

Membrane-Bound, Pyridine Nucleotide-Independent L-Lactate Dehydrogenase of *Rhodopseudomonas sphaeroides*

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Rhodopseudomonas sphaeroides has a pyridine nucleotide-independent L-lactate dehydrogenase associated with the membrane fraction of cells grown either aerobically or phototrophically. The dehydrogenase is present in cells grown on a variety of carbon sources, but at levels less than 20% of that found in cells grown with DL-lactate. The dehydrogenase has been purified 45-fold from membranes of strain L-57, a non-photosynthetic mutant, by steps involving solubilization with lauryl dimethylamine oxide and three anion-exchange chromatography steps. The purified enzyme was specific for the L-isomer of lactate. The K_m of the purified enzyme for L-lactate is 1.4 mM, whereas that of the membrane-associated enzyme is 0.5 mM. The enzyme activity was inhibited competitively by D-lactate and non-competitively by oxalate and oxamate. Quinacrine, a flavin analog, also inhibited the activity. The inducible enzyme may serve as a marker of membrane protein in studies of membrane development.

Rhodopseudomonas sphaeroides and related *Rhodospirillaceae* use a variety of organic compounds as the source of carbon for growth under phototrophic conditions and as substrates for aerobic growth in the dark (11, 29). Information about the primary dehydrogenases concerned in the oxidation of these materials is limited. Succinate dehydrogenase (EC 1.3.99.1) has been purified from chromatophore membranes (intra-cytoplasmic membranes containing the photosynthetic apparatus) of *Rhodospirillum rubrum*, and its properties were found to resemble those of the classical flavoprotein in bovine mitochondria (12). This enzyme has been used frequently as a marker of the aerobic cytoplasmic membrane (5-7, 22, 24), but its distribution in membrane fractions from phototrophically grown *Rhodospirillaceae* is less clear. In *R. rubrum*, succinate dehydrogenase activity was found only in the chromatophore fraction (6), whereas it was detected in both the chromatophore and cell envelope fraction from phototrophically grown *R. sphaeroides* (22). The pyridine nucleotide-independent *sn*-3-glycerolphosphate dehydrogenase (EC 1.1.99.5) in *R. sphaeroides* is also found in membranes from cells grown phototrophically or under aerobic dark conditions (26).

Lactate is used as a respiratory substrate by many bacteria, including *Rhodospirillaceae*. The first step in its dissimilation is presumed to be an oxidation, catalyzed by pyridine nucleo-

tide-independent dehydrogenases associated with the cytoplasmic membrane. Such an enzyme has been purified to homogeneity from the cytoplasmic membrane of *Escherichia coli*; this dehydrogenase is a flavoprotein, specific for D-lactate (10, 13). We have found a pyridine nucleotide-independent L-lactate dehydrogenase activity in membranes of *R. sphaeroides*, and this paper concerns some properties of the partially purified enzyme together with aspects of its regulation by induction.

MATERIALS AND METHODS

Organisms and growth conditions. The wild-type strain of *R. sphaeroides* NCIB 8253 and mutant L-57, which does not form photosynthetic pigments, have been described previously (3, 17, 28). Stock cultures and inocula were grown in malate-glutamate medium with 0.2% yeast extract as before (17, 18). Bulk cultures were grown phototrophically or aerobically at 30°C in basal medium of a composition identical to malate-glutamate except for the substitution of DL-malate with 20 mM sodium DL-lactate or other substrates as indicated. The phototrophic cultures were in bottles filled to the neck and illuminated with 75-W incandescent strip lamps giving an intensity of approximately 700 footcandles (ca. 7,532 lux); aerobic cultures were incubated in Fernbach flasks under conditions of high aeration (18). Cultures were harvested in the early stationary phase of growth, attained at 18 to 24 h after inoculation unless otherwise stated.

Preparation of cell extracts. Harvested cells were washed in 40 mM tris(hydroxymethyl)amino-methane (Tris)-hydrochloride buffer (pH 7.5) and suspended in this buffer or in 40 mM potassium phosphate buffer (pH 7.5) to an absorbance of 40 at 680

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nm, equivalent to 10 mg of protein per ml. Extracts were prepared with the French press or by lysis of spheroplasts. With the former method, cells in phosphate buffer with 1 mM $MgCl_2$ and 10 μg each of deoxyribonuclease and ribonuclease per ml were passed twice through the pressure cell at 16,000 lb/in², the large debris were removed by centrifugation at 10,000 $\times g$ for 10 min, and the supernatant fraction was used for enzyme assays. Spheroplasts and lysates were prepared from cells suspended in 40 mM Tris-hydrochloride buffer. The following additions were made sequentially to 10 ml of cell suspension: water, 18.5 ml; 40% (wt/vol) sucrose, 15 ml; 1 M Tris-hydrochloride buffer (pH 7.5), 5 ml; lysozyme (10 mg/ml), 0.5 ml; 0.1 M ethylenediaminetetraacetic acid, 1 ml. The mixture was stirred gently for 10 min at room temperature (25°C) and then centrifuged at 6,000 $\times g$ for 5 min. The pellet was suspended in 20 to 50 ml of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM $MgCl_2$ and 10 μg each of deoxyribonuclease and ribonuclease per ml and lysed in a Teflon glass homogenizer. The lysate was centrifuged at 40,000 $\times g$ for 15 min, and the pellet, designated as the membrane fraction, was suspended to a protein concentration of 5 to 10 mg/ml in 40 mM potassium phosphate buffer (pH 7.25) containing 25% (vol/vol) glycerol and 0.2 mM dithiothreitol.

Enzyme assays. Enzymes were assayed spectrophotometrically at 25°C with a Gilford spectrophotometer. One unit of enzyme activity is defined as the amount required to convert 1 μmol of substrate to product per min under the standard conditions of assay. The reaction mixture for L-lactate dehydrogenase (L-lactate:phenazine methosulfate oxidoreductase) contained in 1 ml (in micromoles): potassium phosphate buffer (pH 7.5), 50; phenazine methosulfate, 1; 2,6-dichloroindophenol, 0.125; potassium cyanide, 2; lithium L-lactate, 400; and rate-limiting amounts of the enzyme. The reaction was monitored at 600 nm. The same system was used for the assay of succinate dehydrogenase (EC 1.3.99.1), with sodium succinate instead of lactate as the substrate. The reaction mixture for the assay of malate dehydrogenase (EC 1.1.1.37) contained in 1 ml (in micromoles): potassium phosphate buffer (pH 7.5), 100; reduced nicotinamide adenine dinucleotide (NADH), 0.25; oxaloacetate, 0.2; and rate-limiting amounts of enzyme. The reaction was followed at 340 nm. Alkaline phosphatase (EC 3.1.3.1) was assayed at 410 nm in a mixture containing in 1 ml (in micromoles): Tris-hydrochloride buffer (pH 8.7), 900; *p*-nitrophenylphosphate, 0.9, and rate-limiting amounts of enzyme.

The lactate oxidase system activity was measured at 25°C with an oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) in 2 ml of reaction mixture containing (in micromoles): potassium phosphate buffer (pH 7.5), 50; DL-sodium lactate, 40; and membranes, 1.5 to 3 mg of protein.

Electron acceptor specificity of L-lactate dehydrogenase. The coupling of L-lactate dehydrogenase with various electron acceptors was measured in the standard assay system, omitting phenazine methosulfate and 2,6-dichloroindophenol. Reactions with NAD⁺ and NADP⁺ were monitored at 340 nm. Other reactions were measured in anaerobic Thunberg cu-

vettes twice evacuated and flushed with argon. They were monitored in a Cary model 14R spectrophotometer at the indicated wavelength: 2,6-dichloroindophenol, 600 nm; $K_3Fe(CN)_6$, 420 nm; horse heart cytochrome c, 550 nm; riboflavin 5'-phosphate (FMN), 445 nm; and flavine adenine dinucleotide (FAD), 450 nm.

Protein determinations. Crude extracts were assayed for protein by the Lowry method (20). This procedure was not satisfactory during the purification because of interference from detergents and potassium ferrocyanide. The protein in such preparations was precipitated with trichloroacetic acid and deoxycholate (2) and then determined by a modification of the Lowry method, in which the alkaline reagent contains 0.5% sodium deoxycholate (8). Bovine serum albumin was used as the standard.

Molecular weight estimation. A sample of the L-lactate dehydrogenase, together with calf intestine alkaline phosphatase (molecular weight, 100,000) (9), was layered on top of a 4.8-ml gradient of 8 to 33% (wt/vol) glycerol in 0.04 M potassium phosphate buffer (pH 7.25) containing 0.2 mM dithiothreitol and 0.5 mM $K_4Fe(CN)_6$. Centrifugation was in a Beckman SW50.1 rotor for 16.25 h at 35,000 rpm. Fractions of approximately 0.13 ml were collected from the bottom of the gradient, and the molecular weight was calculated from the distances migrated (21).

Other methods. Isoelectric focusing was as previously described (1), with a glycerol gradient to stabilize the focused ampholyte. Electrophoresis on gels of 7.5% polyacrylamide, containing sodium dodecyl sulfate and 2-mercaptoethanol, was by the procedure of Laemmli (15). The preparation of anion-exchange materials was as described by Peterson and Sober (25). Before reuse, the columns were washed with 1 M KCl in 1% (wt/vol) lauryldimethylamine oxide (LDAO) to remove tightly bound material. In an attempt to produce an affinity adsorbant for the L-lactate dehydrogenase, various compounds were covalently coupled to adipic acid dihydrazide-substituted Sepharose 4-B (16).

The oxidation product of L-lactate dehydrogenase was shown to be pyruvate by characterization of the 2,4-dinitrophenylhydrazone derivative. In these experiments, 40 ml of the standard assay mixture containing approximately 0.6 U of enzyme was run until no further change in adsorbance at 600 nm occurred. The 2,4-dinitrophenylhydrazone was isolated and identified by ascending paper chromatography on Whatman no. 1 paper with either 0.05 N NaOH or *n*-butanol saturated with 3% (vol/vol) ammonia as the solvent (19). The derivative of authentic pyruvate was used as the standard.

Materials. Lithium L-lactate, oxidation-reduction cofactors and dyes, epichlorohydrin triethanolamine (ECTEOA)-cellulose, and calf intestine alkaline phosphatase (EC 3.1.3.1) were purchased from Sigma Chemical Co., St. Louis, Mo. DL-Lactic acid was from Matheson, Coleman and Bell, New York, N.Y., and diethylaminoethyl (DEAE)-cellulose was from Whatman Biochemicals Ltd., Kent, England. LDAO was generously provided by the Oxyx Chemical Co., Jersey City, N.J. Other materials were from standard commercial sources.

RESULTS

L-Lactate dehydrogenase in cell extracts. The activity of various dehydrogenases were assayed in French press extracts of wild-type *R. sphaeroides* grown with different substrates under phototrophic or aerobic conditions (Table 1). L-Lactate dehydrogenase activity was found in all types of cells, but the highest levels occurred in lactate-grown organisms. The specific activity of the enzyme in such cells was at least five times that with other substrates and was similar in preparations from phototrophic or aerobic organisms (Table 1). In contrast to the L-lactate dehydrogenase, the levels of succinate and malate dehydrogenase did not vary significantly in cells grown under the various conditions.

Mutant strain L-57 had L-lactate dehydrogenase activity similar to the wild type when grown aerobically with DL-lactate as the substrate. Preparations from this strain were used for the subsequent purification of the enzyme to avoid complications attributable to the photosynthetic pigments.

(i) Intracellular location of enzyme activities. The distribution of enzyme activity between membrane and cytoplasm was determined by centrifugation of lysed spheroplasts of the wild type grown phototrophically with lactate. The L-lactate dehydrogenase and succinate dehydrogenase activities were located predominantly in the membrane fraction, whereas most of the malate dehydrogenase was found in the cytoplasm (Table 2). This distribution of enzyme

TABLE 1. *Dehydrogenase activities in wild-type R. sphaeroides*^a

Carbon source (mM)	Conditions	U/mg of protein		
		L-Lactate dehydrogenase	Succinate dehydrogenase	Malate dehydrogenase
Acetate, 20	Aerobic	0.017	0.070	6.0
	Phototrophic	0.022	0.067	3.8
Pyruvate, 20	Aerobic	0.026	0.062	5.0
	Phototrophic	0.018	0.044	4.2
Succinate, 10	Aerobic	0.031	0.038	6.0
	Phototrophic	0.016	0.034	4.4
D-Glucose, 10	Aerobic	0.035	0.065	5.2
	Phototrophic	0.016	0.088	4.1
DL-Malate, 20	Aerobic	0.023	0.064	4.6
	Phototrophic	0.048	0.107	4.0
DL-Lactate, 20	Aerobic	0.156	0.046	4.3
	Phototrophic	0.122	0.047	4.2

^a Organisms were grown aerobically in the dark or phototrophically in basal medium with substrates added at the indicated final concentration. They were harvested at 18 to 24 h after inoculation in the stationary phase, except for the glucose cultures, which required incubation for 40 h. Enzyme activities were assayed in extracts prepared by the French press as described in the text.

TABLE 2. *Intracellular location of dehydrogenases in phototrophically grown cells*^a

Carbon source	Fraction	L-Lactate dehydrogenase		Succinate dehydrogenase		Malate dehydrogenase	
		U/mg of protein	Total U	U/mg of protein	Total U	U/mg of protein	Total U
DL-Malate	Lysate	0.049	2.9	0.045	2.8	2.4	141
	Cytoplasm	<0.005	<0.01	0.016	0.19	7.2	87
	Membrane	0.064	1.7	0.056	1.9	2.2	73
DL-Lactate	Lysate	0.160	8.2	0.062	3.1	2.7	138
	Cytoplasm	0.006	0.09	0.013	0.19	9.3	130
	Membrane	0.259	5.5	0.062	2.5	1.7	68

^a Spheroplast lysates were prepared from the wild-type strain grown phototrophically on DL-malate or DL-lactate; they were separated into membrane and cytoplasmic fractions by centrifugation as described in the text. Results are given in terms of protein in the fraction, or as total units in each fraction.

activities was also found in preparations of mutant L-57 grown aerobically with lactate.

(ii) K_m value. The apparent K_m of the enzyme for L-lactate in membranes from strain L-57 was 0.5 mM. The same value was found with membranes derived from cells grown on DL-lactate or DL-malate (Fig. 1).

(iii) Lactate oxidase system activity. Membranes from lysed spheroplasts of the wild-type strain grown with lactate under phototrophic or aerobic conditions oxidized lactate with rates of oxygen consumption from 19 to 22 nmol/min per mg of protein. The lactate oxidase system was inhibited by cyanide, antimycin A, and 2-heptyl-4-hydroxyquinoline-*N*-oxide, indicating participation of the respiratory chain.

Solubilization and purification of L-lactate dehydrogenase. Preliminary experiments were with membranes from the wild-type strain grown phototrophically or aerobically with DL-lactate. The dehydrogenase activity and the photosynthetic pigments were solubilized by treatment with several detergents, including Brij 58, Triton X-100, and LDAO. Higher yields with greater stability were found in preparations solubilized with LDAO, and this detergent was

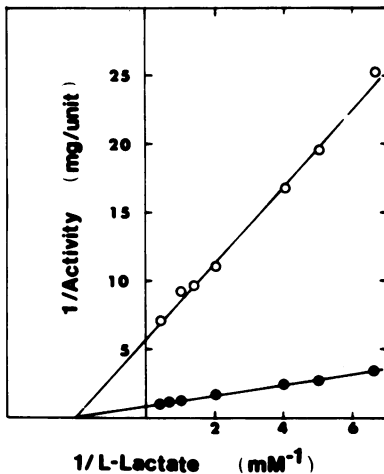


FIG. 1. Lineweaver-Burk plot of membrane-bound L-lactate dehydrogenase. Strain L-57 was grown aerobically on DL-malate (○) or DL-lactate (●). Membranes were prepared from lysed spheroplasts as described in the text.

subsequently used. Previously, LDAO has been used in the purification of reaction center complexes of bacteriochlorophyll from *R. sphaeroides* and other photosynthetic bacteria (4, 23).

The wild-type organisms, even when grown aerobically, formed photosynthetic pigments, and the detergent-solubilized extracts rapidly lost L-lactate dehydrogenase activity when exposed to light. Such lability was not found in similar preparations from mutant L-57, and this organism was consequently used as the source of the enzyme for purification by the procedures described below and summarized in Table 3.

(i) Solubilization. Membranes were prepared from lysed spheroplasts of the mutant grown aerobically on DL-lactate. The membranes from 4 liters of culture were suspended to a protein concentration of 5 mg/ml in "standard buffer," containing 40 mM potassium phosphate buffer (pH 7.25), 25% (vol/vol) glycerol, and 0.2 mM dithiothreitol. Sufficient LDAO was added from a concentrated stock solution to give a final concentration of 0.5% (wt/vol). The mixture stood at 20°C for 30 min and was then centrifuged at 40,000 × *g* for 15 min at 0 to 4°C; subsequent procedures were at the same temperature.

(ii) Chromatography on DEAE-cellulose. The supernatant fraction was applied to a column of DEAE-cellulose (2 by 13 cm; Whatman DE-52) previously equilibrated with the standard buffer. After loading, the column was washed with 50 ml of standard buffer containing 0.15 M KCl and 0.1% (wt/vol) LDAO. Adsorbed protein was eluted with a linear gradient of 0.15 to 0.4 M KCl in 250 ml of the standard buffer containing 0.1% (wt/vol) LDAO. Fractions of approximately 3.1 ml were collected. L-Lactate dehydrogenase activity eluted after the bulk of the protein and the succinate dehydrogenase activity (Fig. 2). Fractions 25 to 55 were combined and dialyzed overnight against 500 ml of the standard buffer containing 0.1% (wt/vol) LDAO and 0.1 mM potassium ferrocyanide.

(iii) Chromatography on ECTEOLA-cellulose. The dialyzed activity was applied to a column (2 by 11 cm) of ECTEOLA-cellulose previously equilibrated with standard buffer containing 0.1% (wt/vol) LDAO and 0.1 mM $K_4Fe(CN)_6$. The column was washed with 50 ml

TABLE 3. Purification of L-lactate dehydrogenase from strain L-57

Fraction	Volume (ml)	U/ml	Protein (mg/ml)	Sp act ^a	Yield (%)
Crude membranes	102	2.57	11	0.23	100
LDAO extract	99	2.98	6.6	0.45	112
DEAE-cellulose	90	1.22	1.2	1.0	42
ECTEOLA-cellulose	51	1.68	0.6	2.8	33
DEAE-Sephadex	90	0.74	0.07	10.5	25

^a Specific activity is units per milligram of protein.

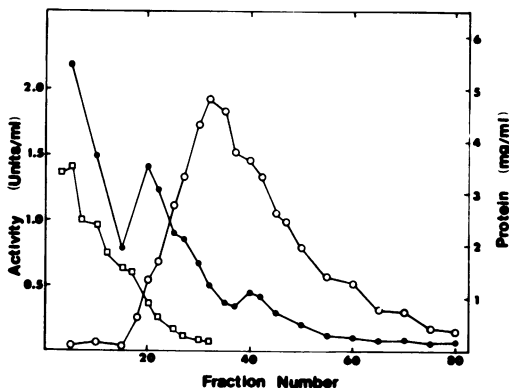


FIG. 2. Elution profile of the DEAE-cellulose column. Symbols: ●, protein; □, succinate dehydrogenase; ○, L-lactate dehydrogenase.

of standard buffer containing 0.1% (wt/vol) LDAO and 0.5 mM $K_4Fe(CN)_6$ and eluted with a linear gradient of 0 to 0.3 M KCl in 250 ml of the same buffer. The L-lactate dehydrogenase activity eluted slightly ahead of the bulk of the protein. Fractions eluting between 0.075 and 0.15 M KCl, containing the majority of the enzyme, were combined.

(iv) **Chromatography on DEAE-Sephadex.** The pooled fractions were applied at a rate of 0.75 ml/min to a column (1.5 by 5 cm) of DEAE-Sephadex A-25 previously equilibrated with the standard buffer containing 0.1% (wt/vol) LDAO and 0.5 mM $K_4Fe(CN)_6$, and the column was then washed with 40 ml of the same buffer. The bulk of the activity did not adsorb to the column, whereas most of the protein did adsorb. The enzyme was concentrated for further study in an Amicon Diaflo apparatus, using compressed nitrogen and an XM-100 membrane. The overall purification was 45-fold over the crude membrane fraction (Table 3). However, electrophoresis of the purified dehydrogenase on a polyacrylamide gel containing sodium dodecyl sulfate revealed that the preparation was still impure and contained at least five protein bands.

(v) **Other purification attempts.** Further attempts at purification were hampered by poor recovery or low purification. Attempted procedures included: chromatography on phosphocellulose, carboxymethyl cellulose, and hydroxyapatite; isoelectric focusing; ammonium sulfate fractionation; acid precipitation; and adsorption to bentonite or calcium phosphate gel. Chromatography was also performed without retention or retardation on Sepharose columns with the following bound ligands: FAD or FMN, chromatographed with and without L-lactate in a variety of buffers, and glyoxylate, malonic sem-

ialdehyde, pyruvate, or α -ketoglutarate. All attempts to utilize gel filtration with gels composed of dextran (Sephadex G-100), polyacrylamide (Bio-Gel P-150), or agarose (Sephacrose 4-B) resulted in poor recovery (0 to 5%) of the applied activity. This phenomenon is not understood, but it does not appear to be due to the loss of a small-molecular-weight component as rapid chromatography on Sephadex G-25 (exclusion limit, 5,000) resulted in only a 20% loss of activity and concentration by compressed nitrogen with an Amicon XM-100 membrane (exclusion limit, 100,000) resulted in less than a 5% loss of activity.

(vi) **Stability.** The purified dehydrogenase lost approximately 15% of its activity after 1 week at 3°C in standard buffer, with 0.1% (wt/vol) LDAO and 0.5 mM $K_4Fe(CN)_6$; storage in the freezer almost completely inactivated the enzyme. The enzyme in its membrane-associated form retained its activity indefinitely when stored in the freezer. During purification, the inclusion of glycerol, dithiothreitol, and 0.1% (wt/vol) LDAO in the buffers was found to be necessary for the maximum recovery of enzyme activity. During chromatography on EC-TEOLA-cellulose, higher yields of enzyme were obtained with $K_4Fe(CN)_6$ present, and the inclusion of this compound lightly improved the stability of the enzyme during dialysis or storage. The presence of $K_4Fe(CN)_6$ during chromatography on DEAE-cellulose caused the enzyme to elute at a lower concentration of KCl. Ferrocyanide was not included in the first DEAE chromatography step since it caused a coincident elution of the lactate dehydrogenase and the bulk of the protein. The procedure adopted gave enhanced purification in the second DEAE step, but recovery was sacrificed. The effects of $K_4Fe(CN)_6$ are not understood, but it was included routinely in buffer systems.

Properties of L-lactate dehydrogenase. Some properties of the L-lactate dehydrogenase were determined with material from mutant L-57 purified through the DEAE-Sephadex stage.

(i) **pH optimum.** The optimum pH for enzyme activity was 7.5 in potassium phosphate buffer. Lower activities were observed in Tris-hydrochloride buffer of the same pH.

(ii) **Substrates and inhibitors.** The apparent K_m of the purified L-lactate dehydrogenase for L-lactate was 1.4 mM (Fig. 3). Surprisingly, this value was higher than that found for the membrane-associated form, which had an apparent K_m of 0.5 mM (Fig. 1). D-Lactate was not a substrate at concentrations up to 100 mM, but it inhibited competitively with a K_i of 22 mM. The L-lactate dehydrogenase was inhibited by oxalate and oxamate, and the kinetics indicated

a noncompetitive type of inhibition (Fig. 3). The K_i values were calculated to be 0.03 and 0.96 mM, respectively, for oxalate and oxamate.

The dehydrogenase activity was inhibited significantly by quinacrine, a flavin analog; at concentrations of 1 and 10 mM, respectively, the degree of inhibition was 18 and 73%. Little or no inhibition was observed with 5 mM ethylenediaminetetraacetic acid, 1 mM *o*-phenanthroline, or 5 mM *N*-ethylmaleimide.

Alternative electron acceptors were examined in the standard assay system without phenazine methosulfate and 2,6-dichloroindophenol. The latter alone was effective, as was potassium ferri-cyanide, but the rates of reduction were less than $\frac{1}{10}$ those observed with phenazine methosulfate (Table 4). Slight reduction was observed with horse heart cytochrome *c*, but neither FAD nor FMN served as an electron acceptor (Table 4). The pyridine nucleotides NAD^+ and NADP^+ were also inactive at concentrations of 0.1 and 1 mM.

(iii) **Molecular weight estimation.** Centrifugation of the L-lactate dehydrogenase in a gradient of 8 to 33% (wt/vol) glycerol revealed that the activity migrated slightly faster than did intestinal alkaline phosphatase, which has a molecular weight of 100,000. From these data, the molecular weight of the L-lactate dehydrogenase was estimated to be $107,000 \pm 3,000$.

(iv) **Identification of the reaction product.** The oxidized product of the reaction catalyzed by the L-lactate dehydrogenase was isolated and identified as pyruvate by paper chromatography. The R_f values in 0.05 M NaOH solvent, in which two spots are characteristic for pyruvate, were 0.50 and 0.65 for the derivatized product and 0.52 and 0.67 for the derivatized

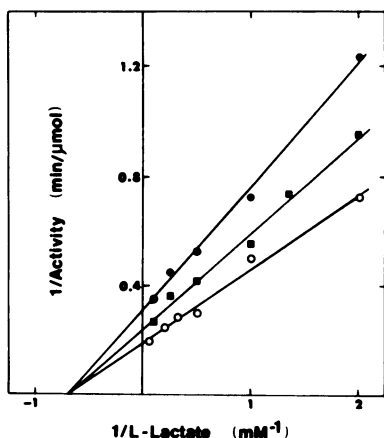


FIG. 3. Kinetics of purified L-lactate dehydrogenase without inhibitor (○) and with the addition of 0.02 mM oxalate (●) or 0.3 mM oxamate (■).

TABLE 4. Activity of L-lactate dehydrogenase with alternate electron acceptors^a

Compound	Concn (μM)	nmol reduced/min ^b
2,6-Dichloroindophenol	40	0.76
	100	2.74
	200	16
$\text{K}_3\text{Fe}(\text{CN})_6$	400	2.4
	1,000	5.2
	4,000	15
Cytochrome <i>c</i>	40	0.028
	100	0.080
	400	0.064
FAD or FMN	40	0
	100	0
	400	0

^a The enzyme was from mutant L-57, purified through the DEAE-Sephadex step (Table 3).

^b The amount of L-lactate dehydrogenase added reduced 266 nmol of phenazine methosulfate per min in the standard assay.

pyruvate standard. When a butanol-ammonia solvent was used, the R_f values were 0.37 for the derivatized product and 0.36 for the derivatized standard.

DISCUSSION

We have shown the L-lactate dehydrogenase of *R. sphaeroides* to be an inducible enzyme associated with the membrane fraction of cells grown phototrophically or aerobically in the dark. Significant L-lactate dehydrogenase was also found in cells grown with substrates other than lactate, but the activity was at least five times higher in organisms grown with lactate. There was no evidence that growth on lactate induced a second form of the L-lactate dehydrogenase. The apparent K_m value for L-lactate was the same in membranes from cells grown with DL-lactate or with DL-malate (Fig. 1), and multiple fractions of enzyme activity were not observed during purification. The *sn*-3-glycerol-phosphate dehydrogenase is another example of an inducible membrane enzyme in *R. sphaeroides* (26). The distribution of these dehydrogenases between chromatophore and cytoplasmic membranes in the phototrophic cell has not been established yet.

The instability of the solubilized L-lactate dehydrogenase frustrated attempts to purify it to homogeneity. The procedures described in this paper achieved 45-fold purification from the membranes, but the enzyme is not yet pure enough to prepare antisera. Specific antisera to the enzyme would be valuable in studies of membrane assembly in *R. sphaeroides* by approaches involving immunoprecipitation.

Certain properties of the L-lactate dehydro-

genase of *R. sphaeroides* are similar to those of the D-lactate dehydrogenase purified to homogeneity from the cytoplasmic membrane of *E. coli* and shown to be a flavoprotein with FAD as the prosthetic group (10, 13). The activity of the dehydrogenase from *R. sphaeroides* with artificial electron acceptors such as phenazine methosulfate and the inhibition by quinacrine are consistent with its being a flavoprotein, but this has yet to be definitely established. The enzyme was also inhibited by oxalate and oxamate, but in a noncompetitive fashion. The D-lactate dehydrogenases of *E. coli* and other organisms are inhibited competitively by these compounds (13, 27).

Interestingly, the K_m value for the solubilized enzyme from *R. sphaeroides* was higher than that found with the membrane-associated form. Such behavior is the reverse of that found for the D-lactate dehydrogenase and the *sn*-3-glycerol phosphate dehydrogenase from the cytoplasmic membrane of *E. coli* (10, 13, 30). In these cases, the K_m values were considerably decreased upon solubilization. The significance of these observations requires more information about the interactions of enzyme and substrate within the membrane milieu of the various organisms.

The incorporation of the L-lactate dehydrogenase into the membrane raises particularly interesting problems in the case of a facultative prototroph such as *R. sphaeroides*. The development of the chromatophore membrane involves the formation and integration of the proteins associated with the light harvesting and reaction center forms of bacteriochlorophyll. These are the major quantitative components of the membrane, and their formation may influence the incorporation of inducible enzymes such as the L-lactate dehydrogenase into the developing membranes of the phototrophic cell. Similar types of questions have been considered by Kung and Henning (14) with respect to cytoplasmic membrane proteins in *E. coli*. Evidence was found of competition for binding sites between the pyridine nucleotide-independent lactate dehydrogenase and other membrane dehydrogenases.

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