activity} ) ( \text{L-LDH Activity} ) ( \text{ratio} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>100 ( \mu \text{mol D-lactate} )</td>
<td>1.062</td>
</tr>
<tr>
<td>100 ( \mu \text{mol L-lactate} )</td>
<td>0.058</td>
</tr>
<tr>
<td>Algal sample</td>
<td>0.418</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>Blank (no enzyme fraction)</td>
<td>0</td>
</tr>
<tr>
<td>Control (no pyruvate)</td>
<td>0</td>
</tr>
<tr>
<td>Product of pyruvate reductase assay</td>
<td>0.150</td>
</tr>
</tbody>
</table>

**Table III. Purification Procedure for the Pyruvate Reductase from C. reinhardtii**

Procedures are described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \text{mol/min-mg protein} )</td>
<td>( -\text{fold} )</td>
<td>( % )</td>
</tr>
<tr>
<td>Yeda press extract</td>
<td>0.015</td>
<td>1.0</td>
</tr>
<tr>
<td>Pellet from 40-65% saturated (( \text{NH}_4 )(_2)\text{SO}_4) precipitation</td>
<td>0.050</td>
<td>3.3</td>
</tr>
<tr>
<td>Eluate from G-25 column</td>
<td>0.060</td>
<td>4.0</td>
</tr>
<tr>
<td>Affi-Gel Blue affinity column and concentra-</td>
<td>0.22</td>
<td>15</td>
</tr>
<tr>
<td>tion through Amicon filter PM-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate from Sephacryl</td>
<td>0.71</td>
<td>47</td>
</tr>
<tr>
<td>S-300 column</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
lactate (Table IIa) is uncertain. The d-lactate used reportedly contained 0.01% of the L-isomer (Sigma) which does not account for the 20% contamination observed. Also, activity (18%) was observed with the algal samples and the L-LDH. No contamination of the *Chlamydomonas* was observed when aliquots of the culture were inoculated on agar plates supplemented with acetate and algal medium.

**Partial Purification of the NADH:Pyruvate Reductase from Chlamydomonas.** The steps used to partially purify the pyruvate reductase from *C. reinhardtii* are detailed "Materials and Methods" and a summary of the procedure is presented in Table III. After cell breakage through a Yeda press, the crude enzyme extract was used to prepare a (NH₄)₂SO₄ cut of 40 to 65% saturation. (NH₄)₂SO₄ inhibited the pyruvate reductase activity, but this inhibition was reversed by desalting the enzyme fraction through a Sephadex G-25 column. The enzyme was not stable in (NH₄)₂SO₄ and rapid desalting resulted in a higher yield of activity.

Before chromatography on an Affi-Gel Blue affinity column, the pyruvate reductase activity was not stable at 4°C. However, after elution from the dye-ligand column with a KCl gradient, the preparation was stable and retained 70% of the original activity after storage at 4°C in phosphate buffer, pH 7.0, with 0.4 to 0.6 M KCl for 1 month. Dialysis of this fraction against 25 mM K-phosphate buffer at pH 7.0 after elution from the dye-ligand column resulted in a 50% decrease in the pyruvate reductase activity within a few hours, suggesting that the high concentration of salt was necessary to stabilize the pyruvate reductase.

The last step towards purification of the enzyme was gel filtration chromatography on a Sephacryl S-300 column (Fig. 2). KCl (0.2 M) was included in the elution buffer, since high salt was apparently required for enzyme stability. This step was the first in which the pyruvate reductase activity was separated from the main peak of the hydroxypyruvate reductase activity. Previously, Gruber *et al.* (10) concluded that separate reductases for pyruvate and hydroxypyruvate existed in Chlorella from studies based on differences in enzyme stability. In *Chlamydomonas*, there are also similar differences in the stability of these two enzymes. For example, 50% of the original hydroxypyruvate reductase activity in a crude extract remained after 52 h of storage at 4°C, whereas the pyruvate reductase activity in the same extract declined to only 13% of the original activity. Consistent with hydroxypyruvate reductase from *C. reinhardtii* (28), this enzyme in *Chlamydomonas* catalyzes the reduction of both hydroxypyruvate and glyoxylate; however, the apparent *Kₘ* (glyoxylate) for this activity in crude extracts from *Chlamydomonas* was unphysiologically high at 8 mM.

Some hydroxypyruvate reductase activity coeluted with the peak of pyruvate reductase activity (Fig. 2). Since hydroxypyruvate is a good substrate for mammalian L-LDH which forms L-glyceraldehyde (17), it is possible that the algal pyruvate reductase could also use hydroxypyruvate. Because the product of the algal pyruvate reductase is d-lactate, the product from hydroxypyruvate reduction should be d-glyceraldehyde, which is the isomer found in algae and higher plants. The hydroxypyruvate reductases from higher plants (25) or from *Chlamydomonas* (data not shown) do not catalyze a dehydrogenase reaction with d-or L-lactate as a substrate and, in *Chlamydomonas*, no pyruvate reductase activity was associated with the main peak of hydroxypyruvate reductase activity after gel filtration chromatography (Fig. 2).

Further attempts to purify the pyruvate reductase from *Chlamydomonas* have, as of yet, been unsuccessful. The enzyme does not bind to DEAE-cellulose or DEAE-Sephadex (pH 6.2-8.0), TEAE-cellulose (pH 7.0-8.0), calcium-phosphate gel (pH 6.4 or 7.0), cellulose phosphate (pH 7.0), carboxymethyl cellulose (pH 7.0), or oxamate-agarose or red dye-ligand affinity columns. These various columns were tested with crude algal extracts, with enzyme fraction after the (NH₄)₂SO₄ cut and desalting, and with the pooled fractions after elution from the Affi-gel blue column. Elution of the reductase activities from the Affi-gel blue column with NADH or NADPH (1 mM) resulted in poor recoveries, and the activity only slowly eluted from the column in a volume of several hundred ml.

**Properties of the Partially Purified Pyruvate Reductase from Chlamydomonas.** The pH optimum for the activity in the direction of pyruvate reduction was maximal around pH 7.0 (Fig. 3). At pH 7.0, the reductase activity in extracts of *Chlamydomonas* was 30 ± 4 (n = 4) µmol NADH oxidized/h·mg Chl, which is to be compared with the maximal *Chlamydomonas* photosynthetic rate of approximately 120 to 160 µmol CO₂ fixed/h·mg Chl. The reductase activity was dependent on NADH; no activity was observed with equivalent amounts of NADPH.

The activity in the direction of d-lactate oxidation was tested in the pH range between 6.2 and 9.6 and in all cases was extremely low. A maximum rate of only 1 to 3% of the pyruvate...
reduction rate was observed at pH 9.4. No oxidation of L-lactate occurred. In Chlorella, \( \text{D-lactate} \) oxidation has also been reported to be low compared to the reductase reaction and the apparent \( K_m \) for \( \text{D-lactate} \) was 40 mM (10). Apparently, \( \text{D-lactate} \) oxidation by this enzyme does not occur under physiological conditions in algae and, therefore, it seems appropriate to name the activity as a pyruvate reductase rather than a \( \text{D-lactate} \) dehydrogenase.

The apparent \( K_m \) (pyruvate) for the pyruvate reductase activity in the crude homogenate was 1.1 mM (Fig. 4b); however, after partial purification of the enzyme, this value was 0.5 ± 0.1 mM (Fig. 4a). The higher apparent \( K_m \) (pyruvate) for activity in crude extracts suggests that there is an inhibitor(s) of the enzyme in the algal extract. Further evidence for this was that the pyruvate reductase assay was not linear with respect to the volume of crude extract added; activity was inhibited if more than 50 \( \mu \)l of extract were included in the assay. However, after the enzyme had been partially purified, the assay was linear with respect to the volume of enzyme fraction added.

Apparent substrate inhibition occurred at pyruvate concentrations above 2 mM for pyruvate reductase in crude homogenates (Fig. 4b). Similar inhibition was observed with the partially purified enzyme at pH values less than 6.8 (data not shown). Substrate inhibition by pyruvate has been observed for both \( \text{D-} \) and \( \text{L-} \) LDHs from other sources and has been attributed to the formation of an abortive ternary complex among enzyme, pyruvate, and NAD (5, 27).

Evidence for the Constitutive Nature of the Reductase. \( \text{D-Lactate} \) production was observed immediately after \( \text{Chlamydomonas} \) cells became anaerobic (Fig. 1). In the rapid centrifugation experiments, the algae were presumably anaerobic for no more than 45 s in the pellet before they were resuspended in methanol. However, lactate had already accumulated significantly in these cells (Table I). This evidence strongly suggests that the pyruvate reductase was present constitutively in the aerobic cells. However, during harvesting of the algae, the cells had been pelleted during centrifugation after two washings. During this time of temporary anaerobic conditions, synthesis of the pyruvate reductase could have been induced. To evaluate this possibility, \( \text{Chlamydomonas} \) cells in the log phase of growth were incubated with either 1 mM (281 \( \mu \)g/ml) cycloheximide or 2.3 mM (743 \( \mu \)g/ml) chloramphenicol for 30 min prior to harvesting. The cells were then harvested as usual and a crude extract prepared. In the extracts from both cycloheximide- and chloramphenicol-treated cells, the pyruvate reductase activity was the same as that from untreated cells. A recent paper of Roessler and Lien (23) showed that cycloheximide at a level of 15 \( \mu \)g/ml did enter \( \text{Chlamydomonas} \) cells and inhibited protein synthesis by greater than 98% as measured by \([^{14}C] \)arginine incorporation into protein. They also showed that the induction of hydrogenase, which occurs under anaerobic conditions, was inhibited 70% by this level of cycloheximide, although chloramphenicol at 500 \( \mu \)g/ml was less effective (23). This data indicates that the pyruvate reductase is constitutive in \( \text{C. reinhardtii} \).

Inhibition of the Pyruvate Reductase by Various Compounds. ATP inhibits the L-LDH from higher plants (4) or mammalian sources (5). The ATP inhibition was noncompetitive with respect to pyruvate and competitive with respect to NADH. The partially purified pyruvate reductase from \( \text{Chlamydomonas} \) was inhibited by ATP (Table IV) at \( K_m \) concentrations of pyruvate and saturating levels of NADH, and this inhibition was more severe at lower levels of NADH.

Although oxamate inhibits LDH from various sources (5), it inhibited the pyruvate reductase activity of \( \text{Chlamydomonas} \) only at concentrations greater than 1 mM (Table IV). Oxalate was also a poor inhibitor of this activity. A slight stimulation by cyanide was noted for the pyruvate reductase activity from \( \text{Chlamydomonas} \). EDTA was also slightly stimulatory. o-Phenanthroline did not significantly inhibit the pyruvate reductase activity (Table IV). The LDH of yeast (9) and the mammalian \( \text{D-}\alpha\text{-hydroxy acid dehydrogenase} \) (30) are zinc-requiring enzymes and are inhibited by oxalate, EDTA, o-phenanthroline, and cyanide, all of which reportedly interact with the essential zinc moiety. There is also a report of a zinc-dependent \( \text{D-LDH} \) in \( \text{Euglena gracilis} \) (21). None of these compounds inhibited the pyruvate reductase reported here, and therefore, it seems unlikely that this enzyme from \( \text{Chlamydomonas} \) requires a zinc moiety.

In \( \text{E. coli} \), the enzymes involved in the synthesis and oxidation of \( \text{D-lactate} \) have labile sulfhydryl groups which must be in a reduced state for enzyme activity (13, 26). The pyruvate reductase activity from \( \text{Chlamydomonas} \) was rapidly lost in the absence of sulfhydryl reagents. To evaluate the effects of these reagents, cells were grown to the log phase of growth and then labeled with [\( ^{14} \text{C} \)]arginine. After harvesting, these cells were incubated either in buffer or buffer containing various concentrations of reducing agents (Table IV). In this manner, it was possible to show the effects of these reagents on the specific activity of the enzyme. The data indicate that the reductase activity was not affected by treatment with the reducing agents.

Table IV. Effects Of Various Compounds on the Pyruvate Reductase Activity from \( \text{C. reinhardtii} \)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Per Cent of Control Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>%</td>
</tr>
<tr>
<td>ATP</td>
<td>1</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>1*</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Oxamate</td>
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<td>92</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Oxalate</td>
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<tr>
<td></td>
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<td>KCN</td>
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<tr>
<td>o-Phenanthroline</td>
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<td>78</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>120</td>
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</tbody>
</table>
D-LACTATE FORMATION AND PYRUVATE REDUCTASE IN CHLAMYDOMONAS

D-LACTATE FORMATION AND PYRUVATE REDUCTASE IN CHLAMYDOMONAS

sence of DTT or β-mercaptoethanol. This activity could be partially restored with addition of sulfhydryl reagents (data not shown). Gruber et al. (10) showed that in crude extracts prepared from Chlorella pyrenoidosa without added sulfhydryl reagents, the pyruvate reductase activity decreased by greater than 90% within 90 min at 4°C.

Effect of Potassium Phosphate on the Pyruvate Reductase. Addition of K-phosphate to cell extracts of Chlamydomonas resulted in stimulation of the pyruvate reductase activity. If crude extracts were prepared in 25 mm Mops buffer at pH 7.3, only low activity was observed. This activity increased with addition of increasing amounts of K-phosphate up to 0.2 m, after which inhibition was observed (data not shown). The effect of this activation was even greater at lower pH values (Fig. 5). The specificity of this activation has not yet been determined.

DISCUSSION

The pyruvate reductase in C. reinhardtii catalyzes d-lactate formation in an irreversible reaction. The lactate only accumulates in the cells during anaerobic conditions, suggesting that the enzyme functions during anaerobic metabolism to reoxidize NADH. A second explanation for anaerobic lactate accumulation may be that if reoxidation of the lactate was coupled to mitochondrial respiration in Chlamydomonas, this process would not occur under anaerobic conditions. In Chlamydomonas, limited D-lactate or glycolate dehydrogenase activity is associated with the mitochondrial membrane (1, 18). The electron carriers for this dehydrogenase reaction are unknown, but may be coupled to mitochondrial respiration (3, 20). Once lactate was made by Chlamydomonas cells grown on 5% CO2, it was turned over only very slowly in the light or dark. This is consistent with the pyruvate reductase being an essentially irreversible enzyme. Also, the algal glycolate or D-LDH is present only in trace levels in Chlamydomonas grown with high CO2 (18). Air-grown cells have higher levels of the glycolate dehydrogenase or D-LDH and would be expected to have a more rapid rate of metabolism of the accumulated D-lactate. Preliminary experiments indicated that this was the case (DW Husic and NE Tolbert, unpublished data). The cellular effects of the accumulation of this lactate are unknown.

In E. coli, D-lactate is produced by a cytosolic D-LDH (13, 26) which, like the pyruvate reductase in Chlamydomonas, catalyzes an irreversible reaction in the direction of pyruvate reduction (27). The lactate is then oxidized by a membrane-bound D-LDH coupled to the bacterial electron transport system to provide energy for the uptake of nutrients (13).

In higher plants, accumulation of L-lactate is limited by aerobic conditions which promote NADH oxidation by the mitochondria. In the light, high levels of ATP and P-enolpyruvate may inhibit glycolysis and have been shown to inhibit the L-LDH of higher plants (4). Under anaerobic conditions for germinating seeds and roots, L-lactate production can occur, subsequently causing a decrease in cellular pH (22). Since low cellular pH values are undesirable, overproduction of lactate under these conditions must also be controlled. This is accomplished in two ways (4). At slightly acidic pH values, NADH and ATP are potent and cooperative inhibitors of the L-LDH whereas at alkaline pH, ATP only slightly inhibits the enzyme and the inhibition is competitive with respect to NADH. Second, under anaerobic conditions, the decrease in pH approaches the pH optimum of pyruvate decarboxylase which decreases the amount of pyruvate available to be converted to lactate. Once the pyruvate decarboxylase is activated, the cells begin to produce ethanol instead of lactate (22). Because of this control mechanism and the ability to regenerate NADH without acid production, plants are able to tolerate anaerobic conditions for much longer time periods than animal cells.

It is not known if this same sort of control mechanism exists in unicellular green algae, but some differences have already been noted. Primarily, the D-isomer of lactate is formed as compared to L-lactate in higher plants. D-Lactate is found in other lower forms of life such as yeast and bacteria, suggesting an evolutionary difference for the formation of either D- or L-lactate in organisms. In Chlamydomonas, the main products of long term anaerobic fermentation are formate and acetate (7, 16) rather than ethanol which is produced in anaerobic tissue of many higher plants. Finally, the pyruvate reductase reported here catalyzes an essentially irreversible reaction in the direction of pyruvate reduction; whereas the L-LDH of higher plants catalyzes reversible reactions (4).

In long term studies (1–6 h) of the anaerobic fermentation products of starch in Chlamydomonas, significant D-lactate accumulation has not been previously observed (7, 16). The lactate we observed in Chlamydomonas cells was labeled with newly fixed 14C from the sugar phosphate pools during short time periods under anaerobic conditions. It is suggested that during the initial minutes of anaerobic conditions, the glycolytic pathway is active to produce energy for the cell. Reducing equivalents (NADH) formed in the glycolytic reactions could be reoxidized by the pyruvate reductase. ATP is required to activate the glycosyl units of starch for further metabolism to occur (16). After initiation of starch fermentation, the subsequent metabolism is apparently switched from D-lactate formation to formate formation as was observed in the long-term anaerobic experiments. D-Lactate formation was only observed in long-term fermentation experiments when starch degradation to formate was blocked with hypophosphite, an inhibitor of the pyruvate-formate lyase reaction (16). How the flow of carbon may be switched from

Fig. 5. Activation of the pyruvate reductase in crude extracts of C. reinhardtii by K-phosphate. The crude extract was prepared by breaking the cells in 3 volumes (w/v) of 50 mm Mops buffer, pH 7.0, containing 1 mm DTT and 5 mm EDTA. The suspension was centrifuged at 35,000 g for 15 min, and an aliquot of the resulting supernatant was added to 50 mm buffers: acetic acid (pH 4.9); Mes (pH 5.8, 6.4, 6.7); Mops (pH 7.8); Epps (pH 8.5). Phosphate, when included in the assays, was adjusted to the appropriate pH with KOH and added to give an approximate concentration of HPO42− of 5 mm per assay. Endogenous phosphate was determined to be 40.2 μg Pi per assay.
initial d-lactate formation to the formate pathway with the onset of starch breakdown is unknown.

Since the pyruvate reductase activity is apparently constitutive in the algal cells, it is interesting to speculate on its regulation in vivo. Under aerobic conditions, NADH might be limiting and ATP levels high. It was shown that the enzyme was inhibited by ATP. Once the cells become anaerobic, the ATP levels should drop and NADH would become more readily available for pyruvate reduction as it can no longer be oxidized by the mitochondria. Another consideration is that during aerobic conditions, the high Km (pyruvate) of the reductase may channel pyruvate towards other pathways. It is possible that, under anaerobic conditions, pyruvate levels may begin to increase and the pyruvate reductase would then become a significant reaction and lead to the observed accumulation of d-lactate. [14C]Pyruvate is never a significant product of the photosynthetic labeling experiments (0–2% of the acid stable counts), and in the anaerobic experiments, pyruvate accounts for only 1 to 3% of the fixed 14C. Finally, phosphate, as well as the redox state of possible essential sulphydryls on the pyruvate reductase, may also play a role in the regulation of this activity.

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LITERATURE CITED