Mechanisms of Active Transport in Isolated Bacterial Membrane Vesicles

XV. PURIFICATION AND PROPERTIES OF THE MEMBRANE-BOUND D-LACTATE DEHYDROGENASE FROM ESCHERICHIA COLI

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SUMMARY

The membrane-bound D-lactate dehydrogenase of Escherichia coli ML 308-225 has been solubilized and purified to homogeneity. The enzyme has a molecular weight of 75,000 ± 7% and contains approximately 1 mole of flavin adenine dinucleotide per mole of enzyme. Its pH optimum before exposure to Triton X-100 is 7.5 to 8.0; after exposure it is 8.0 to 9.5. The purified enzyme has a \( K_m \) value of 1.4 \( \times 10^{-5} \) M for D-lactate and 3.0 \( \times 10^{-2} \) M for L-lactate before exposure to Triton X-100; after exposure its \( K_m \) values are 0.5 \( \times 10^{-5} \) M and 2.1 \( \times 10^{-2} \) M for the same substrates, respectively. The enzyme is not active with D-glycerate, L-glycerate, succinate, malate, D-tartrate, L-tartrate, meso-tartrate, 1-propanol, or isopropanol as substrates and oxidized diphosphopyridine nucleotide has no effect on the catalytic conversion of D-lactate to pyruvate. The enzyme is not inhibited by p-chloromercuribenzoate, n-ethylmaleimide, or 5,5'-dithiobis(2-nitrobenzoic acid) but is inactivated by treatment with 2-hydroxy-3-butynoic acid.

Recent experiments (17) demonstrate that guanidine extracts from wild type vesicles containing D-lactate dehydrogenase activity are able to reconstitute D-lactate-dependent oxygen consumption and active transport in membrane vesicles from an E. coli mutant deficient in D-lactate dehydrogenase. In the present report, the purification of D-lactate dehydrogenase from E. coli ML 308-225 is described. It is demonstrated that the purified, homogeneous enzyme is a flavoprotein containing FAD, and several of its properties are characterized. In subsequent reports structure-function relationships of the purified enzyme with regard to reconstitution of active transport will be examined in detail.

MATERIALS AND METHODS

All chemicals were obtained from commercial sources and were used without further purification. D-lactate and L-lactate were lithium salts obtained from Calbiochem.

Growth of the Bacteria—E. coli ML 308-225 (\( \psi^{-+} \)) was grown overnight at 37°C (late exponential phase) in a medium containing the following per 10 liters: dibasic potassium phosphate, 65 g; monobasic potassium phosphate, 35 g; ammonium sulfate, 10 g; magnesium sulfate, 0.48 g; sodium citrate, 1 g; yeast extract, 0.1 g; and glycolate, 3 g. Cells were harvested at 0-4°C using a Sharples continuous flow centrifuge and were washed twice with 0.02 M potassium phosphate, pH 7.2. Cells stored at −90°C were stable for at least 3 months.

Standard Enzyme Assays—Two assays were used to monitor enzyme activity. The first, the dichlorophenolindophenol assay, was monitored at 600 mp using a Beckman model DU spectrophotometer equipped with a Gilford cuvette changer. Absorbance decreases were monitored for a 3-min period at 25°C; cuvettes with a 1 cm light path were used. The assay mixture contained the following in a final volume of 1.0 ml: 100 mm potassium phosphate, 65 g; monobasic potassium phosphate, 35 g; ammonium sulfate, 10 g; magnesium sulfate, 0.48 g; sodium citrate, 1 g; yeast extract, 0.1 g; and glycolate, 3 g. Cells were harvested at 0-4°C using a Sharples continuous flow centrifuge and were washed twice with 0.02 M potassium phosphate, pH 7.2. Cells stored at −90°C were stable for at least 3 months.

The abbreviation used is: MTT, [3-(4,5-dimethylthiazolyl-2)-2,5]diphenyl tetrazolium.
were monitored as in the first assay, i.e., for a 3-min period at 25° using 1-cm light path cuvettes. The assay mixtures contained the same components as the dichlorophenolindophenol assay with the exception that 60 μg per ml of phenazine methosulfate and 30 μg per ml of the tetrazolium dye replaced dichlorophenolindophenol.

One unit of enzyme activity in the dichlorophenolindophenol assay is defined as a decrease in absorbance at 600 nm of 1.0 optical density unit per min. One unit of enzyme activity in the tetrazolium dye assay is defined as the amount of enzyme that results in the reduction of 1 μmole of MTT per min, using an ε value of 17 mm⁻¹ cm⁻¹ (18, 19). Specific activity is expressed as units per mg of protein. Protein was measured colorimetrically (20) with crystalline bovine serum albumin as a standard. All fractions for protein analyses were first precipitated with 10% trichloroacetic acid and solubilized by heating in 0.1 M sodium hydroxide for 30 min.

Polycrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate was carried out using a pH 9.5 system (21). After electrophoresis, each gel was halved longitudinally with a mechanical slicing device and one-half was stained for protein with Coomassie blue (22) while the other half was stained for enzyme activity. The enzyme activity stain contained the following components per 100 ml: 1.0 M potassium phosphate, pH 7.8, 10 ml; 0.1 M d-lactate, 10 ml; nitroblue tetrazolium, 150 mg; phenazine methosulfate, 40 mg; and water, 80 ml. The gel halves subjected to enzyme staining were rinsed with 0.1 M potassium phosphate, pH 7.8, before incubating them in the assay mixture; incubations were carried out in the dark.

Analytical disc gels containing sodium dodecyl sulfate were performed according to the method of Neville (23); enzyme solutions to be run on these gels were prepared as described (23).

Ultracentrifugation—Sedimentation equilibrium experiments were performed at 4-6° in a Beckman model E ultracentrifuge equipped with a temperature control unit and an electronic speed control. Experiments were conducted at 25° in a double sector, artificial boundary cell of the capillary spill type; Rayleigh optics were used to evaluate the concentration changes. Protein was prepared for these experiments by dialysis at 2° against at least three successive portions of 0.1 M potassium phosphate, pH 7.5, containing 1 mM mercaptoethanol.

Resolution of Flavin from Enzyme and Its Quantitative Determination—The presence of flavin in enzyme preparations was monitored fluorometrically as described by Bessey et al. (24). Measurements were made using either microcuvettes (25) or cuvettes with a 1-cm light path in an Aminco-Bowman spectrophotometer. Control solutions were buffers against which the enzyme had been dialyzed and which were subjected to the same resolution procedures. Quantitative determinations were made by comparisons with standard FAD solutions. Flavin was extracted from the enzyme by either acid extraction with 10% trichloroacetic acid (26) or acid ammonium sulfate precipitation (27). For acid extraction, enzyme solutions at 0-2° containing 3 mg per ml of bovine serum albumin were mixed with enough 50% trichloroacetic acid to yield a final trichloroacetic acid concentration of 10%. The precipitated protein was removed by centrifugation at 18,000 × g for 10 minutes and the supernatant was extracted four times with 3 volumes of chloroform or ether. This procedure extracted 85 to 98% of the flavin from the enzyme.

Acid ammonium sulfate extraction was performed using a modification of the procedure described by Strittmatter (27). At 0°, 0.5-ml aliquots of the enzyme solution were rapidly mixed with 0.5 ml of 3 M potassium bromide and 1 ml of 4 M ammonium sulfate containing 0.055 ml of 1.0 N HSO₄. The precipitate which formed was rapidly sedimented (1-min centrifugation at 18,000 × g) at 0° and subsequently dissolved in 1.0 ml of 0.1 M potassium phosphate, pH 7.5. The supernatant was assayed for flavin as described above.

Miscellaneous Procedures—Absorption spectra were measured in a Cary model 11 recording spectrophotometer.

RESULTS

Enzyme Purification

A summary of a typical purification is presented in Table I. All steps were carried out at 0-4° unless otherwise noted.

**Step 1: Crude Extract—**Frozen cells (150 g) were suspended in 3 times their weight (450 g) of 0.02 M potassium phosphate, pH 7.2, containing 1 mM mercaptoethanol and 100 mg per liter of phenylmethanesulfonyl fluoride. After thawing at 4°, the cells were disrupted by two passages through a Gaulin homogenizer at 12,000 to 13,000 p.s.i. The extract was centrifuged at 10,000 x g for 10 min and the precipitate was discarded.

**Step 2: Ammonium Sulfate Precipitation—**Ammonium sulfate, 17.6 g per 100 ml, was added to the crude extract (620 ml) and allowed to equilibrate for 1 hour with constant stirring. The precipitate was recovered by centrifugation at 30,000 x g for 30 min and was suspended in 0.05 M Tris-chloride, pH 8.0.

**Step 3: Sodium Perchlorate Extraction—**The suspension described in Step 2 (150 ml) was adjusted to 0.02 M d-lactate by addition of 290 mg of lithium d-lactate and enough 8.0 M sodium perchlorate (7.9 ml) was added to yield a final concentration of 0.4 M. The suspension was allowed to stand for 20 minutes at 0-2° and was centrifuged for 90 min at 24,000 rpm in a Beckman model I ultracentrifuge using an SW 25.2 rotor. The precipitate was discarded.

**Step 4: DEAE-cellulose Chromatography—**The supernatant from Step 3 was passed through a Sephadex G-25 (medium) column (4 × 40 cm) equilibrated with 0.05 M Tris-chloride, pH

| Step               | Volume | Total protein | Dichlorophenol-indophenol assay | Tetrazolium dye assay | Recovery* |
|--------------------|--------|---------------|---------------------------------|----------------------|-----------|
| Crude extract      | 620    | 47,800        | 10,280                          | 0.22                 | 1,902     | 0.04    | 96      |
| Ammonium sulfate, 0 to 35% | 150    | 18,200        | 9,850                           | 0.54                 | 1,820     | 0.1     | 96      |
| Sodium percolate   | 217    | 3,700         | 6,720                           | 2.4                  | 1,591     | 0.43    | 34      |
| DEAE-cellulose     | 760    | 5,980         | 7.9                             | 980                  | 1.29      | 55      |
| DEAE-cellulose in Triton X-100 | 29.7  | 4,010         | 135                             | 656                  | 22.1      | 37      |
| DEAE-cellulose in Triton X-100 | 2.5   | 5.2           | 3,300                           | 302                  | 75.4      | 22      |

* Average of results from the two assays.
7.4, containing 1 mM mercaptoethanol, 0.01 mM N-lactate, and 100 mg per liter of phenylmethylsulfonyl fluoride. The protein in the excluded volume was applied to a microgranular DEAE-cellulose column (4 x 50 cm) equilibrated with 0.05 M Tris-chloride, pH 7.4. After washing the column with 750 to 1000 ml of equilibrating buffer, it was developed using a 5000 ml linear gradient of sodium chloride (from 0 to 0.35 M) in equilibrating buffer (Fig. 1A).

Step 5: DEAE-cellulose Chromatography—The fractions from Step 4 which contained the peak of enzyme activity (Fig. 1A, black bar) were pooled and precipitated with ammonium sulfate, 30 g per 100 ml. The precipitate was recovered by centrifugation for 15 min at 30,000 × g and dissolved in 0.05 M Tris-chloride, pH 7.5, containing 1 mM mercaptoethanol and 100 mg per liter of phenylmethylsulfonyl fluoride. The solution was dialyzed for 12 hours against the same buffer, adjusted to 1% Triton X-100, and applied to a column of microgranular DEAE-cellulose (2.0 x 10 cm) previously equilibrated with 0.05 M Tris-chloride, pH 7.4, containing 1% Triton X-100. After the column was washed with approximately 200 to 400 ml of equilibrating buffer, a 1400-ml linear gradient from 0 to 0.20 M sodium chloride (in equilibrating buffer) was applied (Fig. 1B).

Step 6: DEAE-cellulose Chromatography—The fractions from Step 5 containing the peak of enzyme activity were pooled and concentrated in an Amicon ultrafiltration apparatus using a UM-10 membrane. The concentrate was dialyzed overnight against 0.02 M potassium phosphate, pH 7.0, containing 1% Triton X-100 and 1 mM mercaptoethanol. The dialysate was applied to a column of microgranular DEAE-cellulose (0.9 x 25 cm) previously equilibrated with 0.02 M potassium phosphate, pH 7.0, containing 1% Triton X-100. After washing with 200 ml of equilibrating buffer, the column was developed using a linear gradient arranged so that the reservoir contained 500 ml of 0.02 M potassium phosphate, pH 7.0, 1% Triton X-100, and 0.25 M sodium chloride, while the mixing chamber contained 500 ml of 0.02 M potassium phosphate, pH 7.0 and 1% Triton X-100 (Fig. 1C). The fractions with maximal enzyme activity (Fig. 1C, black bar) were concentrated by ultrafiltration using Sartorius colloidion bags. The enzyme was stored in liquid nitrogen and in the presence of 25% glycerol. Under these conditions, the preparation lost less than 10% of its activity in 1 month.

Criteria of Purity

When subjected to gel electrophoresis on standard analytical disc gels (21), the enzyme exhibited a single protein band which could be stained with either Coomassie blue or after incubation in an enzyme assay mixture (Fig. 2A).

The enzyme also migrated as a single protein species on sodium dodecyl sulfate gels (Fig. 2A) and exhibited linear characteristics in plots of sedimentation equilibrium data (see below).

Membrane vesicles (17−19) or enzyme extracts which had not been exposed to Triton X-100 contained only a small amount of this enzyme species and a large amount of activity remained in the stacking gel (Fig. 2B, Gels 1 and 2). After exposure of the vesicles or enzyme extracts to Triton X-100, however, the major portion of the enzyme activity migrated exactly as did the purified enzyme, whereas the enzyme activity previously trapped in the stacking gels disappeared (Fig. 2B, Gels 2 and 4). Finally, dialysis of the Triton X-100-treated extract caused the activity to remain in the stacking gel (Gel 5).

Physical Properties

Molecular Weight—Compared to standards (i.e., albumin dimer, phosphorylase, albumin, catalase, and ovalbumin), the enzyme had a molecular weight of 74,000 ± 5,000 when it was electrophoresed in the presence of sodium dodecyl sulfate. Assuming a partial specific volume (6) of 0.73, molecular weights of 73,500 and 76,000 could be calculated from high and low speed sedimentation equilibrium data (Fig. 3). As noted above, the linearity of this plot (Fig. 3) indicated that only one species of protein was present in the homogeneous preparation with respect to molecular weight.

Absorption Spectrum—The pure enzyme exhibited a peak of absorption at 440 to 480 μM (Fig. 4). In the presence of 10 mM N-lactate or 25 mM N-lactate, the absorption in this area was markedly reduced (Fig. 4, inset, •—• and ○—○). Acid ammonium sulfate precipitation of the enzyme completely abolished the absorption in this area (Fig. 4, ---).

Identification of FAD in N-Lactic Dehydrogenase—The absorption spectrum of the enzyme (Fig. 4) suggested that the enzyme was a flavoprotein. Moreover, the absorption spectrum of the

2 In association with the Triton-induced shift in mobility, a cytochrome band was noted to migrate into the gels to a point, lower than the n-lactate dehydrogenase activity. After dialysis the cytochrome band migrated with the n-lactate dehydrogenase activity in the stacking gels.
flavin obtained by either acid ammonium sulfate or trichloroacetic acid treatment of the enzyme suggested it was FAD.

The flavin extracted from the n-lactate dehydrogenase was able to restore n-amino acid oxidase activity, an FAD-specific function (29). By paper chromatography it migrated in two solvent systems (27) as did authentic FAD and its pH-dependent fluorescence was the same as FAD (data not shown).

The FAD content of the pure enzyme was determined fluorometrically (see "Materials and Methods" and Table II). An average value of 1.21 moles per mole of enzyme using the observed molecular weight of 75,000 for the enzyme was determined.

Characteristics of the Enzyme Reaction

pH Optima—The pH optimum of the purified enzyme was between 7.8 and 8.7 (Fig. 5A). By comparison, the pH optimum of the enzyme preparation prior to Triton X-100 treatment was 7.2 to 8.0 (Fig. 5B), and the pH optimum for n-lactate oxidation in intact membrane vesicles was 6.2 to 6.8 (Fig. 5C). Of interest was the finding that Tris-chloride buffers significantly altered the activity of intact membrane preparations (Fig. 5C) and enzyme preparations not exposed to Triton X-100 (Fig. 5B), whereas they had negligible effects on the purified enzyme (Fig. 5A).

Specificity, Kinetics, and Product of Reaction—The enzyme was active with n-lactate but not with n-glycerate, L-glycerate, succinate, malate, D-tartrate, L-tartrate, meso-tartrate, L-propanol, or isopropanol at concentrations as high as 0.1 M. NAD, NADP, FMN, and added FAD had no effect on enzyme activity. l-Lactate was a substrate for the purified enzyme, but the $K_m$
TABLE II

| Experiment | Protein | Flavin |
|------------|---------|--------|
| 1          | 1       | 0.93   |
| 2          | 1       | 1.45   |
| 3          | 1       | 1.31   |
| 4          | 1       | 1.25   |
| 5          | 1       | 1.27   |
| 6          | 1       | 1.08   |
| Average    | 1       | 1.21   |

* Measured by colorimetric analyses standardized to albumin and using 75,000 as the molecular weight.

* Fluorometric assay (24).

Fig. 5. pH profile of purified enzyme (A), Step 2 enzyme not exposed to Triton X-100 (B), and membrane vesicles (C). Activity was assayed as described under "Materials and Methods" using the dichlorophenolindophenol assay in A and B, and as D-lactate-dependent oxygen uptake (2) in C.

TABLE III

| Substrate       | Relative rate | K_m (mM) |
|-----------------|---------------|----------|
|                 | Purified enzyme | Step 2 enzyme | Membrane vesicles |
| D-lactate       | 100           | 9.0 × 10^{-4} | 1.4 × 10^{-3} | 1.4 × 10^{-2} |
| L-lactate       | 17            | 1.0 × 10^{-3} | 2.2 × 10^{-2} |

* Based on V_max of purified enzyme.

* These values were obtained using both the dichlorophenolindophenol and tetrazolium enzyme assays described under "Methods."

value was significantly higher and the V_max was significantly lower than that observed with D-lactate (Table III). The specificity for D-lactate was also observed with intact membranes and in enzyme preparations not exposed to Triton X-100, and analogous differences in K_m values for D- and L-lactate also were detected in these preparations.

Pyruvate was the product of the enzymatic reaction using either D-lactate or L-lactate as substrate. In both cases the product reacted with 2,4-dinitrophenylhydrazine and the resultant hydrazone was chromatographed with the authentic hydrazone of pyruvate (Table IV).

Inhibition—p-Chloromercuribenzoate, N-ethylmaleimide, and iodoacetate in concentrations as high as 0.01 M did not affect enzyme activity as measured by dichlorophenolindophenol or tetrazolium dye reduction. The effects of sulfhydryl reagents such as mercaptoethanol or dithiothreitol could not be tested since they interfered with enzyme assays.

The purified enzyme was irreversibly inhibited by treatment with 2-hydroxy-3-butyropropionate as described previously for partially purified enzyme preparations (7). Oxamate and oxalate were competitive inhibitors with K_i values of 3.4 and 0.9 μM, respectively.

DISCUSSION

The membrane-bound D-lactate dehydrogenase in E. coli has been purified to homogeneity as judged by gel electrophoresis and equilibrium ultracentrifugation. The enzyme contains approximately 1 mole of FAD per mole of enzyme and has a molecular weight of approximately 75,000. FAD can be released from the enzyme to yield apoenzyme; however, attempts to reactivate the apoenzyme with FAD have been unsuccessful thus far. This enzyme is different from the pyridine nucleotide-dependent D-lactate dehydrogenase previously described by Tarmy and Kaplan (32, 33) in that it catalyzes the conversion of D-lactate to pyruvate; it is not dependent upon addition of NAD or NADH; it is not inactivated by sulfhydryl inhibitors; and it is localized on the cytoplasmic membrane. Since the pyridine nucleotide-dependent enzyme catalyzes the conversion of pyruvate to D-lactate and the flavin-linked enzyme the conversion of D-lactate to pyruvate, it seems likely that the former enzyme generates D-lactate in the intact cell which is then utilized by the membrane-bound enzyme to drive active transport and perhaps other processes. Alternatively, D-lactate may be produced from methylglyoxal as suggested recently by Cooper and Andersen (34).

The differences in pH optima and K_m values before and after exposure to Triton X-100 may represent conformational or structural alterations resulting from environmental changes, i.e. removal of the enzyme from an organized array of proteins and phospholipids in the membrane to a less organized aqueous environment. Just as likely, these alterations could be the result of tightly bound detergent which could alter the conformation of the enzyme secondary to changes in hydrophobic and hydrogen bond interactions. One final possibility that might account for differences in the K_m values before and after exposure to Triton X-100 could be the localization of the enzyme in or on the membrane in its native state. Diffusion barriers and local pH changes causing kinetic differences in membrane-
bound enzymes have been demonstrated in model systems (35, 36).

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