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[35] Monoclonal Affinity Purification of D-Lactate Dehydrogenase from *Escherichia coli*

By EUGENIO SANTOS and H. RONALD KABACK

Active transport of many different solutes by right-side-out cytoplasmic membrane vesicles from various bacteria is driven by a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline) generated by means of substrate oxidation via a membrane-bound respiratory chain.[1-3] Although vesicles have the capacity to oxidize a variety of substrates, generation of $\Delta\bar{\mu}_{H^+}$ is relatively specific for certain electron donors. Thus, D-lactate is the most effective physiological electron donor for generating $\Delta\bar{\mu}_{H^+}$ in *Escherichia coli* ML 308-225 right-side-out membrane vesicles, even though its rate of oxidation is slower than that of other electron donors, such as NADH or succinate.[4-6]

Escherichia coli membrane vesicles containing D-lactate dehydrogenase (D-LDH) catalyze the stoichiometric conversion of D-lactate to pyruvate,[7,8] and electrons derived from the reaction are transferred to oxygen through membrane-bound respiratory intermediates. Concomitant with electron flow, a transmembrane $\Delta\bar{\mu}_{H^+}$ is generated by a mechanism(s) that is (are) not completely understood.[3] In any case, D-LDH is readily solubilized from the membrane with chaotropic agents or nonionic detergents and readheres to the membrane in a functional manner upon dilution of the solubilizing agent.[9-12] D-LDH has been purified to homogeneity[13-16]; it exhibits an M_r of about 65 kDa on sodium dodecyl sulfate–

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polyacrylamide gel electrophoresis (SDS–PAGE) and is composed of a single polypeptide chain containing 1 mol of tightly bound flavin adenine dinucleotide per mol of protein. D-LDH is highly specific for D-(−)-lactic acid and is inactivated by 2-hydroxy-3-butynoic acid, which acts as a "suicide substrate."[17]

The gene encoding D-LDH has been cloned,[18] allowing amplification of the enzyme, and the amino acid sequence of the protein has been deduced from DNA sequencing.[19,20] Furthermore, studies on the *in vitro* transcription and translation of *dld* show that the enzyme is synthesized in mature form and binds to the membrane without a leader sequence.[21]

Polyclonal antibodies directed against purified D-LDH have been prepared and characterized.[22] By utilizing these antibodies in inactivation[11] and immunoadsorption experiments,[23,24] it has been demonstrated that D-LDH is associated with the cytoplasmic surface of the bacterial plasma membrane. More recently, monoclonal antibodies (Mab) against D-LDH have been described.[25] One of the Mabs (1B2a) binds exclusively to the intact D-LDH molecule and does not bind to proteolytic fragments from the enzyme.

In this chapter, a Mab immunoaffinity chromatographic procedure is described that allows purification of D-LDH to apparent homogeneity in a single step.

Experimental Procedures

D-*LDH Assay*

Oxidation of D-lactate by D-LDH is monitored by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm in the presence of phenazine methosulfate (PMS).[14] The typical assay is performed in a final volume of 1.0 ml containing 100 μM DCIP, 250 μM PMS, 20 mM lithium D-lactate, and an appropriate amount of the sample to be assayed (pH is adjusted to 7.8 with phosphate or Tris buffers). The reaction is carried out at room temperature in a 1 × 1 cm cuvette. The enzyme sample is added to the assay mixture (minus D-lactate), and a base line is

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recorded. The reaction is then initiated by addition of D-lactate with rapid mixing. Specific activity of D-LDH is expressed as nmol DCIP reduced/min/mg protein. The molar extinction coefficient of DCIP under these conditions is 16.5×10^3 cm^{-1} M^{-1}.

Protein Assays

For membrane vesicles or extracts thereof containing D-LDH, the method described by Lowry *et al.*[26] is used with crystalline bovine serum albumin as standard. For IgG, absorbance at 280 nm is used. A 1 mg/ml solution of IgG at neutral pH exhibits an absorbance of 1.46 at 280 nm over a light path of 1 cm. For dilute samples, such as the eluates from immunoaffinity columns, the method of Shaffner and Weissmann[27] is used.

Growth of Hybridoma Cell Line 1B2a

1B2a hybridoma cells are grown in RPMI 1640 medium (Gibco 320-1875) supplemented with 15% heat-inactivated fetal bovine serum (Gibco 200-6140), 1% glutamine (Gibco 320-5030), 1% pyruvate (Gibco 320-1360), 1% penicillin-streptomycin (Gibco 600-5140), and 50 μM 2-mercaptoethanol (Sigma).[25] Frozen stock cultures of the cells (10^7 cells in 1 ml of complete medium supplemented with 15% dimethyl sulfoxide) are thawed quickly at 42°, washed at 37° in complete medium (minus dimethyl sulfoxide), and seeded in a T25 tissue culture flask containing 4 ml of complete medium with a feeder layer of 10^4 mouse macrophages (obtained by peritoneal washing of BALB/c mice). Cultures are incubated in a tissue culture incubator set at 37° with 85–90% relative humidity and 10% CO_2. As the number of cells increases, they are transferred successively to T75 and T175 tissue culture flasks.

Ascites Fluid

BALB/c mice are primed by intraperitoneal inoculation of 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane) (Aldrich). Hybridoma cells [$1–5 \times 10^7$ in 1 ml of phosphate-buffered saline (PBS)] are injected 15–30 days later.[25] Ascites fluid is collected after 10 days with an 18-gauge needle and every third or fourth day thereafter until the animals expire. A

[26] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).

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total volume of 10–15 ml (20–30 mg of protein/ml) of fluid is collected from each animal. The ascites fluid is clarified by low-speed centrifugation (1000 *g* for 10 min) and stored at −20° until use.

Purification of IgG

IgG produced by 1B2a hybridoma cells is purified by either of two procedures, according to need.

Small-Scale Purification. Highly purified IgG is obtained from small volumes of ascites fluid by affinity chromatography on protein A-Sepharose 4B.[28] Essentially, 2 ml of 0.1 *M* sodium phosphate (pH 8.0) are added to 4 ml of ascites fluid, and the pH is adjusted to pH 8.1 by adding a few drops of 1 *M* Tris–HCl (pH 9.0). A column [1.5 × 15 cm (Pharmacia) or a 10-ml glass pipette] is packed to a bed volume of ~6 ml with protein A-Sepharose 4B (Pharmacia), equilibrated in 0.1 *M* sodium phosphate (pH 8.0) and washed with 30 ml of the same buffer. The sample is applied to the column, and the column is then washed with equilibration buffer until the absorbance of the eluant at 280 nm is zero (about five times the bed volume). Bound IgG is then eluted sequentially with 30 ml each of 0.1 *M* sodium citrate at pH 6.0, 5.5, 4.5, and 3.5 in order to elute IgG_1, IgG_{2a}, and IgG_{2b} isotypes, respectively. Mab 1B2a is an IgG_{2b} isotype and elutes at pH 3.5.[25] This fraction is dialyzed against 100 volumes of PBS (pH 7.2) and stored at −70° after adjusting the protein concentration to 3–5 mg/ml.

Large-Scale Purification. Clarified ascites fluid is adjusted to an absorbance of 14 at 280 nm (~10 mg of protein/ml) with PBS, and ammonium sulfate is added to 50% saturation (31.3 g/100 ml). The mixture is kept in the cold for a few hours with occasional stirring. The precipitate is collected by centrifugation and washed once with 1.75 *M* ammonium sulfate and dissolved in 10 m*M* sodium phosphate (pH 6.8) to a volume equal to that of the original ascites sample prior to precipitation. The solution is then dialyzed against 100 volumes of 10 m*M* sodium phosphate (pH 6.8) with two or three changes at 4°. The sample is applied to a column of DEAE-cellulose (DE-52; Whatman) previously equilibrated with the same buffer. The bed volume of the DEAE-cellulose column is at least as great as the volume of the original ascites sample. After sample application, the column is washed with equilibration buffer, and IgG which does not bind to the resin washes through the column and is collected. The pooled eluate is adjusted to a desired protein concentration with 10 m*M* sodium phosphate (pH 6.8) and stored at −70°.

[28] P. D. Ey, S. J. Prowse, and C. R. Jenkins, *Immunochemistry* **15,** 429 (1979).

Preparation of Immunoaffinity Resin

Affi-Gel 10 (Bio-Rad) is used as the gel matrix for immunoadsorbent chromatography.[25] Prior to use, the commercial gel slurry is washed three times with one volume of isopropanol and three times with cold water (on a sintered glass filter); fines are removed after each wash. The gel slurry is then transferred to 50-ml polypropylene Falcon tubes, and an equal volume of a solution of Mab 1B2a (20 mg/ml of protein in 0.2 *M* $NaHCO_3$/0.2 *M* NaCl, pH 8.0) is added. Coupling is achieved by gentle mixing on a rocker table at 4° overnight. The suspension is washed thoroughly with 0.1 *M* $NaHCO_3$ containing 0.1 *M* NaCl (pH 8.0) until the absorbance of the wash solution at 280 nm is zero. Unreacted sites are blocked by incubating the gel with one volume of 0.1 *M* ethanolamine–HCl (pH 8.0) for 60 min at room temperature with gentle stirring. The gel is then washed with an excess of 50 m*M* PBS containing 0.1% Triton X-100 and 0.01% sodium azide (pH 7.4). As described, the affinity gel bears about 15 mg of IgG/ml of wet gel matrix and can be stored for several months in the cold without detectable loss of affinity.

Enzyme Purification

Bacterial Growth

Escherichia coli ML 308-225 is grown aerobically at 37° in minimal medium with 1% sodium succinate (hexahydrate) as a carbon source.[13] Cells are grown until late exponential phase ($A_{560} \simeq 2.0$) and harvested by centrifugation. Alternatively, *E. coli* IY83, an overproducer strain carrying the *dld* gene on a multicopy recombinant plasmid,[18] may be used.

Preparation of Membranes

Plasma membranes contain all of the D-LDH activity in *E. coli* and are used as starting material for purification of the enzyme. Right-side-out[11,29] or inside-out[30] membrane vesicles or the membrane fraction obtained by passing cells through a French pressure cell at 20,000 psi[13] may be used.

Solubilization of D-LDH

Membranes are resuspended at a concentration of 8 mg of protein/ml in PBS (pH 7.4) containing 0.1% Triton X-100 and 0.5 μM phenylmeth-

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PURIFICATION OF D-LDH BY IMMUNOAFFINITY CHROMATOGRAPHY[a]

Fraction	Protein (mg)	Specific activity (nmol DCIP reduced/min/ mg/protein)	Total activity (nmol DCIP reduced/min)	Purification factor	Yield of enzyme activity (%)
Membrane vesicles	64(100%)	107	6848	1	100
Triton X-100 extract	8.2(12.8%)	638	5232	6	76
Fractions 40–55 (Fig. 1)	0.118(0.18%)	24,600	2903	230	42

[a] Data from Santos *et al.*[25]

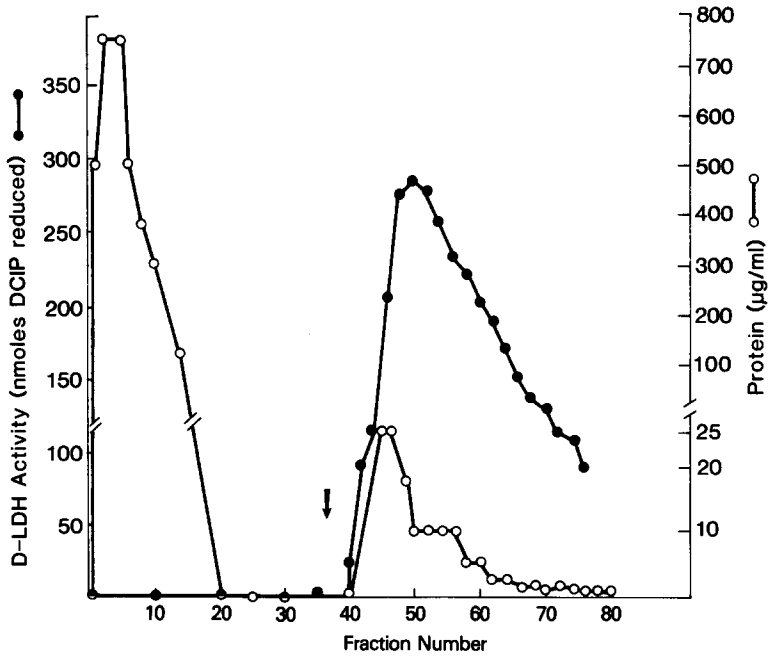

FIG. 1. Immunoaffinity chromatography of D-LDH. Right-side-out membrane vesicles from *E. coli* ML 308-225 (64 mg of protein) were resuspended in 8.0 ml of PBS/0.1% Triton X-100/0.5 μM phenylmethylsulfonyl fluoride (pH 7.4), and incubated for 1 hr at 0°. The suspension was then centrifuged in a 60Ti Beckman rotor for 60 min at 45,000 rpm. The supernatant (~8 ml) was carefully aspirated and applied to the top of a 1B2a/Affi-Gel 10 column (8.0 ml, bed volume), prepared as described. Unadsorbed material was eluted with five volumes of PBS/0.1% Triton X-100 (pH 7.4). The arrow indicates where elution with 0.1 *M* ethanolamine/1.1 *M* guanidine/0.1% Triton X-100 (pH 11.0) was begun. The fractions (1.4 ml) were immediately neutralized by adding 0.7 ml of 1.0 *M* Tris–HCl (pH 7.0). Protein content (○) and D-lactate : DCIP reductase activity (●) were then determined. From Santos *et al.*[25]

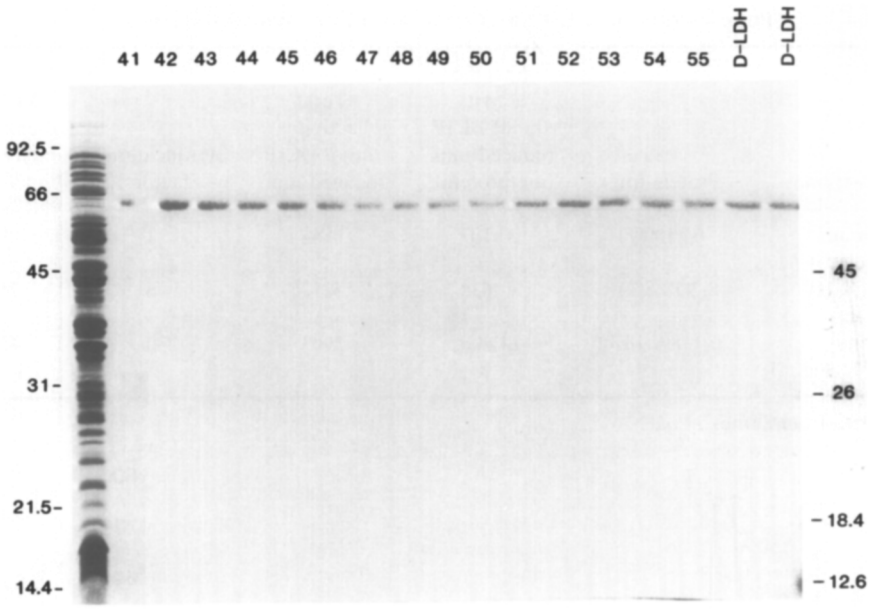

FIG. 2. SDS–PAGE of D-LDH purified by immunoaffinity chromatography. Aliquots (75 μl) of neutralized immunoaffinity chromatography fractions 41–55 (cf. Fig. 1) were subjected to SDS–PAGE with molecular weight standards (indicated on left side), 15–20 μg of protein from the original Triton X-100 extract of ML 308-225 membrane vesicles (first lane on left), and 1 μg of purified D-LDH (last two lanes on right). The gel was stained with silver. From Santos *et al.*[25]

ylsulfonyl fluoride (PMSF). The mixture is incubated at 4° for 1 hr with gentle agitation on a magnetic stirrer. Solubilized D-LDH is then separated from the debris by centrifugation at 4° in a Beckman 60Ti rotor for 60 min at 45,000 rpm. The supernatant, containing solubilized D-LDH, is used for immunoaffinity chromatography.

Immunoaffinity Chromatography

A column of appropriate dimensions is packed with Mab 1B2a immunoaffinity gel prepared as described and washed with five times the bed volume of PBS (pH 7.4) containing 0.1% Triton X-100. The Triton X-100 extract containing D-LDH is applied to the column in a volume equal to that of the column bed. After the extract has entered the column, the outlet is closed, and D-LDH (antigen) is allowed to interact with the affinity matrix for 2 hr at 4° (alternatively, the extract is incubated with the immunoaffinity resin in batch prior to pouring the column). Unadsorbed material (~95% of the total protein in the extract) is eluted from the

column by washing with five volumes of PBS (pH 7.4) containing 0.1% Triton X-100. Adsorbed D-LDH is then eluted by washing with 5–10 volumes of 0.1 *M* ethanolamine containing 1.1 *M* guanidinium hydrochloride and 0.1% Triton X-100 (pH 11.0). Fractions (1.4 ml) eluted under these conditions are neutralized immediately by adding a half volume of 1.0 *M* Tris–HCl (pH 7.0) and assayed for D-lactate : DCIP reductase activity.

A typical purification of D-LDH from right-side-out membrane vesicles of *E. coli* ML 308-225 is summarized in the table. On extraction of vesicles with 0.1% Triton X-100, about 13% of the membrane protein containing about 80% of the D-lactate : DCIP reductase activity is solubilized from the membrane, and the specific activity of D-LDH increases about 6-fold in the supernatant. The extract is then applied to a 1B2a immunoaffinity column as described, and the effluent analyzed for protein and D-LDH activity (Fig. 1). During elution with PBS/0.1% Triton X-100, a major peak containing about 95% of the protein, but no D-LDH, emerges. When the column is then washed with 0.1 *M* ethanolamine/1.1 *M* guanidine/0.1% Triton X-100 at pH 11.0, ~0.2% of the original protein and over 50% of the D-LDH activity applied to the column is eluted in a single peak (fractions 40–55), and the specific activity of D-LDH in these fractions is over 200-fold higher than that of the original membrane vesicles (see the table). Furthermore, when the peak fractions from the column are subjected to SDS–PAGE, followed by silver staining, it is apparent that they contain a single major component that comigrates with authentic D-LDH (Fig. 2). The procedure described allows purification of the enzyme to apparent homogeneity in a single chromatographic step with an overall yield of over 40%.

[36] Fumarate Reductase of *Escherichia coli*

By BERNARD D. LEMIRE and JOEL H. WEINER

Introduction[1]

When the facultative anaerobe *Escherichia coli* is grown anaerobically on a glycerol-fumarate medium, a very simple electron-transport chain consisting of the anaerobic glycerol-3-phosphate dehydrogenase, a *b*-type

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