Membrane-Bound D-Lactate Dehydrogenase from *Escherichia coli*: Purification and Properties†

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ABSTRACT: The membrane-bound enzyme D-lactate dehydrogenase has been purified from *Escherichia coli* W3110*trp*A33. The enzyme from this strain of *E. coli* has been compared with that purified by M. Futai [(1973) *Biochemistry 12*, 2468] and by L. D. Kohn & H. R. Kaback [(1973) *J. Biol. Chem. 248*, 7012] from the ML strain 308-225. The published purification procedures for D-lactate dehydrogenase from the ML strain cannot be used for that from the W strain. However, the present procedure (with minor modification) can be used for the isolation and purification of this enzyme from both strains. The enzyme is purified from sonicated cell extracts and is separated from contaminating proteins by Sephadex G-200 and hydroxylapatite chromatography in the presence of 1% Triton X-100 and 0.1% sodium dodecyl sulfate. Detergent can be removed by acetone precipitation. The activity of the purified detergent-free enzyme is increased fivefold by Triton X-100. The Michaelis–Menten constant of the enzyme in the sonicate is 1.8×10^{-3} M; on addition of Triton X-100, it is $3\text{–}4 \times 10^{-4}$ M, as is that of the purified enzyme either in aqueous solution or in Triton X-100. The pH optimum of the enzyme from both strains is 9–9.5 in both crude extract and purified form, in the presence and absence of Triton X-100. Enzymes from both strains react identically with antibody prepared against enzyme from strain W3110*trp*A33. The purified enzyme is quite heat stable at 60 °C in aqueous solution, but is inactivated more readily in the presence of Triton X-100. Enzyme from the *E. coli* ML 308-225 strain appears somewhat more heat stable than that from strain W3110*trp*A33. Activity of the purified detergent-free enzyme is destroyed by freezing and thawing but can be stable up to 6 months at 4 °C.

As part of a study of interactions among membrane components, we have purified the membrane-bound enzyme D-lactate dehydrogenase of *Escherichia coli*. This enzyme catalyzes the oxidation of D-lactate in electron transfer reactions, coupled to active transport of various amino acids and sugars into *E. coli* cells (Barnes & Kaback, 1971). It has been purified and characterized by Kaback and his co-workers (Kohn & Kaback, 1973) and by Futai (1973), using the ML 308-225 strain of *E. coli*. We have used strain W3110*trp*A33, which requires tryptophan (Drapeau et al., 1968) and can incorporate fluorotryptophan (Pratt & Ho, 1975), a potentially useful spectroscopic marker for study of protein components, and their interactions with lipids. When 4-fluorotryptophan has replaced 75% of the tryptophan in cell protein, D-lactate dehydrogenase shows a specific activity twice that of the control. When 50% of the tryptophan is replaced by 5- or 6-fluorotryptophan, the specific activity is one-half the normal value (Pratt & Ho, 1975). Thus these spectroscopic probes may give information about the active site of the enzyme.

In the course of purifying the protein, it was noted that the detergent Triton X-100, used for solubilization, also increases the enzymatic activity. When the detergent is removed, phospholipids can interact with the enzyme and increase activity. Similar results have been reported by Tanaka et al. (1976) for D-lactate dehydrogenase (D-LDH)[1] isolated from the ML strain. We find that the enzymes from the ML and W strains are essentially identical, biochemically and immunologically, so that work done with the two systems should be directly comparable. However, the purification procedures worked out for the enzyme from the ML strain could not be used for the W strain.

D-Lactate dehydrogenase may be considered an integral membrane protein (Singer, 1974); since detergents are required to solubilize it, it associates with phospholipids when solubilized and forms aggregates in neutral aqueous buffers (Kohn & Kaback, 1973; Futai, 1973; Tanaka et al., 1976; Fung et al., 1979). Although lipids do not appear to be required for enzymatic activity, phospholipids and detergents increase activity up to tenfold beyond that in aqueous solution. Thus this should be an excellent system in which to study interactions between membrane lipids and proteins. Biochemical and biophysical studies of protein–lipid interactions are described in the following paper (Fung et al., 1979).

Experimental Section

Growth of Bacteria. Strains used were *E. coli* W3110*trp*A33, obtained from Dr. C. Yanofsky, and *E. coli* ML 308-225, obtained from Dr. H. R. Kaback. The medium used was M-9 (Miller, 1972) supplemented with 1% casein hydrolysate (Peptone No. 5, Gibco Diagnostics), 0.4% succinate, and 10^{-4} M L-tryptophan. Bacteria were grown in 5-gal carboys, containing 15 L of medium, with aeration through two fritted filter tubes, $^5/_8 \times 4$ in. Turbidity was measured with a Fisher electrophotometer at 525 nm. A reading of 8 corresponds to 1×10^8 bacteria/mL. Cells were harvested by centrifugation at 13000*g* for 10 min and washed once with 0.01 M Tris, pH 7.2, containing 0.01% β-mercaptoethanol.

Assay of D-Lactate Dehydrogenase. The assay mixture contained 0.1 M potassium phosphate, at pH 8.0, 10 mM D-lactate, 60 μg of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and 120 μg of phenazine methosulfate (PMS; Futai, 1973; Kohn & Kaback, 1973). In

† From the Department of Biological Sciences, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received April 18, 1978.* Supported by research grants from the National Institutes of Health (GM-18698) and the National Science Foundation (PCM 76-21469). L.W.-M.F. was a recipient of a National Research Service Award, National Institutes of Health (GM-05164), during 1975–1977. This paper was presented in part at the 61st Annual Meeting of the Federation of American Societies for Experimental Biology, April 3–8, 1977, Chicago, Illinois.

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[1] Abbreviations used: D-LDH, D-lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; DNase, deoxyribonuclease; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/79/0418-0312$01.00/0

addition, 1% Triton X-100 (Rohm and Haas) was added routinely to bring the volume to 1 mL (usually 0.58 mL of Triton or a concentration of 0.58% Triton in the assay mixture). The reaction was started by addition of an appropriate dilution of enzyme. The increase in absorbance at 570 nm was followed for 3 min at 23 °C in a Zeiss PMQ II spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme giving a reduction of 1 μmol of MTT per min, using an extinction coefficient of 17 mM^{-1} cm^{-1} (Kistler & Lin, 1971). Specific activity is expressed as units per mg of protein. Protein was measured by the method of Lowry et al. (1951) with 0.3% sodium dodecyl sulfate ($NaDodSO_4$) added to the alkaline copper tartrate to solubilize the precipitate formed in the presence of Triton X-100 (Yu & Steck, 1975).

Preparation of Antibody to D-*Lactate Dehydrogenase.* Antiserum was prepared in two young adult (2.5 kg) San Juan strain rabbits. Each rabbit was injected with a total of approximately 0.65 mg of enzyme purified from the W3110*trp*A33 strain of *E. coli*. The immunization schedule was as follows: 0.27 mg of enzyme per rabbit was incorporated into complete Freund adjuvant (Difco) and injected into the toe pads of both hind feet. After 28 days, 0.22 mg of enzyme per rabbit was incorporated into incomplete Freund adjuvant [equal volumes of aqueous enzyme and a mixture of 3 parts of Arlacel-A (Sigma) and 7 parts of mineral oil (Marcol 52, Imperial Oil Co.)]. The very firm emulsion was injected into the subscapular region. A trial bleeding 10 days later gave sera which reacted with the immunizing antigen. To increase the antibody content of the sera, a third injection in incomplete Freund adjuvant of 0.16 mg of protein per rabbit was given again in the subscapular region, 19 days following the second injection. The rabbits were bled 10 days later and again 4 days later. The sera from these final bleedings were pooled from both animals and used for all experiments.

Immunodiffusion. Commercially prepared agar plates (type I, Cordis Laboratories) were used for double immunodiffusion experiments according to the method of Wadsworth (1957).

Other Procedures. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was carried out according to the method of Studier (1973).

The ammonium cobalt thiocyanate assay of Garewal (1973) was used to measure Triton X-100.

Visible absorption spectra were measured with an Aminco Model DW-2 spectrophotometer.

Materials. The following were obtained from Sigma: D-lactate (lithium salt), MTT, PMS, DNase, RNase. 2-Methyllactic acid and methyl DL-lactate were obtained from Eastman. DE52 was obtained from Whatman, Sephadex G-200 from Pharmacia, hydroxylapatite from Bio-Rad, and Triton X-100 from Rohm and Haas. Other chemicals were reagent grade from commercial sources and were used without further purification.

Results

Growth of Bacteria. When bacteria in stationary phase are inoculated into fresh medium, the specific activity of D-lactate dehydrogenase increases fourfold during early growth and then remains constant (Figure 1). At the end of exponential growth, the specific activity gradually declines again. Under our conditions, the cells had to be harvested in exponential phase, at a density of less than 2×10^9 cells/mL. Above this level, although the specific activity was the same, there was excessive loss of enzyme during the first steps of the purification, suggesting a change in the environment of the enzyme. Cells from four carboys, or 60 L of cells (240 g of wet cell paste), were grown and purified at one time.

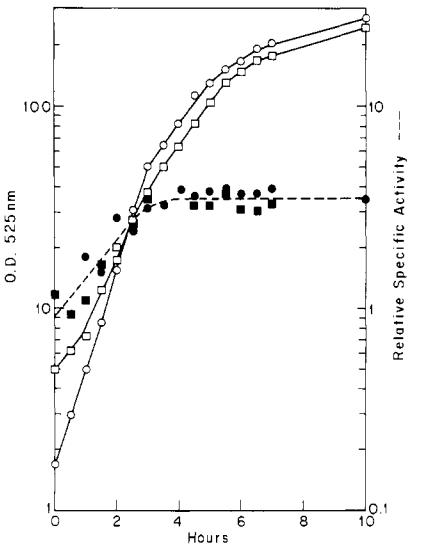

FIGURE 1: Increase during cell growth in OD and D-lactate dehydrogenase activity of *E. coli* ML 308-225 and W3110*trp*A33. OD: ML 308-225 (○—○); W3110*trp*A33 (□—□). Specific activity: ML 308-225 (●- - -●); W3110*trp*A33 (■- - -■).

Purification. All steps were carried out at 0–4 °C unless otherwise stated. Washed cells (240 g) were immediately resuspended in 800 mL of 0.01 M Tris-HCl at pH 8.0 containing 0.01% β-mercaptoethanol. Two-hundred-milliliter batches were sonicated for 10 min with a Sonifier Cell Disrupter (Heat Systems Co.) at the highest setting of the standard probe. The pooled sonicates were adjusted to pH 8 with 3.5% ammonium hydroxide, and 169.4 g of ammonium sulfate was added (30% saturation), while maintaining the pH at 8. After 30 min of stirring, the mixture was centrifuged at 39000*g* for 30 min. The pellet was resuspended in 400 mL of the Tris-HCl buffer. One gram of deoxycholate and 5.88 g of NaCl in 160 mL of H_2O were added to the suspension, which was then stirred for 30 min and centrifuged at 39000*g* for 30 min. The supernatant was brought to 0.02 M with $MgCl_2$, treated with 1 μg/mL each of DNase and RNase, and incubated for 2 h at 37 °C. To the chilled preparation, 0.7 volume of acetone at 0 °C was added and the mixture was immediately centrifuged at 13000*g* for 10 min. The pellet was resuspended in 120 mL of 0.05 M sodium phosphate buffer, pH 7.2, containing 0.01% β-mercaptoethanol (buffer A) and recentrifuged at 39000*g* for 10 min. The pellet was resuspended in 120 mL of buffer A plus 0.84 g of deoxycholate and stirred for 2 h. Six milliliters of 8 M sodium perchlorate was added; the mixture was stirred for 30 min and centrifuged at 39000*g* for 30 min. The supernatant was dialyzed overnight against 4 L of buffer A containing 1% Triton (buffer B).

DE52 Column Chromatography. The dialyzed acetone fraction was applied to a 2.6 × 35 cm DE52 anion-exchange column equilibrated with buffer B and then washed with 600 mL of buffer B containing 2.4 g/L of NaCl. The activity was eluted with 500 mL of buffer B containing 7.3 g of NaCl. Fractions containing more than 6 units/mL were pooled and precipitated with an equal volume of acetone at 0 °C. The precipitate was recovered by centrifugation at 13000*g* for 10 min and resuspended in approximately 10 mL of buffer B containing 0.1% $NaDodSO_4$ (buffer C) and dialyzed against 1 L of buffer C for 6 h.

Sephadex G-200. The concentrated fraction from the DE52 column was applied to a 2.2 × 110 cm column of Sephadex G-200 equilibrated with buffer C, and the activity was eluted

Table I: Purification of D-Lactate Dehydrogenase from 250 g of Wet Cell Paste of *E. coli*

fraction	W3110*trp*A33 total units	W3110*trp*A33 units per mL	W3110*trp*A33 total protein (mg)	W3110*trp*A33 protein (mg/mL)	W3110*trp*A33 yield of enzyme (%)	W3110*trp*A33 sp act. (units/mg)	ML 308-225 sp act. (units/mg)
sonicate	2560	3.2	19 600	24.5	100	0.13	0.12
ammonium sulfate	2360	6.0	9 800	24.5	88	0.24	0.23
deoxycholate	2120	4.2	3 131	6.2	83	0.68	0.35
acetone	1620	15.6	426	4.1	63	3.8	4.5
DE52	1130	19.5	122	2.1	44	9.3	29.7
Sephadex G-200	880	23.3	19	0.5	34	46.6	151
hydroxylapatite	620	20.6	3	0.1	24	206	230

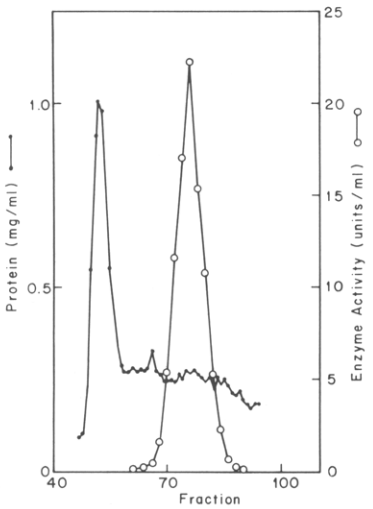

FIGURE 2: Sephadex G-200 chromatography of D-lactate dehydrogenase in the presence of 1% Triton X-100 and 0.1% $NaDodSO_4$. Each fraction contained 3.8 mL and was eluted in 10–15 min.

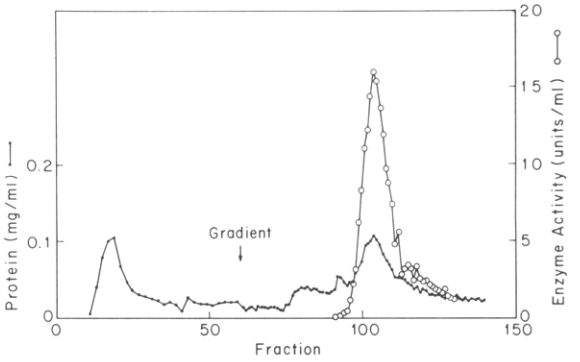

FIGURE 3: Hydroxylapatite chromatography of D-lactate dehydrogenase in the presence of 1% Triton X-100 and 0.1% $NaDodSO_4$. Each fraction contained 2.5 mL and was eluted in 10–15 min.

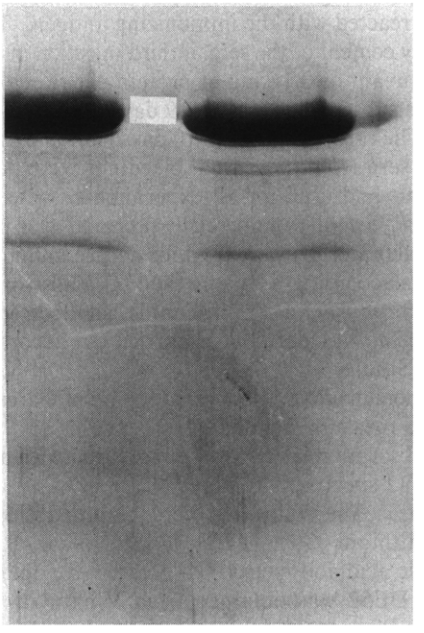

FIGURE 4: Polyacrylamide gel electrophoresis of D-lactate dehydrogenase purified from *E. coli* ML 308-225 (left) and W3110*trp*A33 (right).

wih buffer C. A cloudy yellowish material was eluted at the void volume, before the peak of enzyme activity (Figure 2). Fractions containing more than 6 units/mL were pooled.

Hydroxylapatite. The pooled fractions from the Sephadex G-200 column were applied to a 2.6 × 20 cm hydroxylapatite column equilibrated with buffer C. The column was washed with 250 mL of buffer C and the activity was eluted with a gradient prepared with 250 mL of buffer C in the mixing chamber and 250 mL of buffer C plus 0.2 M potassium phosphate, pH 7.2, in the reservoir (Figure 3). Fractions containing more than 6 units/mL of enzyme were pooled and precipitated with an equal volume of acetone. After centrifugation at 39000*g* for 30 min, the pellet was resuspended in the smallest possible volume of 0.05 M sodium phosphate, pH 7.2, plus 0.01% β-mercaptoethanol and dialyzed for 6 h against 1 L of the same buffer. The concentrated enzyme was destroyed by repeated freezing and thawing but could remain stable for 6 months at 4 °C.

The purification of D-LDH from 240 g of wet cell paste of strain W3110*trp*A33 is summarized in Table I. There is a 2000-fold purification with 20% recovery of enzyme. All assays of purification steps contained 0.58% Triton X-100.

Purification of D-Lactate Dehydrogenase from Strain ML 308-225. Table I shows that the methods worked out for purification of enzyme from W3110*trp*A33 worked just as well for enzyme from ML 308-225, if not even better. The reverse was not true, however. As shown by Futai (1973) and by Kohn & Kaback (1973), DEAE column chromatography is the method of choice for purifying the enzyme from the ML strain. The enzyme from the W strain could not be purified further using DE52 column chromatography, and a gradient was no more successful than the single stepwise column. The following modifications were found necessary for the purification of enzyme from the ML strain: (i) 37% saturation with ammonium sulfate instead of 30%; (ii) precipitation with 0.8 volume of acetone instead of 0.7 volume; and (iii) dilution of the dialyzed acetone fraction with an equal volume of buffer B before application to the DE52 column.

Visible Absorption Spectrum. The visible absorption spectrum of the enzyme purified from W3110*trp*A33 shows a broad peak at around 450 nm and a shoulder at around 480 nm, suggesting a flavoprotein. This is identical with the results found by Futai (1973) and by Kohn & Kaback (1973) for enzyme from the ML strain.

Polyacrylamide Gel Electrophoresis. Bands formed by polyacrylamide gel electrophoresis in the presence of

Table II: Values of Michaelis-Menten Constant, K_m, for D-Lactate Dehydrogenase

	K_m (mol/L)	
	W3110*trp*A33	ML 308-225
sonicate		
H_2O	1.8×10^{-3}	1.9×10^{-3}
Triton	3×10^{-4}	4×10^{-4}
purified enzyme		
H_2O	4×10^{-4}	3×10^{-4}
Triton	4×10^{-4}	3×10^{-4}

$NaDodSO_4$ are shown in Figure 4. Enzymes from the two strains give identical bands, with a molecular weight around 75000. The estimated purity of the D-LDH is 90–95%.

Immunological Characterization. Immunodiffusion of antiserum made to the enzyme from the W strain against the same enzyme produced two precipitin lines. When enzyme from ML was placed in a neighboring well, precipitin lines formed with both antigens and fused, indicating antigenic identity of both precipitin systems.

To show that one of the precipitin lines represented the enzyme–antienzyme system, the plastic template of the immunodiffusion plate was removed, the agar layer was briefly washed with 0.9% NaCl, and then the substrate mixture used for enzyme assay was poured over the surface of the plate. In a brief time the purple reaction product was visible around the enzyme wells as a zone ending sharply over the precipitin line closer to the antiserum well. Thus this precipitin line indicates the enzyme–antienzyme system; the other precipitin line is presumably an antigenic contaminant, although identical for both strains.

Effect of Detergents on Enzyme Activity. In the final acetone-precipitated hydroxylapatite fraction, Triton X-100 could not be detected by the method of Garewal (1973), indicating less than 0.005% Triton remaining, or less than 6 mol of Triton X-100 per mol of protein. The amount of $NaDodSO_4$ remaining was not measured but is expected to be low also. The concentrated enzyme was diluted 1:5000 in H_2O for assay. When Triton X-100 was omitted from the assay solution, the activity was three- to fivefold lower than in the presence of 1% Triton as shown also by Tanaka et al. (1976). The detergent Lubrol at a concentration of 2% enhanced the basal activity up to sixfold. In the sonicate, activity was increased about twofold by Triton X-100. The effect of phospholipids in enhancing enzyme activity will be discussed in the following paper (Fung et al., 1979).

Optimum pH. The optimum pH for the oxidation of D-lactate was between 9 and 9.5 for D-LDH from both ML and W strains, in the presence and absence of Triton, in both the sonicate and purified form (Figure 5). Assays were routinely done at pH 8, following Futai (1973) and Kohn & Kaback (1973).

Kinetics. The values of the Michaelis–Menten constant (K_m) for D-lactate are shown in Table II. The same value of $3\text{–}4 \times 10^{-4}$ M is found for purified enzymes from both strains in the presence and absence of Triton. The K_m value in sonicates without Triton added is higher as noted also by Tanaka et al. (1976) and Kohn & Kaback (1973) for enzyme in membrane vesicles. The compound 2-methyllactate is not a substrate and inhibits only about 10% at 0.1 M. Methyl DL-lactate is a substrate at 0.1 M, with V_{max} almost equal to that of D-lactate.

Heat Inactivation. Concentrated enzyme (1 mg/mL) diluted 1:50 into H_2O and incubated at 60 °C was remarkably stable, losing 35% of its activity in an hour, when assayed by a further 1:50 dilution into an assay solution containing Triton.

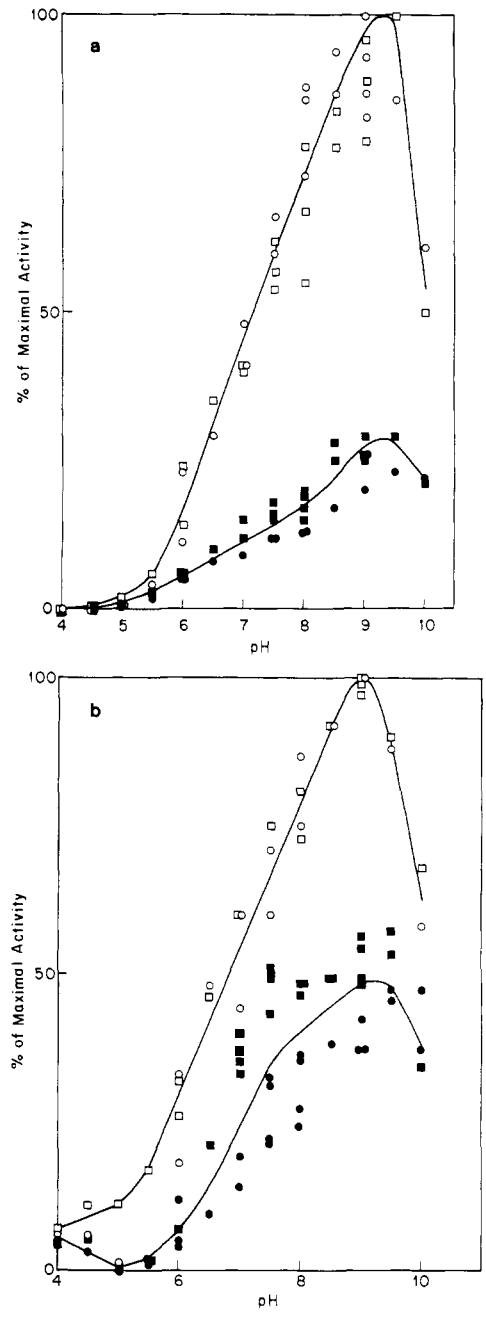

FIGURE 5: pH profile of (a) purified enzyme and (b) sonicate, in presence (top curve) and absence (bottom curve) of Triton X-100. *E. coli* ML 308-225 (O—O or ●—●); *E. coli* W3110*trp*A33 (□—□ or ■—■). Buffers used were: pH 4–6, sodium acetate; pH 6–8, potassium phosphate, pH 7–9, Tris-HCl; pH 9–10, glycine.

On the other hand, activity was lost rather rapidly when dilution was made into 1% Triton, followed by incubation at 60 °C. D-LDH from the ML strain seemed to be somewhat more stable than that from the W strain (Figure 6).

Discussion

We have purified the D-lactate dehydrogenase from *E. coli* strain W3110*trp*A33 and compared it with the enzyme derived from strain ML 308-225, on which previous work has been carried out. Our purification differs from that of Kohn & Kaback (1973) and Futai (1973) in that the starting material is a sonicate rather than a homogenate or membrane preparation and DE52 column chromatography in the presence of Triton X-100 could not be used for final separation of the enzyme from contaminating proteins. It was necessary to use

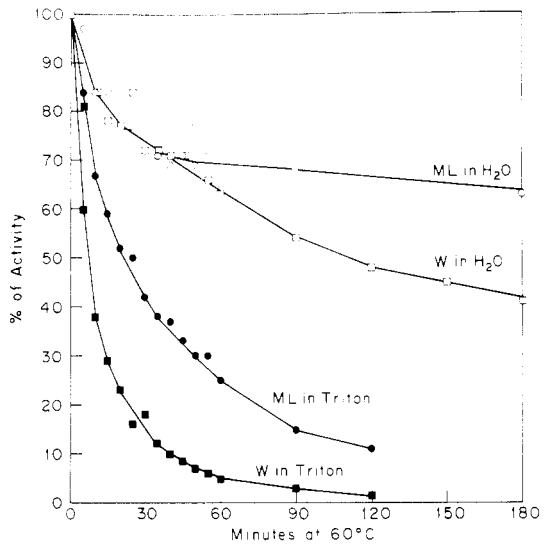

FIGURE 6: Heat inactivation of purified D-lactate dehydrogenase. Purified detergent-free enzyme (1 mg/mL) was diluted 1:50 into water or 1% Triton X-100 and incubated at 60 °C. Aliquots were taken at intervals and diluted 1:50 into standard assay solution containing Triton.

Sephadex G-200 and hydroxylapatite in the presence of 1% Triton X-100 and 0.1% $NaDodSO_4$. This method was successful with enzyme from either strain ML 308-225 or W3110*trp*A33 prepared from sonicated cell extracts. Differences from the purifications worked out by Kohn & Kaback (1973) and Futai (1973) for enzyme from strain ML 308-225 suggest differences in the environment of the enzyme, possibly as a result of different forms of cell extract preparation or in the relationship of lipopolysaccharides to the inner membrane. Thus membrane vesicles prepared from strain ML 308-225 have less contamination with lipopolysaccharides than those prepared from other strains (Kaback, 1972). Once purified, enzymes from the two strains appear to be biochemically and immunologically identical, although differences in heat inactivation suggest that there may be some amino acid substitutions or binding of other cell components to the enzyme. Nevertheless, it appears that work done with the two systems should be directly comparable.

The membrane-bound D-lactate dehydrogenase of *E. coli* is an important component of the cytoplasmic membrane, involved in the respiratory chain and active transport. It is distinct from the cytoplasmic D-lactate dehydrogenase described by Tarmy & Kaplan (1968) and from the inducible membrane-bound L-lactate dehydrogenase purified by Futai & Kimura (1977). It is functional in membrane vesicles, and vesicles from a mutant lacking active enzyme can be reconstituted for active transport by addition of the purified enzyme (Futai, 1974; Short et al., 1974). Although essentially as active in supporting transport as normal membrane vesicles, the reconstituted vesicles apparently have enzyme attached to the outside rather than to the normal site on the inside surface (Futai & Tanaka, 1975; Short et al., 1975). Nevertheless this may be a useful system for studying structure–function relationships in membranes.

Triton X-100 used in purification of D-LDH can be rapidly removed to a level less than 0.005% and the enzyme concentrated by precipitation with acetone, so that studies may be made of the properties of the enzyme in aqueous solution and of the effects of lipids and detergents. Activity of the purified detergent-free enzyme is enhanced by phospholipids, suggesting an important role for lipid–enzyme interaction in the membrane. Thus this enzyme should be a useful one with which to study membrane protein–lipid interaction. Biochemical and magnetic resonance studies of D-lactate dehydrogenase and phospholipids are reported in the following paper (Fung et al., 1979).

Acknowledgments

We are indebted to Dr. G. W. Stemke for preparing antibody and carrying out the immunodiffusion experiments. We wish to thank Dr. Lap-Chee Tsui for the polyacrylamide gel electrophoresis of our samples and Dr. C. Yanofsky and Dr. H. R. Kaback for providing bacterial strains.

References

Barnes, E. M., Jr., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5518.
Drapeau, G. R., Brammar, W. J., & Yanofsky, C. (1968) *J. Mol. Biol. 35*, 357.
Fung, L. W.-M., Pratt, E. A., & Ho, C. (1979) *Biochemistry 18* (following paper in this issue).
Futai, M. (1973) *Biochemistry 12*, 2468.
Futai, M. (1974) *Biochemistry 13*, 2327.
Futai, M., & Tanaka, Y. (1975) *J. Bacteriol. 124*, 470.
Futai, M., & Kimura, H. (1977) *J. Biol. Chem. 252*, 5820.
Garewal, H. S. (1973) *Anal. Biochem. 54*, 319.
Kaback, H. R. (1972) *Biochim. Biophys. Acta 265*, 367.
Kistler, W. S., & Lin, E. C. C. (1971) *J. Bacteriol. 108*, 1224.
Kohn, L. D., & Kaback, H. R. (1973) *J. Biol. Chem. 248*, 7012.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Miller, H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Pratt, E. A., & Ho, C. (1975) *Biochemistry 14*, 3035.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1461.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291.
Singer, S. J. (1974) *Annu. Rev. Biochem. 43*, 805.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237.
Tanaka, Y., Anraku, Y., & Futai, M. (1976) *J. Biochem. (Tokyo) 80*, 821.
Tarmy, E. M., & Kaplan, N. O. (1968) *J. Biol. Chem. 243*, 2579.
Wadsworth, C. (1957) *Int. Arch. Allergy Appl. Immunol. 10*, 355.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9170.