JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1562–1566
0021-9193/83/031562-05$02.00/0

Vol. 153, No. 3

Identification of Two Distinct Lactate Dehydrogenases in *Rhodospirillum rubrum*

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Received 1 November 1982/Accepted 14 December 1982

The activities of pyridine nucleotide-independent D- and L-lactate dehydrogenases were detected in membranes from *Rhodospirillum rubrum* grown under aerobic and phototrophic conditions. Crossed immunoelectrophoretic analysis revealed two antigenically distinct enzymes that were further distinguished by specificity for D- and L-stereoisomers of lactate and by the sensitivity of the D-lactate dehydrogenase to inhibition by oxamate and oxalate.

Rhodospirillum rubrum is a facultative phototroph. In the presence of sufficient levels of oxygen, this bacterium carries out oxidative, chemotrophic metabolism. If the level of oxygen is reduced, *R. rubrum* forms a specialized intracytoplasmic membrane (12), the chromatophore membrane (17), in which the photochemical components are located. A by-product of phototrophic metabolism in *R. rubrum* is molecular hydrogen (8, 18). Hydrogen has been suggested as an alternative fuel to replace fossil hydrocarbons (16), and the photobiological production of hydrogen by photosynthetic bacteria has been proposed as a means of solar energy conversion (24). Hillmer and Gest (10) have found that lactate is an efficient carbon source for hydrogen production during phototrophic growth of the related organism *Rhodopseudomonas capsulata*. Zürrer and Bachofen (26) have further demonstrated the feasibility of using *R. rubrum* cultures to produce hydrogen from lactic acid and lactic acid-containing materials, such as yogurt waste and whey. Unfortunately, little is known about the enzymes involved in the oxidation of lactate by *R. rubrum*. In this communication, we report that both L- and D-lactate dehydrogenase (lactate:phenazine methosulfate [PMS] oxidoreductase) activities are present in *R. rubrum* membranes. Crossed immunoelectrophoresis (CIE) was used to demonstrate that these activities are the result of two unique enzymes.

(This work was presented in part at the 13th International Congress of Microbiology, Boston, Mass., August 1982.)

R. rubrum S1 was grown in the medium of Ormerod et al. (18). For phototrophic growth, 0.6% (wt/vol) L-glutamate was included as both the carbon and nitrogen source. One-liter stock bottles were completely filled and sparged with 95% N_2–5% CO_2 before incubation at 30°C at approximately 2,160 lx. The medium prepared for chemotrophic growth contained 0.1% (wt/vol) L-glutamate and 0.6% (wt/vol) malate. The 750-ml cultures were incubated at 30°C in 2.8-liter Fernbach flasks and aerated by shaking at 300 rpm in a gyratory shaker. Bacterial growth was monitored by measurements of optical density at 680 nm on a Beckman DU spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.; modified by Update Instruments, Madison, Wis.).

Phototrophic cultures were harvested in late exponential growth, and chemotrophic cultures were harvested in mid-exponential growth. Cells were washed twice in 10 mM Tris-hydrochloride buffer (pH 7.5) and broken by two passages through a French pressure cell at 16,000 lb/in^2. After large debris was removed by centrifugation at 2,500 rpm for 10 min, membranes were recovered from the supernatant fluid by centrifugation for 90 min at 50,000 rpm (226,000 × *g*). Membranes were suspended in 10 mM Tris-hydrochloride buffer and stored at −70°C until analysis.

Membrane fractions were assayed for lactate dehydrogenase (LDH) activities spectrophotometrically in a coupled 2,6-dichlorophenol indophenol (DCIP)-PMS assay. The standard reaction mixture contained 5 mM potassium phosphate buffer (pH 7.5), 0.25 mM KCN, 0.033 mM DCIP, 2.0 mM PMS, rate-limiting amounts of enzyme, and either 20 mM D-lactate or 160 mM L-lactate. The activity of LDH was calculated from the initial rate of decrease in the absorbance of DCIP at 600 nm, corrected for the endogenous rate determined in the absence of lactate. The observed rate was directly related to the amount of enzyme added. One unit of enzyme is that amount which catalyzes the reduction of 1 μmol of DCIP per min.

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Membrane proteins were extracted by incubation for 1 h at 0°C with Triton X-100 (detergent/protein ratio, 1). Incubation was in the dark to avoid photoinactivation (15; M. L. P. Collins and C. A. Norton, submitted for publication). The insoluble residue was separated from the detergent extract either by centrifugation at 35,000 rpm (111,000 × *g*) for 1 h or by sedimentation in a Beckman Airfuge at 28 lb/in^2 (approximately 93,000 rpm, 100,000 × *g*) for 10 min.

Membrane fractions and detergent extracts were assayed for protein concentration by the method of Lowry et al. (14). Protein determinations were performed in the presence of 0.1% sodium dodecyl sulfate to solubilize membrane protein and to prevent interference by Triton X-100. Bovine serum albumin was used as the standard.

D-Lactate (lithium salt, grade D-X), L-lactate (lithium salt, grade L-X), tetranitro blue tetrazolium, PMS, DCIP, oxamate, oxalate, and bovine heart L-LDH were obtained from Sigma Chemical Co., St. Louis, Mo. NAD was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Agarose (type HGT) was obtained from Marine Colloids, Rockland, Maine. Triton X-100 was from Research Products International, Elk Grove Village, Ill.

Both D-LDH and L-LDH activities (0.0181 and 0.0333 U/min per mg of protein, respectively) were detected in membrane fractions obtained from phototrophically grown *R. rubrum*. There was no LDH activity in the cytoplasmic fraction (i.e., 226,000 × *g* supernatant fluid). The maximal rate of reduction of DCIP by either D-lactate or L-lactate required both PMS and KCN. Enzyme staining of immunoprecipitated LDH enzymes (see below) did not require KCN; detergent disruption of the membrane and electrophoretic separation of membrane components probably prevent the reduction of endogenous electron acceptors. The pyridine nucleotide requirement of these LDH activities was tested by the addition of 1 to 4 mM NAD to enzyme assays. Neither D-LDH nor L-LDH showed an increase in activity in the presence of NAD. Moreover, enzyme staining of LDH immunoprecipitates did not require NAD (see below; Fig. 2).

The kinetic parameters of LDH activity in the membrane preparation differed for the D- and L-stereoisomers. The values for the apparent K_ms for D- and L-lactate were estimated from the Lineweaver-Burk (23) equation (Fig. 1); the value for D-lactate was found to be 0.106 mM, and that for L-lactate was 153 mM. The apparent K_m calculated for L-LDH activity is probably an overestimate, as this enzyme was inhibited by high levels of substrate (320 mM). Moreover, the K_m apparent for L-lactate, calculated on the basis of the Hofstee (23) equation, was 106 mM. The K_m apparent for D-lactate, estimated by the Hofstee equation, was found to be 0.105 mM.

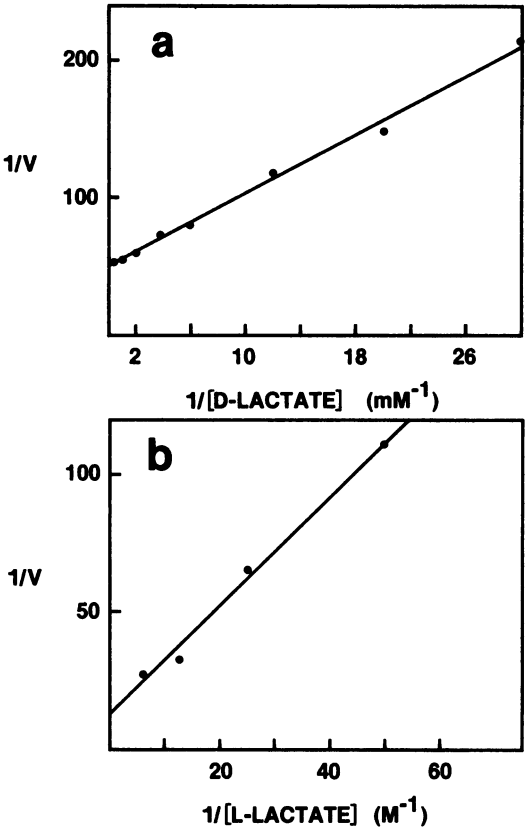

FIG. 1. Lineweaver-Burk double-reciprocal plots for DCIP reduction in the presence of (a) D-lactate and (b) L-lactate. The enzyme preparation consisted of membranes obtained from phototrophically grown *R. rubrum*. The level of D-lactate was varied from 0.033 to 2 mM, and that of L-lactate was varied from 20 to 160 mM. Velocity is expressed in units per milligram of protein.

Dehydrogenase activity measured in the presence of D- and L-lactate together was greater than that in the presence of either stereoisomer alone. This suggested that more than one enzyme was responsible for the oxidation of the D- and L-substrates. The possibility of multiple LDH enzymes was investigated by CIE. This technique can distinguish between two antigenically different enzymes that have the same catalytic activity (4, 21). When Triton extracts of membranes from phototrophically grown *R. rubrum* were analyzed by CIE, two immunoprecipitates that exhibited LDH activity were observed (Fig. 2a). Neither immunoprecipitate stained in the absence of substrate. LDH antigens that form these immunoprecipitates appear to be distinct, as immunological reactions of

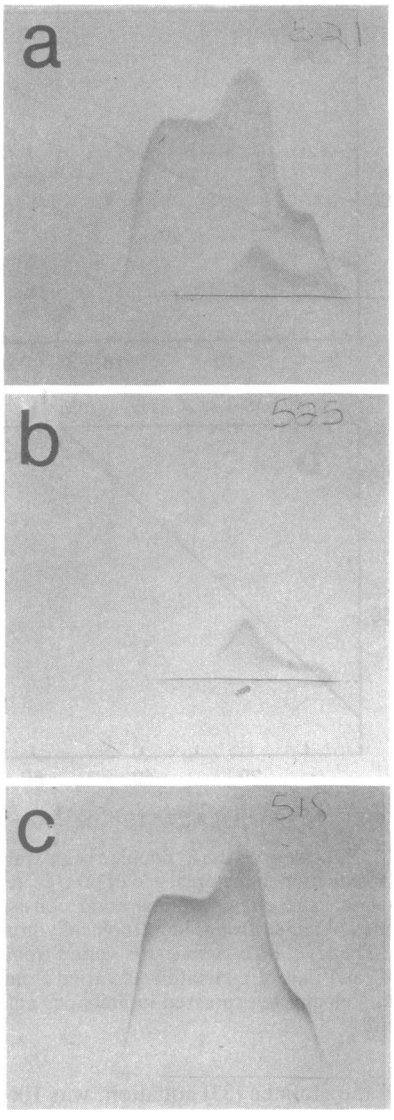

FIG. 2. CIE slides stained for LDH enzymes. Gels of 1% agarose prepared in 1% (wt/vol) Triton buffered by Tris-barbital (4.48 g of Tris, 8.86 g of diethylbarbituric acid per liter [pH 8.6]) were cast on glass slides (5 by 5 cm). A Triton extract of membranes from phototrophic cells (230 μg of protein) was applied to the well at the lower right. Electrophoresis was conducted for 120 min at 180 V in the first dimension and 12 to 16 h at 55 V for the second dimension. Antiserum, raised against phototrophic membranes, was purified and concentrated (5- to 10-fold) by $(NH_4)_2SO_4$ fractionation and dialysis procedures (9). Antiserum (50 μl/ml of agarose) was present in the second-dimension gel. Wet immunoplates were stained for LDH activity by incubation in the following reagent mixture: 0.7 mM tetranitro blue tetrazolium, 100 mM lactate, 0.2 mM PMS, and 40 mM Tris, pH 7.5. The anode was at the left for the first dimension and at the top for the second dimension. The substrates were: (a) D,L-lactate, (b) D-lactate, and (c) L-lactate.

identity or partial identity were not observed. Both immunoprecipitates showed a bimodal shape, indicating heterogeneity of electrophoretic mobility. This has been observed previously in the CIE analysis of antigens from bacterial membranes (2, 3, 19, 21).

These two LDH enzymes of *R. rubrum* were specific for the D- and L-stereoisomers of lactate, as only the cathodic immunoprecipitate stained with an enzyme stain prepared with D-lactate (Fig. 2b), and only the anodic immunoprecipitate stained with L-lactate enzyme stain (Fig. 2c). These two enzymes were further distinguished by inhibitor sensitivity. Enzyme staining of the D-LDH immunoprecipitate was completely inhibited by including oxamate or oxalate in the assay mixture at a molar level equivalent to the D-lactate present. Enzyme staining of the L-LDH was not prevented by either inhibitor (data not shown).

It should be noted that some lots of D-lactate were contaminated by levels of L-lactate sufficient to stain the L-LDH, as well as the D-LDH. Pretreatment to oxidize contaminating L-lactate by incubation with purified bovine L-LDH in the presence of NAD for 1 h at 37°C reduced or eliminated the staining of the L-LDH immunoprecipitate by these D-lactate preparations.

Membranes prepared from aerobically grown *R. rubrum* were found to exhibit both D- and L-LDH activities. CIE analysis of Triton extracts of these membranes identified distinct D- and L-LDH immunoprecipitates (Fig. 3). No LDH activity was detected in the cytoplasmic fraction from aerobically grown cells.

LDHs occur in many diverse bacterial species. In fermenting organisms, the reduction of pyruvate to lactate is coupled to the oxidation of NADH. This is an important step in the regener-

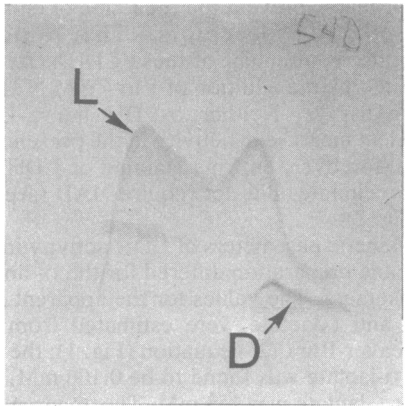

FIG. 3. CIE analysis of LDH enzymes of aerobically grown *R. rubrum*. Immunoprecipitates designated D and L were sequentially stained in reagents (see the legend to Fig. 2) containing D- and L-lactate.

ation of NAD. These pyridine nucleotide-linked LDHs are soluble enzymes located in the cytoplasmic fraction (7).

Pyridine nucleotide-independent LDHs have been identified in several species of bacteria. These enzymes catalyze the oxidation of lactate to pyruvate and not the reverse reaction (7).

The LDH of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* has been purified and characterized (15). This pyridine nucleotide-independent enzyme is specific for L-lactate and is membrane associated. L-LDH has been observed in the CIE analysis of *R. sphaeroides* membranes (2). Antiserum adsorption studies have shown that this enzyme is located on the outer surface (cytoplasmic face) of isolated chromatophores (3).

D- and L-LDH activities have been detected in membrane preparations of *Escherichia coli*. Both L- and D-LDH enzymes have been purified from *E. coli* membranes (5, 6, 13, 22) and have been shown to be flavoproteins (5, 6, 13) that are located on the inner face of the cytoplasmic membrane (6, 20). Both enzymes appear to be primary dehydrogenases that are coupled to the respiratory chain (1, 6). The oxidation of D-lactate generates an electrochemical gradient of protons that energizes the accumulation of amino acids and other solutes by membrane vesicles prepared from *E. coli* (1, 11).

In the present study, D- and L-LDH activities were demonstrated in *R. rubrum*. Both activities were pyridine nucleotide independent and membrane associated. Both D- and L-LDH could be solubilized with Triton X-100. CIE unambiguously identified two enzymes that differed antigenically and in isomeric specificity. They could also be distinguished by inhibitor sensitivity and kinetic properties.

These enzymes catalyze the lactate-dependent reduction of DCIP in vitro and are probably involved in the oxidation of lactate in vivo. One or both of these enzymes may be involved in the utilization of lactate during the photoproduction of hydrogen, but neither enzyme activity is limited to phototrophic growth. The high K_m measured for L-LDH suggests the possibility that this enzyme has another or additional physiological substrates in vivo or that an activator that affects the affinity of L-LDH for L-lactate is present in the cell, but was not present in our enzyme assays. The role of these LDH enzymes in the metabolism of lactate in *R. rubrum* is under further investigation.

This work was supported by grants from the National Science Foundation (PCM-80-24536) and the Graduate School of the University of Wisconsin-Milwaukee.

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