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Monoclonal Antibodies against the Membrane-Bound, Flavin-Linked D-Lactate Dehydrogenase of *Escherichia coli*: Preparation, Characterization, and Use in Immunoaffinity Chromatography

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ABSTRACT: Three mouse hybridoma cell lines are described that produce monoclonal antibodies directed against the membrane-bound, flavin adenine dinucleotide linked D-lactate dehydrogenase of *Escherichia coli*. In contrast to polyclonal antibodies produced in rabbits, none of the monoclonal antibodies inhibits enzyme activity. Immunoblots of D-lactate dehydrogenase proteolytic fragments indicate that each antibody is directed against a different region of the molecule. One monoclonal antibody, 1B2a, reacts with native, undigested D-lactate dehydrogenase only and is used to purify the enzyme in a single step. The protocol involves chromatography of a Triton X-100 extract of membrane vesicles containing D-lactate dehydrogenase on a column made with the monoclonal antibody coupled to a solid support. After the column is washed free of unadsorbed protein, elution at high pH in the presence of guanidine hydrochloride yields a fraction containing highly purified, catalytically active D-lactate dehydrogenase.

Active transport of many different solutes by right-side-out plasma membrane vesicles from various bacteria is driven by a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$,[1] interior negative and alkaline) (Kaback, 1974, 1976, 1983, 1985; Konings & Boonstra, 1977) generated by means of substrate oxidation via a membrane-bound respiratory chain. Although vesicles have the capacity to oxidize a variety of substrates, generation of $\Delta\bar{\mu}_{H^+}$ seems to be 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 membrane vesicles, even though its rate of oxidation is slower than that of other electron donors such as NADH or succinate (Barnes & Kaback, 1971; Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975).

E. coli membrane vesicles containing D-lactate dehydrogenase (D-LDH) catalyze the stoichiometric conversion of D-lactate to pyruvate (Kaback & Milner, 1970; Barnes & Kaback, 1970), and electrons derived from this 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 (Kaback, 1985). In any event, 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 (Reeves et al., 1973; Short et al., 1974, 1975b; Haldar et al., 1982). The enzyme has been purified to homogeneity in at least three different laboratories (Kohn & Kaback, 1973; Kaczorowski et al., 1978; Futai, 1973; Pratt et al., 1979). In each instance, it exhibits an apparent molecular weight of about 65 kdaltons on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and is composed of a single

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; D-LDH, D-lactate dehydrogenase; DCIP, 2,6-dichlorophenolindophenol; Mab, monoclonal antibody; PBS, phosphate-buffered saline; Pen–Strep, penicillin–streptomycin; HAT, hypoxanthine/aminopterin/thymidine; pristane, 2,6,10,14-tetramethylpentadecane; SP-RIA, solid-phase radioimmunoassay; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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polypeptide chain containing 1 mol of tightly bound flavin adenine dinucleotide per mole of protein. D-LDH is highly specific for D(−)-lactic acid and is specifically inactivated by 2-hydroxy-3-butynoic acid, which acts as a "suicide substrate" (Walsh et al., 1972).

Polyclonal antibodies directed against purified D-LDH have been prepared and characterized (Short et al., 1975a). By utilization of these antibodies in inactivation (Short et al., 1975b) and immunoadsorption experiments (Owen & Kaback, 1979a,b), it has been clearly demonstrated that D-LDH is associated with the cytoplasmic surface of the bacterial plasma membrane. Finally, the gene encoding D-LDH has been cloned (Young et al., 1982) and sequenced (Campbell et al., 1984; Rule et al., 1984), and studies on the in vitro transcription and translation of *dld* show that the protein is synthesized in mature form and binds to the membrane without a leader sequence (Santos et al., 1982).

In this paper, the preparation and characterization of three monoclonal antibodies against D-LDH are described. Each antibody recognizes a different epitope in the enzyme, and none of the antibodies has any effect on catalytic activity. In addition, an immunoaffinity chromatographic procedure is described that allows purification of D-LDH to apparent homogeneity in a single step.

Experimental Procedures

Bacterial Growth and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown in minimal medium A (Davis & Mingioli, 1959) supplemented with 1% sodium succinate (hexahydrate). Right-side-out membrane vesicles were prepared from exponentially growing cultures by osmotic lysis (Kaback, 1971; Short et al., 1975b).

Purification of D-LDH, Assay of Enzymatic Activity, and Preparation of Polyclonal IgG. D-LDH was purified and assayed with 2,6-dichlorophenolindophenol (DCIP) as described (Kohn & Kaback, 1973; Kaczorowski et al., 1978). Rabbit antiserum against the purified enzyme was prepared according to Short et al. (1975a). Polyclonal IgG was purified from the serum by ammonium sulfate precipitation, followed by *O*-(diethylaminoethyl) chromatography (Harboe & Ingild, 1973).

Immunization Procedure for Monoclonal Antibodies (Mab). Female, BALB/c mice (4 week old; Charles River Laboratories) were immunized by subcutaneous injection of 25 μg of purified D-LDH emulsified with complete Freund's adjuvant in a final volume of 0.1 mL. On day 15, the animals received an intraperitoneal booster injection (100 μL per animal) of 25 μg of enzyme dissolved in phosphate-buffered saline [PBS (pH 7.4); 137 mM sodium chloride, 2.68 mM potassium chloride, 8.13 mM dibasic sodium phosphate, 1.46 mM monobasic potassium phosphate] containing 0.1% Triton X-100. Antibody titers were tested by double-immunodiffusion analysis (Ouchterlony) of serial dilutions of serum in PBS against purified D-LDH in the center well. All animals exhibited a high titer under these conditions. Two days before sacrifice (day 45), the animals were given another intraperitoneal booster injection similar to that given on day 15. Spleens were removed aseptically, placed in RPMI 1640 medium supplemented with 2% penicillin–streptomycin (Pen–Strep, Gibco), minced with flat-surface, bent tweezers, and passed through nylon mesh to eliminate particulate material. The cells were then washed in RPMI 1640 medium before fusion.

Construction of Hybridomas. Mouse myeloma P3×63Ag8.653 cells were fused with spleen cells by using 50% poly(ethylene glycol) 4000 (Merck) according to the procedure of Fazekas de St. Groth & Scheidegger (1980). Myeloma cells were maintained in RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 1% Pen–Strep.

After fusion, the cells were suspended in the medium given above plus 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) and distributed into 96-well cuiture plates over a feeder layer of mouse macrophages (10^3 per well). Hybridomas were selected and maintained in HAT medium up to day 14, with changes every 2 days; after day 14, HAT medium minus aminopterin was used for 7 more days, and finally, only RPMI 1640 medium supplemented as described above was used. Positive cultures were transferred to 24-well plates over a feeder layer of mouse macrophages (2×10^4 per well) and later expanded through T25 and T75 culture flasks to produce enough cells to inject into animals for production of ascites fluid. Hybridoma clones were frozen in 15% dimethyl sulfoxide (Littlefield, 1964).

Ascites Fluid. BALB/c mice were primed by intraperitoneal inoculation of 0.5 mL of 2,6,10,14-tetramethylpentadecane (pristane) (Kennett et al., 1980). Hybridoma cells [$(5–10) \times 10^7$ in 1 mL of PBS] were injected intraperitoneally 15–30 days later. Ascites fluid was harvested after 10 days, clarified by low-speed centrifugation, and stored at −20 °C.

Antibodies in ascites fluid were purified by affinity chromatography on a protein A–Sepharose 4B column (Ey et al., 1979). To 6.0 mL of ascites fluid containing 100–150 mg of protein 3.0 mL of 0.1 M potassium phosphate (pH 8.0) was added. A few drops of 1 M Tris-HCl (pH 9.0) was added to adjust the pH to 8.1. The sample was applied to a protein A–Sepharose column equilibrated with 0.1 M sodium phosphate (pH 8.0), and the column was washed with the same buffer until no protein was detected in the eluant. Bound IgGs were then eluted stepwise with 0.1 M sodium citrate at pH 6.0, 5.5, 4.5, and 3.5 in order to elute IgG1, IgG2a, and IgG2b isotypes, respectively. Fractions eluted from the column were dialyzed against PBS (pH 7.4), the protein concentrations adjusted to 1 mg/mL, and the samples frozen in liquid nitrogen.

Solid-Phase Radioimmunoassay (SP-RIA). Purified D-LDH (2 μg in 50 μL of PBS/0.1% Triton X-100) was applied to each well of a 96-well microtray culture plate (Costar) and allowed to adsorb overnight at 37 °C. The wells were then emptied, and nonspecific binding sites were blocked by adding 200 μL of 5% bovine serum albumin (BSA; Sigma, fraction V) in PBS (pH 7.4) and incubating for 2 h at 37 °C. The BSA solution was aspirated from the wells, and 50 μL of ascites fluid, culture supernatant or purified IgG was added and incubation continued for 1 h at 37 °C. The antibody solution was aspirated, and the wells were washed 3 times with 1% BSA in PBS. ^{125}I-Labeled protein A (Ey et al., 1979) (2×10^5 cpm/well) dissolved in PBS with 1% BSA was added, and the samples were incubated for an additional 45 min. Unbound protein A was finally removed by aspiration, and the wells were washed as before. Bound radioactivity was detected by autoradiography on Kodak XR-5 film with a Cronex Lightning Plus intensifier screen.

Protease Digestion. Proteolysis of purified D-LDH with *Staphylococcus aureus* V8 protease was carried out as described (Cleveland et al., 1977). Digestion with trypsin was performed in 100 mM Tris-HCl (pH 8.0) at 37 °C with given incubation times and amounts of enzyme. After digestion, the samples were electrophoresed and electroblotted as described.

SDS–PAGE. The procedure of Laemmli (1970) was used throughout, with 13% polyacrylamide in the separating gel and 5% polyacrylamide in the stacking gel. Gels were stained with either Coomassie Brilliant Blue or with silver (Oakley et al., 1980) as indicated. Molecular weight standards included phosphorylase B (92.5 kdaltons), ovalbumin (45 kdaltons), carbonic anhydrase (31 kdaltons), trypsin inhibitor (21.5 kdaltons), and lysozyme (14.4 kdaltons). Prestained markers were obtained from Bethesda Research Laboratories and included ovalbumin (45 kdaltons), λ chymotrypsinogen (25.7 kdaltons), β-lactoglobulin (18.4 kdaltons), and cytochrome *c* (12.3 kdaltons).

Immunoblotting. After separation by SDS–PAGE, polypeptides were electrophoretically transferred to nitrocellulose (BA85, Schleicher & Schuell) (Towbin et al., 1979) with an Electroblot apparatus (E-C Corp.) set at 0.2-A constant current for 3 h. Efficiency of transfer was monitored visually through use of prestained molecular weight markers. The nitrocellulose paper was then incubated for 2 h at 37 °C in 200 mL of 25 mM Tris-HCl (pH 7.4) containing 0.9% sodium chloride and 1% BSA. The paper was then incubated with a 1:20 dilution of a given Mab in 10 mL of the same solution. After incubation overnight at 37 °C with gentle shaking, the paper was washed 3 times with 100 mL of 25 mM Tris-HCl (pH 7.4)/0.9% sodium chloride, and ^{125}I-labeled protein A (10^5 cpm) was added in 10 mL of the same buffer. The samples were incubated for 3 h at 37 °C with gentle shaking, and the paper was washed once with 100 mL of the same buffer, once with 100 mL of the same buffer containing 0.1% Triton X-100, and twice more with buffer lacking detergent. Finally, the paper was dried and exposed for autoradiography.

Immunoaffinity Chromatography. Affi-Gel 10 (Bio-Rad Laboratories) was washed 3 times with 1 volume of 2-propanol and 3 times with cold water; fines were removed after each wash. An equal volume of a solution containing purified 1B2a (20 mg/mL protein in 0.2 M sodium bicarbonate/0.2 M sodium chloride, pH 8.0) was then added to the gel, and coupling was allowed to proceed overnight at 4 °C with gentle mixing. The suspension was washed with 0.1 M sodium bicarbonate (pH 8.0)/0.1 M sodium chloride, until the absorbance of the wash solution at 280 nm was zero. Unreacted sites were blocked by incubating the gel with 1 volume of 0.1 M ethanolamine hydrochloride (pH 8.0) for 60 min at room temperature, and the gel was then washed with an excess of PBS containing 0.5% Triton X-100 and 0.01% azide (pH 7.4). A column 8 cm in length and 1 cm in diameter was poured and washed with 5 volumes of PBS/0.1% Triton X-100 (pH 7.4). A Triton X-100 extract containing D-LDH was applied to the column in a volume equal to that of the column bed (8 mL), and the mixture was incubated for 1 h at room temperature. Unadsorbed material was eluted by washing the column with 5 volumes of PBS/0.1% Triton X-100 (pH 7.4). Adsorbed antigen was then eluted with 5–10 volumes of 0.1 M ethanolamine/1.1 M guanidine hydrochloride/0.1% Triton X-100 (pH 11.0). Fractions eluted under these conditions were neutralized immediately by adding a half-volume of 1.0 M Tris-HCl (pH 7.0).

Protein Determinations. Protein was determined as described by Lowry et al. (1951) with crystalline BSA as standard. Alternatively, the method of Schaffner & Weissman (1973) was used for dilute samples.

Results and Discussion

Preparation and Characterization of Mabs against D-LDH. Female, BALB/c mice were immunized with purified D-LDH, and splenocytes from the immunized animals were fused with P3×63Ag8.653 myeloma cells as described under Experimental Procedures. The cells were distributed into four 96-well plates (250 μL/well), and hybridomas were selected by growth in HAT medium. Individual hybridoma clones appeared in some wells as soon as 5 days after plating. Within 3 weeks, 120 of the wells contained monolayers that originated from single cells, and 20%–30% of the supernatants from these wells gave a positive reaction by SP-RIA with purified D-LDH and ^{125}I-labeled protein A (not shown). Of the wells exhibiting the strongest reactions, 48 were transferred to 24-well plates and grown over a feeder layer of macrophages. After 9 days growth, SP-RIA was repeated, and positive clones were expanded into T5 and T25 culture flasks. For unknown reasons, many of the hybridoma cell lines stopped producing antibody at one of these steps, and only three hybridomas (designated 1B2a, 1B2b, and 1B4) were selected for expansion into T75 flasks and subsequent intraperitoneal injection into pristane-primed mice for ascites fluid production. SP-RIA of ascites fluid from mice injected with all three hybridoma cell lines demonstrated high levels of antibodies against D-LDH (not shown).

The isotypes of the Mabs were determined after purification from ascites fluid by protein A–Sepharose column chromatography. From the elution pattern at various pH values and by Ouchterlony double-diffusion analysis with rabbit antisera against specific mouse IgG isotypes, it was concluded that the Mabs produced by hybridomas 1B2a and 1B2b are of the type IgG2b, while hybridoma 1B4 produces an IgG2a immunoglobulin.

In contrast to rabbit polyclonal IgG directed against purified D-LDH which inhibits D-LDH activity by 70%–80% (Short et al., 1975a,b), none of the Mabs causes significant inhibition of D-lactate:DCIP reductase activity (not shown). It seems unlikely, therefore, than any of these Mabs is directed against the active site of the enzyme.

Immunoreactivity of D-LDH Proteolytic Fragments. In a preliminary effort to localize different epitopes within the D-LDH molecule, purified enzyme was subjected to limited proteolysis, followed by SDS–PAGE and electroblotting on to nitrocellulose. Subsequently, the immunoreactivity of the proteolytic fragments toward a given Mab was assayed with ^{125}I-labeled protein A. As shown by the Coomassie Brilliant Blue stained gel presented in Figure 1A, digestion of D-LDH with V-8 protease from *S. aureus* leads to the appearance of two major fragments with relative mobilities of about 42 and 40 kdaltons and a minor band at about 15 kdaltons (lanes b and c). In contrast, digestion with trypsin leads to the appearance of a major fragment at about 44 kdaltons and two relatively minor fragments at about 22 kdaltons (lanes e–h).

Mab 1B2a recognizes intact D-LDH (Figure 1B, lane d) but does not react with the proteolytic fragments generated by either trypsin or V8 protease (lanes b and c, respectively). In contrast, Mab 1B2b reacts with intact D-LDH (Figure 1C, lane f) and with the tryptic fragment at 44 kdaltons but not with the other fragments (lanes b and c) nor with any of the peptides generated by V8 digestion (lanes d and e). Lastly, Mab 1B4 reacts with intact D-LDH (Figure 1D, lane a) and possibly with some of the low molecular weight fragments generated by both trypsin and V8 digestions (lanes b–e); however, this Mab exhibits a much higher background than the other two, which makes identification of immunoreactive fragments difficult.

Purification of D-LDH by Immunoaffinity Chromatography. In order to simplify the purification of D-LDH, an immunoaffinity resin was prepared by coupling Mab 1B2a, which

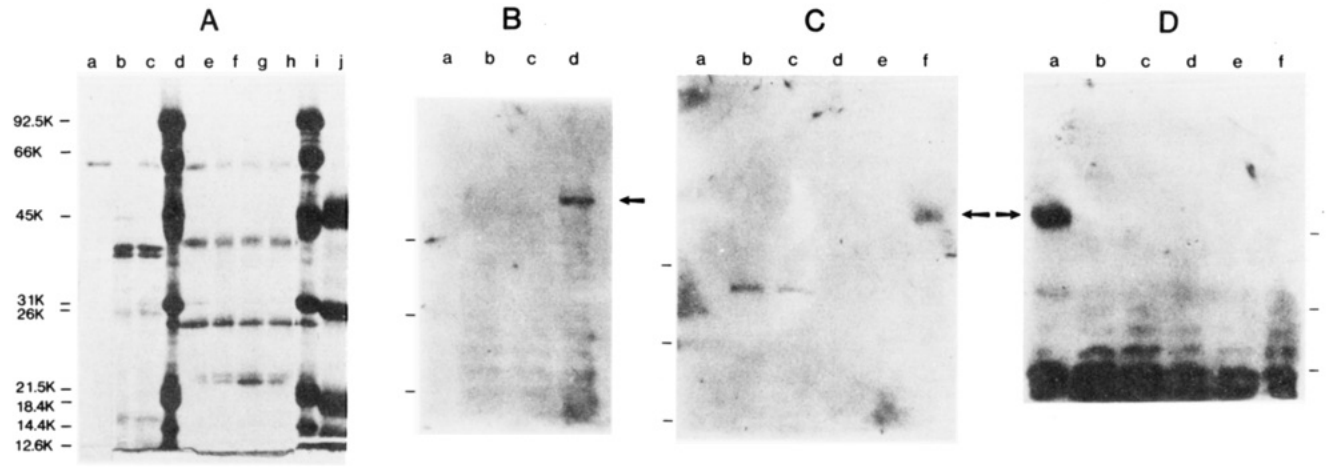

FIGURE 1: Immunoreactivity of D-LDH proteolytic fragments. Samples of purified D-LDH were digested with V8 protease or trypsin and subjected to SDS–PAGE as described under Experimental Procedures. Polypeptide bands were either stained with Coomassie Brilliant Blue (panel A) or electroblotted on to nitrocellulose and assayed for immunoreactivity with Mabs 1B2a (panel B), 1B2b (panel C), or 1B4 (panel D) and ^{125}I-labeled protein A. Digestion with proteases was carried out in a final volume of 30 μL containing 5 μg of purified D-LDH and 2.4 μg of a given proteolytic enzyme for indicated periods of time. (Panel A) (Lane a) Untreated, purified D-LDH; (lanes b and c) purified D-LDH digested with V8 protease for 2 and 1 h, respectively (the molecular mass of V8 protease is 28 kdaltons); (lanes e–h) purified D-LDH digested with trypsin for 30, 60, 105, and 130 min, respectively (the molecular mass of trypsin is 23.5 kdaltons); (lanes d and i) molecular weight standards (cf. Experimental Procedures); (lane j) prestained molecular weight standards (cf. Experimental Procedures). (Panel B) Mab 1B2a immunoblot. (Lane a) Prestained molecular weight standards; (lanes b and c) purified D-LDH digested with trypsin for 1 h with trypsin and V8 protease, respectively; (lane d) untreated, purified D-LDH. (Panel C) Mab 1B2b immunoblot. (Lane a) Prestained molecular weight standards; (lanes b and c) purified D-LDH digested with trypsin for 1 and 2 h, respectively; (lanes d and e) purified D-LDH digested with V8 protease for 1 and 2 h, respectively; (lane f) untreated, purified D-LDH. (Panel D) Mab 1B4 immunoblot. (Lane a) Untreated, purified D-LDH; (lanes b and c) purified D-LDH digested with trypsin for 1 and 2 h, respectively; (lanes d and e) purified D-LDH digested with V8 protease for 1 and 2 h, respectively; (lane f) prestained molecular weight standards. The position of the prestained markers is indicated on the side of the immunoblots with a dash: the position of D-LDH is indicated by an arrow.

Table I: Stability of D-LDH Activity[a]

conditions	% activity: without neutralization	% activity: with dilution/neutralization
PBS/0.1% Triton X-100/20 mM D-lactate (pH 7.4)	100	
+3 M $MgCl_2$	0	0
+100 mM acetate/100 mM NaCl/0.1% Triton X-100 (pH 2.6)	0	0
+0.5 M guanidine hydrochloride	100	100
+1.0 M guanidine hydrochloride	100	100
+2.3 M guanidine hydrochloride	70	70
+2.9 M guanidine hydrochloride	10	10
+3.3 M guanidine hydrochloride	0	0
+0.1 M ethanolamine hydrochloride/0.1% Triton X-100 (pH 11.0)	30[b]	100

[a] A total of 5 μg of purified D-LDH was dissolved in 20 μL of PBS/0.1% Triton X-100 (pH 7.4). Appropriate additions were then made from stock solutions in order to achieve the final salt and D-lactate concentrations and pH values given, the final volume was adjusted to 400 μL, and the samples were incubated at room temperature for 15 min. For those samples assayed without neutralization, 100 μL was diluted into 1.0 mL of PBS/0.1% Triton X-100 (pH 7.4); for those samples assayed with dilution and neutralization, 100 μL was neutralized with an appropriate amount of 1 M Tris-HCl (pH 8.0) and diluted 10-fold into PBS/0.1% Triton X-100 (pH 7.4). The samples were then assayed for D-lactate:DCIP reductase activity as described under Experimental Procedures. [b] The value reported is for the first 30–60 s of the reaction. Thereafter, the rate of D-lactate:DCIP reductase activity decreased to zero.

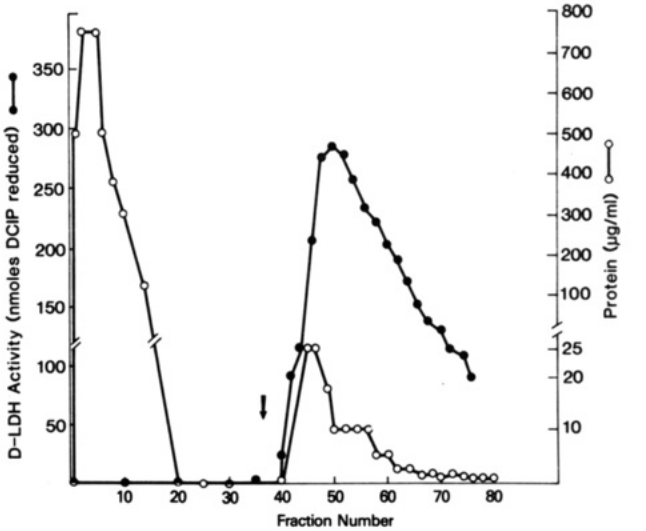

FIGURE 2: Immunoaffinity chromatography of D-LDH. Right-side-out membrane vesicles from *E. coli* ML 308-225 (64 mg of protein) were resuspended in 8.0 mL of PBS/0.1% Triton X-100/0.5 μM phenylmethanesulfonyl fluoride (pH 7.4) and incubated for 1 h at 0 °C. The suspension was then centrifuged in a 60Ti Beckman rotor for 60 min at 45 000 rpm. The supernatant (ca. 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 under Experimental Procedures. Unadsorbed material was eluted with 5 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 started. 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 as described under Experimental Procedures.

reacts with intact D-LDH only (Figure 1B), to Affi-Gel 10. The retention capacity of the gel was then tested in batch by mixing known amounts of purified enzyme with the gel for 30 min at room temperature and assaying the supernatant for D-LDH activity. It was found that 20 μL of gel prepared as described binds a minimum of 50 μg of D-LDH.

Several methods known to release antigens from immunoadsorbants were tested with solubilized, purified D-LDH in order to define conditions that do not inactivate the enzyme (Table I). Treatment of D-LDH for 30 min at low pH (Robb & Strominger, 1976; Dufau et al., 1975) or incubation with 3.0 M magnesium chloride (Shiu & Freisen, 1974; Caldwell & Kuo, 1977) irreversibly inactivates. Treatment with

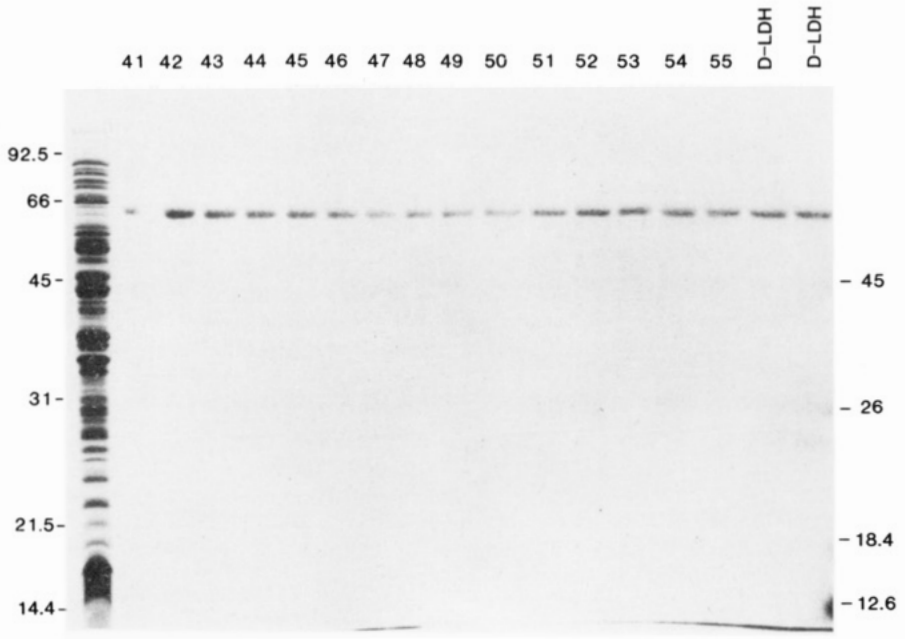

FIGURE 3: SDS–PAGE of D-LDH purified by immunoaffinity chromatography. Aliquots (75 μL) of neutralized immunoaffinity chromatography fractions 41–55 (cf. Figure 2) were subjected to SDS–PAGE with molecular weight standards (indicated on left side), 15–20 μg of protein from the original Triton X-100 extract 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 as described under Experimental Procedures.

Table II: Purification of D-LDH by Immunoaffinity Chromatography

fraction	protein (mg)[a]	D-LDH activity [nmol of DCIP reduced min^{-1} (mg of protein)$^{-1}$]	recovery (%)	purification factor (x-fold)
membrane vesicles	64 (100)	107		
Triton X-100 extract	8.2 (12.8)	638	76	6
fractions 40–55 (Figure 2)	0.118 (0.18)	24600	42	230

[a] Parenthesized values are percentages.

guanidine hydrochloride (Maze & Gray, 1980), on the other hand, does not inactivate D-LDH at concentrations up to 1.0 M; higher concentrations progressively inactivate. D-LDH is solubilized reversibly from the *E. coli* plasma membrane by chaotropic agents (Reeves et al., 1973), and this property of the enzyme has been useful for functional reconstitution of membrane vesicles from mutants defective in D-LDH (Reeves et al., 1973; Short et al., 1974, 1975b; Haldar et al., 1982). Exposure of D-LDH to 50 mM ethanolamine at pH 11.0 for 5 min inhibits D-lactate:DCIP reductase activity by 70% over the first minute of the reaction; subsequently, the rate diminishes to zero. Remarkably, however, neutralization of the sample leads to quantitative recovery of activity. Finally, the enzyme was exposed to pH 11.0 in the presence of increasing concentrations of guanidine hydrochloride, incubated for 10 min at room temperature, and then diluted and neutralized. Enzyme activity remains essentially completely intact after exposure to alkaline pH and guanidine hydrochloride concentrations up to 1.1 M. Importantly, moreover, purified D-LDH bound to the immunoaffinity gel is quantitatively released with no loss in enzymatic activity by exposure to alkaline pH in the presence of 1.1 M guanidine hydrochloride (not shown).

At this point, a crude preparation of D-LDH was subjected to immunoaffinity chromatography. Right-side-out membrane vesicles from *E. coli* ML 308-225 were suspended in PBS (pH 7.4) containing 0.1% Triton X-100 and 0.5 μM phenylmethanesulfonyl fluoride (8 mg of protein/mL) and incubated at 0 °C for 1 h. At this concentration of Triton X-100, 13% of the membrane protein containing about 90% of the D-lactate:DCIP reductase activity is solubilized from the membranes, and the specific activity of D-LDH increases about 6-fold in the supernatant (Table II). The extract was then applied to a 1B2a/Affi-Gel 10 immunoaffinity column as described and the effluent analyzed for protein and D-LDH activity (Figure 2). As shown, when the column is washed with PBS (pH 7.4) containing 0.1% Triton X-100, a major peak containing about 95% of the protein applied to the column but no D-LDH activity is eluted. Strikingly, when the column is then washed with 0.1 M ethanolamine/1.1 M guanidine/0.1% Triton X-100 at pH 11.0, approximately 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 (Table II). Furthermore, when the peak fractions from the column are subjected to SDS–PAGE, followed by silver staining, it is readily apparent that they contain a single major component that comigrates with authentic D-LDH (Figure 3). Thus, the procedure described allows purification of D-LDH to apparent homogeneity in a single step with an overall yield of over 40%.

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