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A BIOCHEMICAL STUDY OF THE RECONSTITUTION OF D-LACTATE DEHYDROGENASE-DEFICIENT MEMBRANE VESICLES USING FLUORINE-LABELED COMPONENTS

E.A. PRATT, JUDITH A. JONES, PATRICIA F. COTTAM, SUSAN R. DOWD and CHIEN HO *

Department of Biological Sciences, Carnegie-Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213 (U.S.A.)

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Fluorine-19 labeled compounds have been incorporated into lipids and proteins of *Escherichia coli*. ^{19}F-Labeled membrane vesicles, prepared by growing a fatty acid auxotroph of a D-lactate dehydrogenase-deficient strain on 8,8-difluoromyristic acid, can be reconstituted for oxidase and transport activities by binding exogenous D-lactate dehydrogenase. ^{19}F-Labeled D-lactate dehydrogenases prepared by addition of fluorotryptophans to a tryptophan-requiring strain are able to reconstitute D-lactate dehydrogenase-deficient membrane vesicles. Thus, lipid and protein can be labeled independently and used to investigate protein-lipid interactions in membranes.

Introduction

Many studies indicate that protein-lipid interactions are important for the activity, organization, and regulation of enzymes in membranes, and suggest that studies of protein-lipid interaction at a molecular level are necessary for understanding the reaction mechanisms involved [1–4]. As part of our program of investigating structure-function relationships in biological membranes by biochemical and biophysical methods, we have been studying the membrane-bound enzyme D-lactate dehydrogenase (EC 1.1.2.4) [5,6], whose activity is enhanced by phospholipids [7–9]. D-Lactate dehydrogenase catalyzes the oxidation of D-lactate in electron transfer reactions coupled to active transport of various amino acids and sugars [10]. These activities can be reconstituted by addition of purified enzyme to D-lactate dehydrogenase-deficient membrane vesicles prepared from *Escherichia coli* strain ML 308-225 *dld*-3 [11,12]. This system allows the possibility of studying the interaction of lipid and protein by labeling independently the membrane lipids and the exogenous D-lactate dehydrogenase. Since the fluorine-19 nucleus can be a useful probe for nuclear magnetic resonance studies of biological molecules [13–22], we have developed methods to incorporate ^{19}F-labeled components into either lipids or proteins of *E. coli* [17–19,23].

Isolation of unsaturated fatty acid auxotrophs has made possible studies of the effects of changing the lipid composition of the membrane [24,25]. An unsaturated fatty acid auxotroph of a D-lactate dehydrogenase-deficient strain has been used to study the effect on reconstitution of changing the lipid composition [26]. We have isolated such a strain and used it to incorporate a ^{19}F-labeled myristic acid into the membrane lipids of this organism [18,19]. Thus, we can prepare ^{19}F-labeled D-lactate dehydrogenase-deficient membrane

* To whom all correspondence should be addressed.

Abbreviations: MTT, 3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate.

vesicles. In addition, we can incorporate 4-, 5-, and 6-fluorotryptophans into the proteins of *E. coli* strain W3110*trp*A33 and isolate ^{19}F-labeled D-lactate dehydrogenase [23]. We have tested these ^{19}F-labeled components to establish whether they are functional in reconstitution of D-lactate dehydrogenase-deficient membrane vesicles.

Materials and Methods

Materials. The fluorinated fatty acid, 8,8-difluoromyristic acid, was synthesized as described previously [18]. Palmitoleic acid was obtained from Nu-Chek Prep, ^{3}H-labeled proline from New England Nuclear, and 4-, 5-, and 6-fluorotryptophans, Brij 58, phenazine methosulfate (PMS), and 3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide (MTT) from Sigma. Triton X-100 was obtained from Rohm and Haas. Other chemicals were reagent grade and were used without further purification.

Bacterial strains. Escherichia coli ML 308-225 [27] and ML 308-225 *dld*-3 [28] were gifts of Dr. H.R. Kaback. The unsaturated fatty acid-requiring mutants ML 308-225 *ufa*-8 and ML 308-225 *dld*-3 *ufa*-2 were isolated following mutagenesis with nitrosoguanidine as described in Ref. 19. W3110*trp*A33, which is a tryptophan auxotroph [29], was the gift of Dr. C. Yanofsky.

Preparation of membrane vesicles. Bacteria were grown in a medium containing 60 mM potassium phosphate buffer (pH 7), 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7\ H_2O$, and 20 mM sodium succinate. Fatty acids were added to the sterile medium at a final concentration of 100 mg/l from a 10% stock in 50% ethanol. Brij 58 was added to a concentration of 300 mg/l. Cells were grown at 37°C with aeration by shaking to a density of 0.5 at 550 nm measured on a Zeiss PMQ II spectrophotometer. A density of 1.0 is equivalent to $1.3 \cdot 10^9$ ML 308-225 cells/ml. To prepare fluorine-labeled membrane vesicles, fatty acid-requiring cells were grown to a density of 0.5 in medium containing oleic or palmitoleic acid, centrifuged, resuspended in medium containing 25 mg/l of 8,8-difluoromyristic acid and reincubated until doubled in density. Membrane vesicles were prepared from all strains essentially as described by Kaback [30] and stored frozen at −80°C.

Preparation of D-lactate dehydrogenase. For preparation of D-lactate dehydrogenase, cells were grown in M-9 medium [31] with 0.4% succinate supplemented with 1% casamino acids (Gibco), and 10^{-4} M tryptophan when needed, to a density of 3. To incorporate fluorine, cells of W3110*trp*A33 were grown to a density of 0.5, centrifuged, resuspended in the same medium with 4-, 5-, or 6-fluorotryptophan in place of tryptophan, and reincubated for 5 h [23]. Enzyme was prepared as described by Pratt et al. [32] through the DE52 column step. Pooled DE52 fractions were precipitated with an equal volume of acetone to remove Triton X-100 used in the purification process, resuspended in a minimum volume of 50 mM potassium phosphate buffer (pH 7.2), and dialyzed against the same buffer containing 0.01% mercaptoethanol for 6 h. The dialyzed enzyme was filtered through a Metricel Ga-6 0.45 μm filter (Gelman Sciences, Inc.) and stored frozen at −80°C.

Assays. D-Lactate dehydrogenase and succinate dehydrogenase were assayed as described previously [32] using phenazine methosulfate and MTT in the presence of Triton X-100, at pH 8 and 21°C.

Protein was measured by the method of Lowry et al. [33] with bovine serum albumin as standard.

Lipid analysis. Lipids were extracted from membrane vesicles by the method of Bligh and Dyer as described by Ames [34], and resolved into individual species by thin-layer chromatography on silica gel (Type 0, New England Nuclear) using chloroform/methanol/water (65 : 25 : 4, v/v), and visualized with iodine vapor. Fatty acids were determined as their methyl esters by gas chromatography (Perkin-Elmer, Sigma 3).

Reconstitution of membrane vesicles. Membrane vesicles were reconstituted essentially as described by Short et al. [12]. Enzyme was mixed with 0.02 ml of 1 M guanidine-HCl and incubated 10 min at 23°C. Membrane vesicles (0.3 mg protein) were added and enough 100 mM potassium phosphate buffer (pH 6.6) to bring the volume to 1.0 ml. After 30 min incubation at 30°C, the membranes were centrifuged at $20000 \times g$ for 10 min. The supernatants were decanted and the pellets resuspended in 1 ml of 100 mM potassium phosphate buffer (pH 6.6).

Oxidase assay. Oxidase activity was determined with a Clark-type oxygen electrode (YSI 5331) inserted into a jacketed 3 ml reaction chamber (Johnson Research Foundation) maintained at 37°C. The reaction mixture contained approx. 0.3 mg vesicle protein, 100 mM potassium phosphate buffer (pH 6.6), 10 mM $MgSO_4$, and 16.7 mM D-lactate or succinate, or 1.4 mM NADH.

Transport assay. Transport of [^{3}H]proline into membrane vesicles was measured at 23°C essentially as described by Kaback [30]. A 1 ml reaction mixture contained 0.4–0.5 mg membrane protein, 100 mM potassium phosphate buffer (pH 6.6), 10 mM $MgSO_4$, 20 mM D-lactate, 20 μm proline, 1 μC [^{3}H]proline, and D-lactate dehydrogenase as indicated. The reaction was started by addition of D-lactate, and 0.1 ml aliquots were diluted into 100 mM LiCl, filtered, washed with 100 mM LiCl, and counted in a Beckman LS 7000 liquid scintillation counter.

Results

Binding and reconstitution with unlabeled components

Fig. 1A shows binding of D-lactate dehydrogenase prepared from strain ML 308-225 to membrane vesicles of strains ML 308-225 and ML 308-225 *dld*-3. There is no difference in binding between the two strains. At least 6 units/mg membrane protein can be bound, or 12-times the level normally found in membrane vesicles of ML 308-225.

Fig. 1B shows increase in oxidase activity in the membrane vesicles containing bound D-lactate dehydrogenase. The two strains show equal increases in oxidase activity for units of D-lactate dehydrogenase bound, and oxidase activity rises to levels 7–8-times that measured in membrane vesicles of ML 308-225 without added D-lactate dehydrogenase. This suggests that the oxidase system is not saturated by the electrons fed into it from the D-lactate dehydrogenase normally present. Table I shows that oxidase activity supported by D-lactate, succinate, and reduced nicotinamide adenine dinucleotide (NADH) is also additive.

TABLE I

OXIDASE ACTIVITY OF MEMBRANE VESICLES OF STRAIN ML 308-225

Substrate	Oxidase activity (ng O_2/min/mg)
D-Lactate	37
Succinate	42
NADH	376
D-Lactate + succinate	91
D-Lactate + succinate + NADH	479

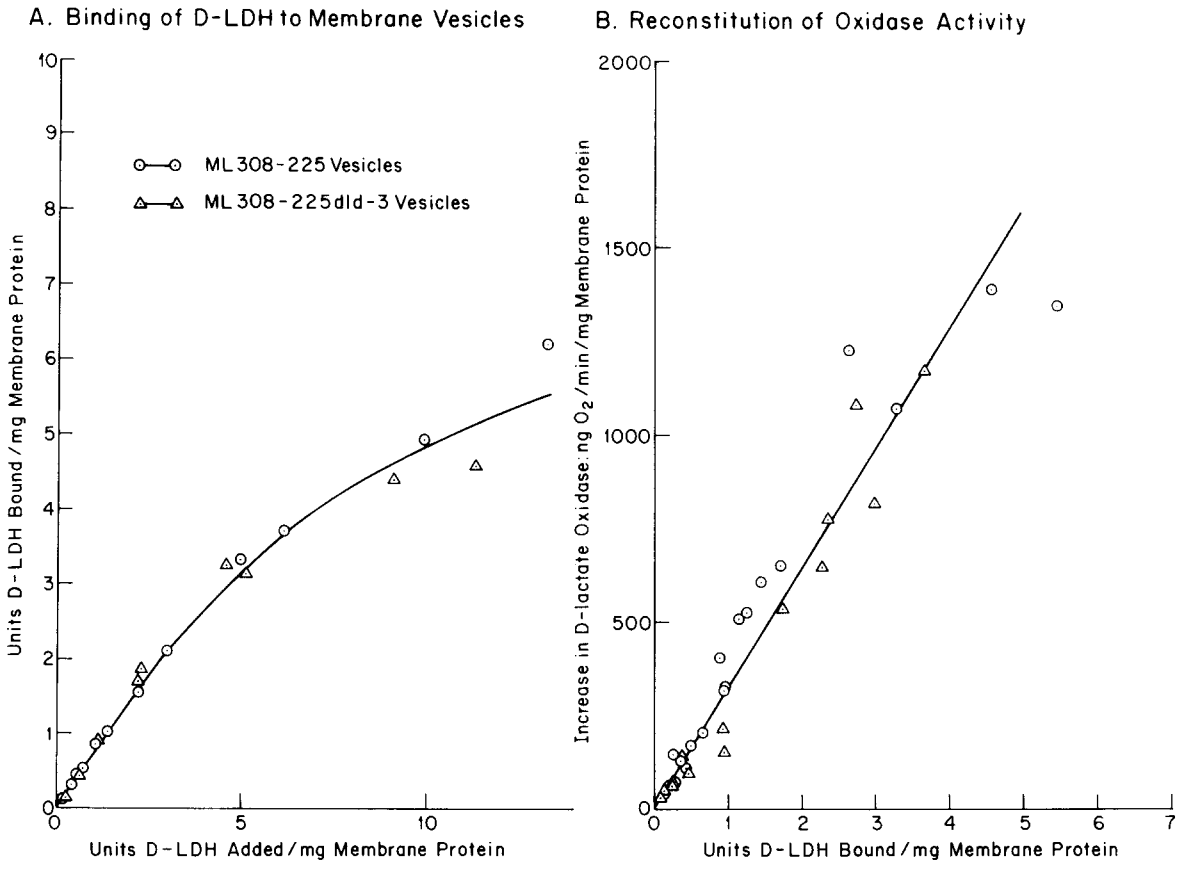
Fig. 1C illustrates uptake of [^{3}H]proline in the presence of increasing amounts of added D-lactate dehydrogenase. The effect is very different from that with oxidase activity. There is little, if any, increase in accumulation of [^{3}H]proline by ML 308-225 membrane vesicles when extra D-lactate dehydrogenase is added, and, in fact, transport is inhibited in these membrane vesicles by levels of enzyme which increase transport activity in ML 308-225 *dld*-3 membrane vesicles. The maximum steady-state level of accumulation of [^{3}H]proline in ML 308-225 *dld*-3 membrane vesicles is 2–3-times the level measured in ML 308-225 membrane vesicles without added D-lactate dehydrogenase.

Incorporation of 8,8-difluoromyristate into lipids

Table II shows the fatty acid composition of membranes prepared from parent cells and from fatty acid-requiring mutants grown in oleate or palmitoleate and transferred to fluorinated fatty acid for one doubling. About 10–20% of the fatty acids become fluorinated. This amount can be increased by longer growth on the fluorinated fatty acid, but the cells become increasingly fragile and difficult to work with [19].

Fig. 2A shows the binding of D-lactate dehydrogenase from strain ML 308-225 to the membrane vesicles described in Table II. Membrane vesicles from the parent strains ML 308-225 and ML 308-225 *dld*-3 bind enzyme better than the fatty acid-requiring mutants. Incorporation of 8,8-difluoromyristate somewhat decreases binding to membrane vesicles from oleic acid-grown cells. Membrane vesicles prepared from palmitoleic acid-grown cells do not bind enzyme as well, and incorporation of 8,8-difluoromyristate increases binding (data not shown).

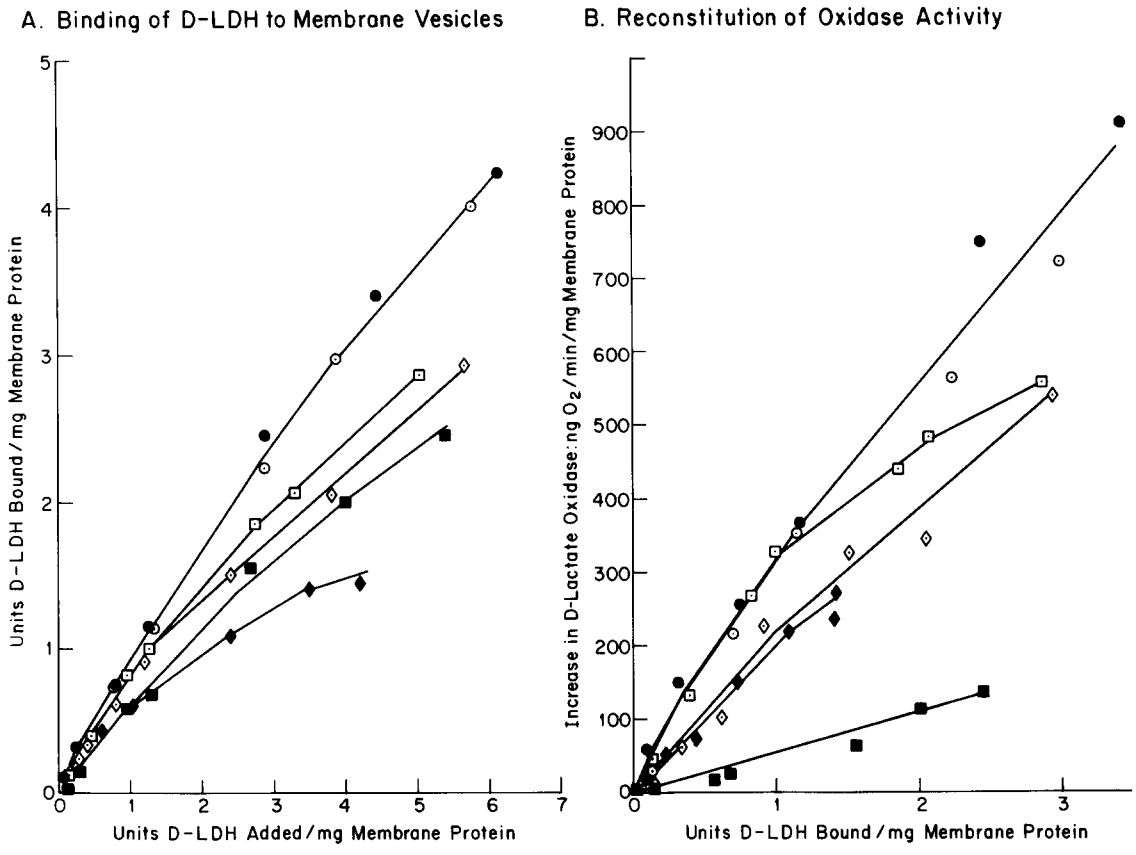
If these membrane vesicles with varying

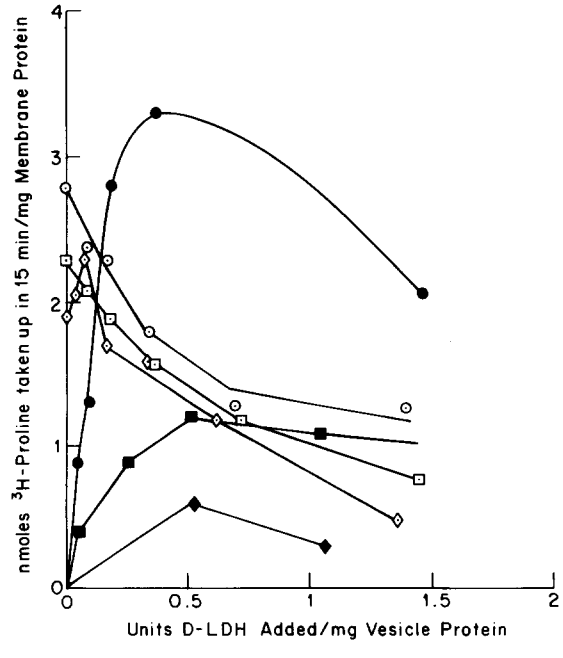


amounts of bound D-lactate dehydrogenase are used for oxidase assays, the results are as shown in Fig. 2B. For a given amount of enzyme bound, the parent strains show greater D-lactate oxidase activity than the fatty acid-requiring mutants. Membrane vesicles from *ufa*-8 cells grown in oleic acid and transferred to 8,8-difluoromyristate function almost as well as those grown in oleic acid. Membrane vesicles from *ufa*-2 cells grown in oleic acid are less effective and their oxidase activity is increased by incorporation of fluorine. Membrane vesicles from cells grown in palmitoleic acid also show low activity, and an increase in activity on incorporation of fluorine (data not shown).

As shown in Fig. 2C, *ufa*-8 membrane vesicles resemble those from the parent ML 308–225 in showing little or no increase in transport activity on addition of exogenous D-lactate dehydrogenase.

Fig. 1. Reconstitution of D-lactate dehydrogenase-dependent activities with unlabeled components. (A) Binding of D-lactate dehydrogenase to membrane vesicles. (B) Reconstitution of oxidase activity. (C) Reconstitution of proline transport. D-Lactate dehydrogenase from *E. coli* ML 308-225. Membrane vesicles from: ○, ML 308-225; △, ML 308-225 *dld*-3.



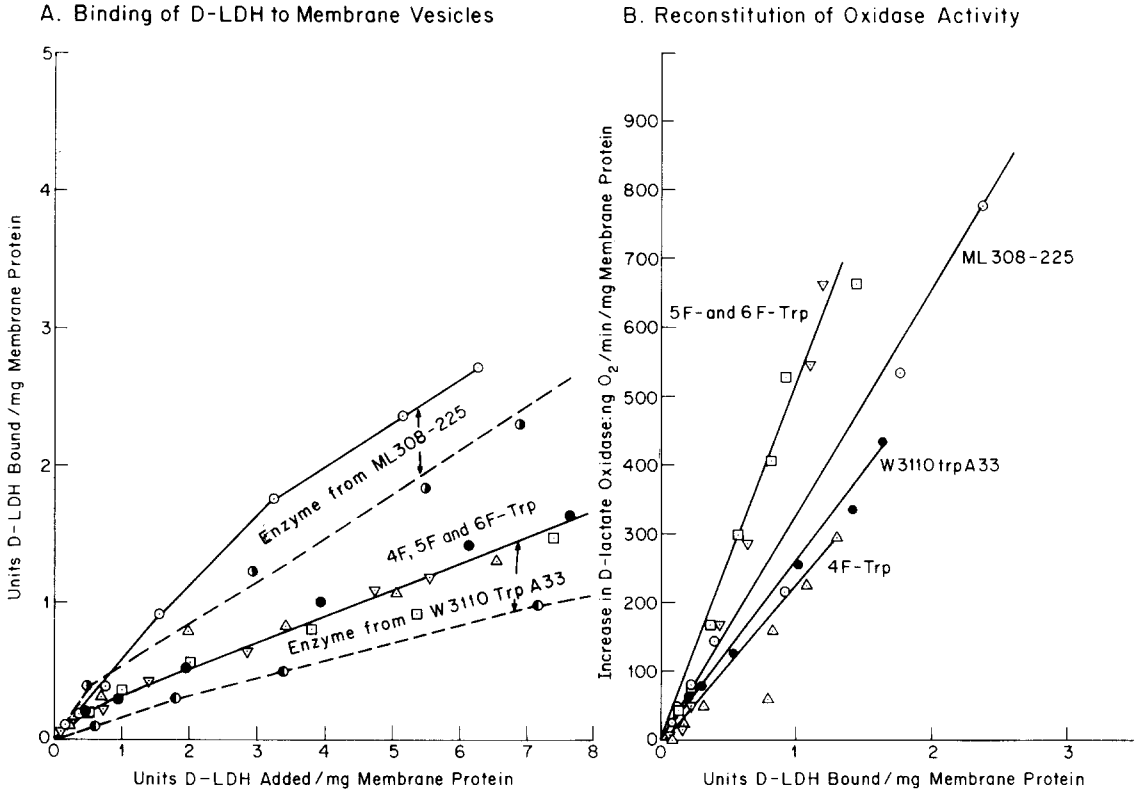


Membrane vesicles from the *dld*-3 *ufa*-2 mutant transport more poorly than the *dld*-3 parent, but do show significant reconstitution (Fig. 2C). In both sets of membrane vesicles, there is decreased transport activity after incorporation of 8,8-difluoromyristate. Membrane vesicles from fatty acid-requiring mutants grown in palmitoleic acid similarly show lower activity than the parents, and the activity is increased on incorporation of 8,8-difluoromyristate (data not shown).

Fluorine-labeled D-lactate dehydrogenase

Table III shows the properties of D-lactate dehydrogenase containing 4-, 5-, or 6-fluorotryptophan. As found previously in the crude extract [23], the partially purified 4-fluorotryptophan-con-

Fig. 2. Reconstitution of D-lactate dehydrogenase-dependent activities with membrane vesicles containing ^{19}F-labeled lipids. (A) Binding of D-lactate dehydrogenase to membrane vesicles. (B) Reconstitution of oxidase activity. (C) Reconstitution of proline transport. D-Lactate dehydrogenase from ML 308-225. Membrane vesicles from: ○, ML 308-225; □, ML 308-225 *ufa*-8 with 18:1; ◇, ML 308-225 *ufa*-8 with 18:1 → 14:0(8,8-F_2); ●, ML 308-225 *dld*-3; ■, ML 308-225 *dld*-3 *ufa*-2 with 18:1; ◆, ML 308-225 *dld*-3 *ufa*-2 with 18:1 → 14:0(8,8-F_2).



taining enzyme has a significantly higher specific activity than the other forms.

Fig. 3A shows that D-lactate dehydrogenase prepared from strain W3110*trp*A33 does not bind as well to membrane vesicles of strain ML 308-225 *dld*-3 as does enzyme from strain ML 308-225. This is not because of incompatibility between strains, however. When membrane vesicles are prepared from strain W3110*trp*A33, enzyme from ML 308-225 binds better than enzyme from W3110*trp*A33 (Fig. 3A). Fluorine-labeled enzymes bind to *dld* membrane vesicles to the same extent as unlabeled enzyme from strain W3110*trp*A33.

If these membrane vesicles with varying

Fig. 3. Reconstitution of D-lactate dehydrogenase-dependent activities with ^{19}F-labeled D-lactate dehydrogenase. (A) Binding of D-lactate dehydrogenase to membrane vesicles. (B) Reconstitution of oxidase activity. (C) Reconstitution of proline transport. Membrane vesicles from *E. coli* ML 308-225 *dld*-3. D-Lactate dehydrogenase from: ⊙, ML 308-225; ●, W3110*trp*A33; △, W3110*trp*A33 with 4F-Trp; ▽, W3110*trp*A33 with 5F-Trp; □, W3110*trp*A33 with 6F-Trp. In (A): Membrane vesicles from *E. coli* W3110*trp*A33, D-lactate dehydrogenase from: ◑, ML 308-225; ◐, W3110*trp*A33.

TABLE II

FATTY ACID COMPOSITION OF MEMBRANES OF PARENTS AND FATTY-ACID REQUIRING MUTANTS

Strain, supplement	Mol% of fatty acids [a]				
	14:0	16:0	16:1	8,8-F_2	18:1
ML 308-225	3	40	32	–	19
ML 308-225 *ufa*-8 oleate, 18:1	3	38	13	–	42
ML 308-225 *ufa*-8 palmitoleate, 16:1	5	33	46	–	8
ML 308-225 *ufa*-8 oleate → 14:0(8,8-F_2)	2	50	9	11	24
ML 308-225 *ufa*-8 palmitoleate → 14:0(8,8-F_2)	5	51	19	11	8
ML 308-225 *dld*-3	3	36	33	–	15
ML 308-225 *dld*-3 *ufa*-2 oleate, 18:1	4	34	13	–	37
ML 308-225 *dld*-3 *ufa*-2 palmitoleate, 16:1	5	42	33	–	2
ML 308-225 *dld*-3 *ufa*-2 oleate → 14:0(8,8-F_2)	7	39	8	23	16
ML 308-225 *dld*-3 *ufa*-2 palmitoleate → 14:0(8,8-F_2)	7	40	20	19	< 0.5

Percentages do not add up to 100% because some minor components have been omitted.

amounts of bound D-lactate dehydrogenase are used for oxidase assays, the results are as shown in Fig. 3B. For a given amount of enzyme bound, 5- or 6-fluorotryptophan-labeled enzymes appear to give a somewhat higher oxidase activity.

Steady-state levels of uptake of proline by ML 308-225 *dld*-3 membrane vesicles in the presence of varying amounts of D-lactate dehydrogenase are shown in Fig. 3C. For a given amount of enzyme added, 5- and 6-fluorotryptophan-labeled enzymes appear to give a higher transport activity. These results with oxidase and transport activity suggest that 5- and 6-fluorotryptophan-labeled enzymes may be functionally more effective than indicated by the PMS-MTT assay.

TABLE III

PROPERTIES OF D-LACTATE DEHYDROGENASE CONTAINING 4-, 5-, OR 6-FLUOROTRYPTOPHAN

D-LDH prepared from	% Tryptophan in fluorinated form	Spc. act.	K_m (M) ($\times 10^4$)
ML 308-225	–	9.0	2.4
W3110*trp*A33	–	11.8	2.6
W3110*trp*A33 + 4F-Trp	59	64.1	2.3
W3110*trp*A33 + 5F-Trp	51	9.9	2.5
W3110*trp*A33 + 6F-Trp	33	16.3	2.9

Discussion

D-Lactate dehydrogenase is located on the inner surface of the cytoplasmic membrane [35,36]. It is an intrinsic membrane protein, requiring detergents for solubilization [37]. Upon reconstitution, the exogenous D-lactate dehydrogenase appears to bind to the external surface of the membrane vesicles [36] and thus may not be in its native position. Nevertheless, it ties into the electron transport chain, allowing D-lactate-supported oxidase and amino acid transport activities to take place. In fact, large quantities of enzyme will bind and produce oxidase activities many times higher than in membrane vesicles prepared from wild-type cells without added enzyme (Figs. 1A and 1B). Membrane vesicles from both wild-type cells and the D-lactate dehydrogenase-deficient mutant show the same increase in oxidase activity for the same amount of enzyme bound. Also, membrane vesicles have been prepared from strain ML 308-225 carrying a plasmid containing the gene for D-lactate dehydrogenase. These membrane vesicles have approx. 13 times as much D-lactate dehydrogenase per mg protein as the parent, and their D-lactate oxidase activity is approx. 12 times as great as that of the parent. Addition of exogenous D-lactate dehydrogenase increases the oxidase activity still further (Pratt, E.A., Rule, G.S. and Ho, C., unpublished data). As shown by these data and Table I, oxygen uptake by membrane vesicles may be

increased either by adding more of one primary dehydrogenase, or by providing substrate for more than one dehydrogenase.

With transport of proline, the effect is quite different (Fig. 1C). Membrane vesicles prepared from ML 308-225 show little if any increase in transport activity in the presence of exogenous D-lactate dehydrogenase, and activity is inhibited by increasing amounts of D-lactate dehydrogenase. In membrane vesicles from ML 308-225 *dld*-3, however, proline transport activity may increase to three times the level in ML 308-225 membrane vesicles. Additional enzyme causes inhibition of transport in *dld*-3 membrane vesicles also. The same effects are seen in membrane vesicles from the fatty acid-requiring mutants *ufa*-8 and *ufa*-2 (Fig. 2C).

Short et al. [12] also find a difference in transport activity between wild-type and reconstituted membrane vesicles. Oxamate causes efflux when added to reconstituted *dld*-3 membrane vesicles previously loaded with lactose, but not when added to wild-type membrane vesicles. Short et al. suggest that this may mean that D-lactate dehydrogenase plays an additional role in the native system which externally bound enzyme cannot fulfill. Our results suggest that the presence of internal D-lactate dehydrogenase prevents transport activity by exogenous D-lactate dehydrogenase. The exogenous D-lactate dehydrogenase cannot provide energy for support of additional transport activity and, in fact, inhibits transport supported by endogenous D-lactate dehydrogenase. The differences in transport activity between reconstituted mutant and wild-type membrane vesicles are in marked contrast to the situation with oxidase activity, where the activities of exogenous and endogenous enzyme seem to be additive and produce greater oxygen uptake. It will be interesting to see if any differences in enzyme-lipid interaction are detectable in the two cases.

The ^{19}F-nucleus has clear advantages as a probe for nuclear magnetic resonance study of interactions in membrane systems [17–19]. Our results indicate that lipids and proteins of *E. coli* can be labeled with ^{19}F, and ^{19}F-labeled components are functional in reconstitution of D-lactate dehydrogenase activity in D-lactate dehydrogenase-deficient membrane vesicles. D-Lactate dehydrogenase labeled with 4-, 5-, or 6-fluorotryptophan is equally as effective as unlabeled D-lactate dehydrogenase from the same strain in binding and reconstitution (Fig. 3). There seems to be a difference between D-lactate dehydrogenase from strains ML 308-225 and W3110*trp*A33 in ability to bind to membrane vesicles. Other small differences between enzymes from these strains have been previously noted [32]. However, for a given amount of enzyme bound, reconstitution of oxidase and proline transport activity is equally effective.

Membrane vesicles prepared from the *ufa*-8 and *ufa*-2 fatty acid-requiring mutants are not as active as the parents in binding, oxidase, or transport (Fig. 2). As shown by George-Nascimento et al. [26], binding is less sensitive to lipid content of the membrane vesicles than is reconstitution of activity. Membrane vesicles prepared from cells grown on palmitoleic acid are less functional than those grown on oleic acid; transferring to 8,8-difluoromyristic acid for one generation generally decreases the activity of oleate-grown cells and increases that of palmitoleate-grown cells. Thus, fluorine-labeled enzyme and membrane vesicles containing fluorine-labeled lipids may be used in studying protein-lipid interaction in reconstitution of D-lactate dehydrogenase-supported activities in D-lactate dehydrogenase-deficient membrane vesicles.

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