RECONSTITUTION OF D-LACTATE DEHYDROGENASE-DEFICIENT MEMBRANE VESICLES USING FLUORINE-LABELED COMPONENTS

AN APPROACH TO INVESTIGATING PROTEIN-LIPID INTERACTIONS IN BIOLOGICAL MEMBRANES

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As part of our investigation of structure-function relationships in biological membranes by biochemical and biophysical methods, we have been studying the membrane-bound enzyme D-lactate dehydrogenase (D-LDH) of *Escherichia coli*, and the effects on its activity of varying the lipid composition of the membrane. D-LDH catalyzes the oxidation of D-lactate in electron transfer reactions coupled to active transport of various amino acids and sugars (Barnes and Kaback, 1971). These activities can be reconstituted by addition of purified enzyme to D-LDH-deficient membrane vesicles from *E. coli* ML 308–225 *dld*-3 (Futai, 1974; Short et al., 1974). Isolation of unsaturated fatty acid auxotrophs makes possible studies of the effects on reconstitution of changing the lipid composition of the membranes (George-Nascimento et al., 1976). Because fluorine-19 can be a useful probe for nuclear magnetic resonance (NMR) studies, we have also incorporated 8,8-difluoromyristic acid into the lipids of unsaturated fatty acid auxotrophs, and have prepared membrane vesicles containing ^{19}F-labeled lipids (Gent et al., 1978 and 1981). In addition, we can incorporate 4-, 5-, and 6-fluorotryptophans into the proteins of *E. coli* W3110*trp*A33 and extract ^{19}F-labeled D-LDH. These ^{19}F-labeled components have been tested for their ability to reconstitute D-LDH-dependent activity in D-LDH-deficient membrane vesicles.

MATERIALS AND METHODS

Fluorotryptophans were incorporated into proteins of *E. coli* strain W3110*trp*A33 as described by Pratt and Ho (1975). These cells can double in density once in the presence of 5- and 6-fluorotryptophan, twice in the presence of 4-fluorotryptophan. Cells grown in the presence of 5- or 6-fluorotryptophan have a specific activity one-half normal; those grown in the presence of 4-fluorotryptophan have a specific activity twice normal. Cells of strain ML 308–225 and of strain W3110*trp*A33, unlabeled or fluorotryptophan-labeled, were used to prepare D-LDH by the method of Pratt et al. (1979) through the DE52 fractionation step. The pooled DE52 fractions were twice precipitated with acetone, and these partially purified enzymes were used to reconstitute membrane

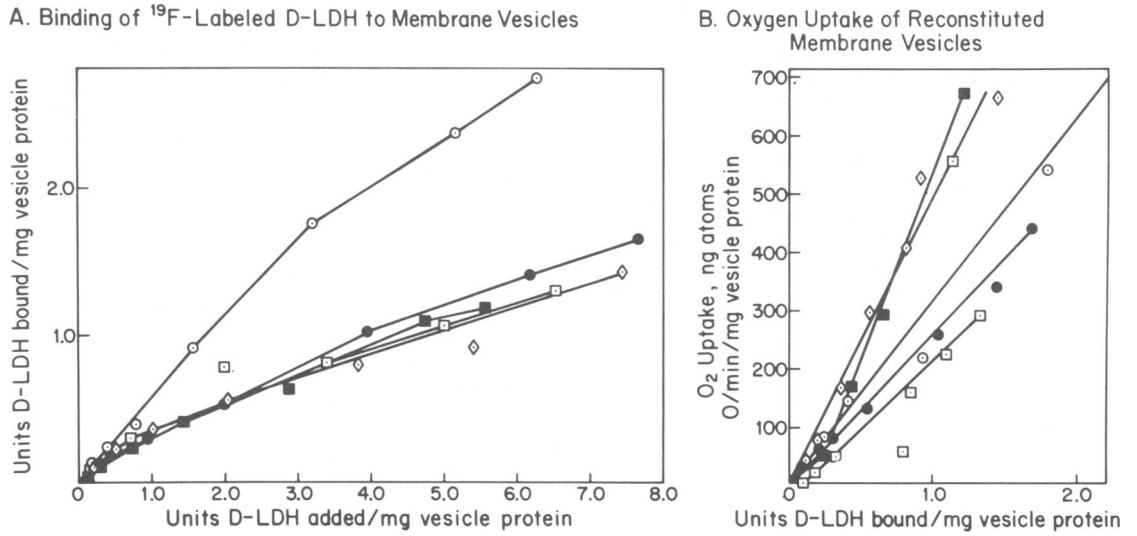

FIGURE 1 *A*, Binding of D-lactate dehydrogenases to membrane vesicles from *E. coli* ML 308–225 *dld*-3; *B*, Reconstitution of D-lactate-dependent oxidation in ML 308–225 *dld*-3 membrane vesicles containing bound D-lactate dehydrogenase. Increase in O_2 uptake over amount without added enzyme vs. amount of enzyme bound. D-LDH from: ⊙—⊙ ML 308–225; ●—● W3110*trp*A33; ⊡—⊡ W3110*trp*A33, 4F-Trp; ■—■ W3110*trp*A33, 5F-Trp; ♦—♦ W3110*trp*A33, 6F-Trp.

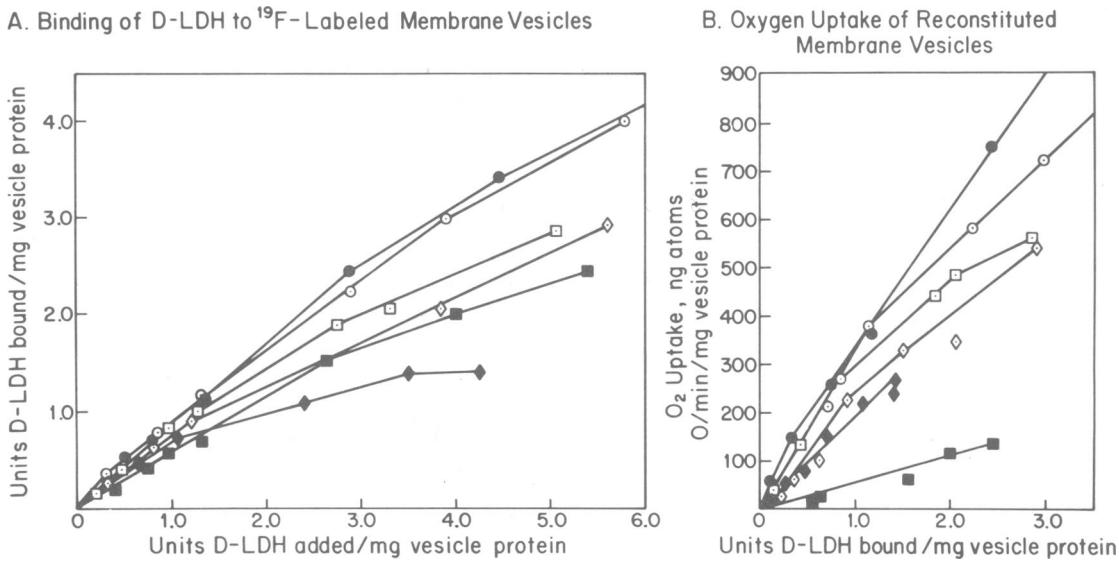

FIGURE 2 *A*, Binding of D-lactate dehydrogenase from *E. coli* ML 308–225 to membrane vesicles from wild type strains and unsaturated fatty acid auxotrophs; *B*, Reconstitution of D-lactate-dependent oxidation in vesicles with bound D-lactate dehydrogenase. Membrane vesicles from: ⊙—⊙ ML 308–225; ●—● ML 308–225 *dld*-3; ⊡—⊡ ML 308–225 *ufa*-8, oleic acid; ◆—◆. ML 308–225 *ufa*-8, oleic→8,8-difluoromyristic acid; ■—■ ML 308–225 *dld*-3 *ufa*-2, oleic acid; ♦—♦ ML 308–225 *dld*-3 *ufa*-2, oleic→8,8-difluoromyristic acid.

vesicles prepared by the method of Kaback (1971) from strain ML 308–225 *dld*-3.

Unsaturated fatty acid auxotrophs derived from strains ML 308–225 and ML 308–225 *dld*-3 can be grown in the presence of oleic or palmitoleic acid, washed, and resuspended in a medium containing 8,8-difluoromyristic acid. The cells grow to a limited extent and incorporate the fluorine-labeled fatty acid (Gent et al., 1978). Membrane vesicles were made from these cells and from the same strains grown in the presence of oleic or palmitoleic acid and from the parent strains.

To test for binding of enzyme to membrane vesicles, varying amounts of enzyme were incubated for 10 min at 23°C with 0.02 ml of 1 M guanidine-HCl. Membrane vesicles were added (~ 0.3 mg of membrane protein), followed by sufficient 0.1 M phosphate buffer pH 6.6 to bring the volume to 1.0 ml. After 30 min at 30°C, the membrane vesicles were centrifuged at 20,000 *g* for 10 min, the supernatant decanted, and the pellet resuspended in 1 ml buffer. Supernatants and pellets were assayed for D-LDH activity by reduction of dimethylthiazolyldiphenyltetrazolium bromide in the presence of phenazine methosulfate (Futai, 1973; Kohn and Kaback, 1973; Pratt et al., 1979). One unit of D-LDH is defined as that amount which reduces 1 μmol of tetrazolium dye/min.

The capacity of the bound enzyme to function was tested with D-lactate oxidase and transport assays. To measure D-lactate oxidase activity, the resuspended pellets with bound enzyme were placed in the chamber of an oxygen electrode with 0.1 M $MgSO_4$ and sufficient 0.1 M phosphate buffer pH 6.6 to bring the volume to 3 ml. D-lactate was added (17 mM) and the rate of uptake of O_2 measured. Transport of ^{3}H-proline into vesicles was measured by mixing 0.4 mg of membrane protein and varying quantities of D-LDH in a final volume of 1 ml, containing 0.1 M phosphate buffer pH 6.6, 0.01 M $MgSO_4$, 10 mM D-lactate and 0.2 mM ^{3}H-proline. Aliquots of 0.1 ml were filtered at various time intervals after addition of D-lactate and counted in a liquid scintillation counter.

RESULTS

Fig. 1 shows binding to ML 308–225 *dld*-3 membrane vesicles and reconstitution of oxidase activity by D-LDH prepared from strains ML 308–225, W3110*trp*A33, and W3110*trp*A33 labeled with 4-, 5-, and 6-fluorotryptophan. Fluorine-labeled D-LDH binds to membrane vesicles as well as unlabeled enzyme prepared from the same strain. For a given amount of enzyme bound to membrane vesicles, there is essentially equal reconstitution of oxidase activity by all enzyme preparations. Similarly, for the same amount of D-LDH, labeled or unlabeled, equal reconstitution of proline transport occurs (data not shown).

Fig. 2 shows binding and reconstitution with D-LDH prepared from strain ML 308–225 and membrane vesicles from strains ML 308–225, ML 308–225 *dld*-3, and unsaturated fatty acid-requiring mutants derived from each. Membrane vesicles prepared from unsaturated fatty acid auxotrophs of ML 308–225 and ML 308–225 *dld*-3 grown on oleate or palmitoleate, or containing 8,8-difluoromyristic acid, although less active than membrane vesicles from the parent strain, bind D-LDH and show reconstitution of oxidase activity. Quantitative comparisons are difficult, as individual vesicle preparations vary in activity. In general, membrane vesicles from cells grown on oleic acid are more active than membranes from cells grown on palmitoleic acid, while incorporation of 8,8-difluoromyristic acid decreases the activity somewhat. However, fluorine-labeled membrane vesicles are clearly functional in binding and oxidase activity.

Thus, D-LDH-deficient membrane vesicles can be reconstituted using fluorine-labeled components and ^{19}F-NMR can be used to study the membrane protein-lipid interactions involved.

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REFERENCES

Barnes, E. M. Jr., and H. R. Kaback. 1971. Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in *Escherichia coli* membrane vesicles. *J. Biol. Chem.* 246:5518–5522.

Futai, M. 1973. Membrane D-lactate dehydrogenase from *Escherichia coli*. Purification and properties. *Biochemistry.* 12:2468–2474.

Futai, M. 1974. Reconstitution of transport dependent on D-lactate or glycerol 3-phosphate in membrane vesicles of *Escherichia coli* deficient in the corresponding dehydrogenases. *Biochemistry.* 13:2327–2333.

Gent, M. P. N., P. F. Cottam, and C. Ho. 1978. Fluorine-19 nuclear magnetic resonance studies of *Escherichia coli* membranes. *Proc. Natl. Acad. Sci. U. S. A.* 75:630–634.

Gent, M. P. N., P. F. Cottam, and C. Ho. 1981. A biophysical study of protein-lipid interaction in membranes of *Escherichia coli*. *Biophys. J.* 33:211–224.

George-Nascimento, C., S. J. Wakil, S. A. Short, and H. R. Kaback. 1976. Effect of lipids on the reconstitution of D-lactate oxidase in *Escherichia coli* membrane vesicles. *J. Biol. Chem.* 251:6662–6666.

Kaback, H. R. 1971. Bacterial membranes. *Methods Enzymol.* 22:99–120.

Kohn, L. D., and H. R. Kaback. 1973. Mechanisms of active transport in isolated bacterial membrane vesicles XV. Purification and properties of the membrane-bound D-lactate dehydrogenase from *Escherichia coli*. *J. Biol. Chem.* 248:7012–7017.

Pratt, E. A., and C. Ho. 1975. Incorporation of fluorotryptophans into proteins of *Escherichia coli*. *Biochemistry.* 14:3035–3040.

Pratt, E. A., L. W. -M. Fung, J. A. Flowers, and C. Ho. 1979. Membrane-bound D-lactate dehydrogenase from *Escherichia coli*: Purification and properties. *Biochemistry.* 18:312–316.

Short, S. A., H. R. Kaback, and L. D. Kohn. 1974. D-Lactate dehydrogenase binding in *Escherichia coli dld* membrane vesicles reconstituted for active transport. *Proc. Natl. Acad. Sci. U. S. A.* 71:1461–1465.

LIPID-PROTEIN INTERACTIONS IN MEMBRANES CONTAINING THE ACETYLCHOLINE RECEPTOR

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Lipid-protein interactions involving the acetylcholine receptor (AChR) from the electric ray *Torpedo californica* were studied in purified native membrane vesicles and in reconstituted membranes prepared from receptor protein and defined lipids. The effect of AChR on membrane lipids has been examined by electron paramagnetic resonance (EPR) techniques using spin-labeled lipids incorporated into the membranes. Reconstituted membranes were used to study the dependence of AChR function on membrane lipid composition.

RESULTS AND DISCUSSION

Interaction of Spin-labeled Lipids with Native AChR Membranes

Fig. 1 *a* shows the EPR spectrum of 16-doxylstearic acid in native AChR membranes. The spectrum contains two components. One is due to spin probes that are almost completely immobilized on the conventional EPR time scale. The splitting between the outermost peaks of this component is 62.5 Gauss. The other component is due to relatively mobile spins and is characteristic of 16-doxylstearic acid in fluid phospholipid bilayers (Fig. 1 *d*). The immobile component was quantitated by doubly integrating the spectrum before and after computer subtraction of a computer-simulated spectrum of the component (1). Fig. 1 *b* shows the simulated relatively immobile spectrum and Fig. 1 *c* is the remaining spectrum of the more mobile component after subtraction. Based on the spectral analysis, the amount of immobile probe was 25% and the amount was not strongly temperature dependent over the range 0–20°C. The maximum splitting of the immobile component was temperature dependent and the simulated spectrum was always calculated to match the maximum splitting. The linewidths in Fig. 1 *c* are slightly larger than those of the pure lipid system indicating a small effect of the protein on the bulk lipids.

In contrast to the results with free 16-doxylstearic acid, a phosphatidylcholine spin label (PCSL) containing the 16-doxyl-stearic acid did not clearly show an immobile component when incorporated into native membranes (Fig. 2 *a*). The spectrum is nearly identical to the spectrum of the same probe in lipid-only membrane vesicles (Fig. 2 *b*). Our spin label results are qualitatively similar to those obtained by Rousselet et al. (2) using *Torpedo marmorata*, and indicate that the fatty acid interacts more strongly with AChR than phosphatidylcholine does. We are now examining the spectral properties of phosphatidylserine, phosphatidylethanolamine and cholesterol spin labels to determine if there are charge or lipid-class specificities.

Reconstituted Membranes

Membranes containing purified AChR and defined lipids were prepared by the cholate dialysis procedure developed by Epstein and Racker (3). Using carbamylcholine-stimu-