Simultaneous Reconstitution of *Escherichia coli* Membrane Vesicles with D-Lactate and D-Amino Acid Dehydrogenases†

Kasturi Haldar, Paula J. Olsiewski,‡ Christopher Walsh, Gregory J. Kaczorowski,§ Amar Bhaduri,‖ and H. Ronald Kaback*

ABSTRACT: Purified preparations of D-amino acid dehydrogenase [Olsiewski, P. J., Kaczorowski, G. J., & Walsh, C. T. (1980) *J. Biol. Chem. 225*, 4487] and D-lactate dehydrogenase [Kohn, L. D., & Kaback, H. R. (1973) *J. Biol. Chem. 248*, 7012] bind independently to right-side-out and inverted *Escherichia coli* vesicles and to phosphatidylcholine liposomes without detectable competition. The reconstituted vesicles catalyze D-lactate- and D-alanine-dependent respiration (O_2 uptake), proton translocation, and proton/lactose symport. The enzymes do not share common sites of association on either face of the *E. coli* membrane, and binding of both enzymes to the bilayer appears to be due to nonspecific affinity for the surface rather than specific binding to proteinaceous receptors. Each enzyme, however, appears to reduce a common proton translocating step in the membrane-bound respiratory chain, and substrate-derived electrons are transferred through a common rate-determining redox component that precedes the site of proton translocation. The results suggest that although binding is nonspecific, there is a common site for proton translocation in the membrane between the flavin-linked dehydrogenases and the cytochromes and that this site is accessible by distinct routes of electron transfer from primary dehydrogenases on either surface of the membrane.

Active transport has been extensively studied in cytoplasmic membrane vesicles prepared from *Escherichia coli* by osmotic lysis (Kaback, 1970, 1974a, 1980). These vesicles have been characterized in detail (Kaback, 1971, 1974a) and exhibit the same polarity and configuration as the membrane of the intact cell (Owen & Kaback, 1978, 1979a,b). The vesicles retain the ability to couple respiration (Kaback, 1976; Ramos & Kaback, 1977a–c) and ATP hydrolysis (Hugenholtz et al., 1981) to the active transport of many different solutes by mechanisms in which chemiosmotic forces (Mitchell, 1966, 1968, 1979) play a central, obligatory role. Accordingly, the immediate driving force for active transport is an electrochemical gradient of hydrogen ion ($\Delta\bar{\mu}_{H^+}$)[1] composed of an electrical potential ($\Delta\Psi$) and a chemical gradient of hydrogen ions (ΔpH).

Despite the role of the various membrane dehydrogenases in establishing the $\Delta\bar{\mu}_{H^+}$ required for active transport, the mechanism of vectorial proton expulsion has been difficult to study experimentally, since little is known about the molecular architecture of the *E. coli* respiratory chain. However, a technique that may provide some insight into this process is reconstitution of proteins involved in respiration. Membrane vesicles have been singly reconstituted with several different purified dehydrogenases (Reeves et al., 1973; Short et al., 1974, 1975; Futai, 1974; Dancey et al., 1976; Schryners et al., 1978; Jaworowski et al., 1981). In particular, reconstitution of D-lactate-dependent transport and oxidase activity has been observed in membrane vesicles incubated with D-lactate dehydrogenase (D-LDH; Reeves et al., 1973; Short et al., 1974) despite enzyme association with the "wrong" surface of the membrane (Short et al., 1974, 1975). More recently, reconstitution of both right-side-out and inside-out vesicles with D-amino acid dehydrogenase (D-ADH) has been reported (Olsiewski et al., 1981). The studies demonstrate that D-ADH, like D-LDH, is able to generate a $\Delta\bar{\mu}_{H^+}$ when bound to either surface of the membrane. In both cases, the $\Delta\bar{\mu}_{H^+}$ generated is similar in magnitude but opposite in polarity, reflecting the orientation of the membrane rather than the direction of electron flow from the primary dehydrogenase. Since the apparent scalar addition of reducing equivalents does not alter the polarity of the $\Delta\bar{\mu}_{H^+}$, it was concluded that the primary dehydrogenase does not play a direct role in vectorial proton translocation (Olsiewski et al., 1981).

As an extension of these studies, reconstitution of membrane vesicles from *E. coli* ML 308-225*dld*-3 has now been performed concurrently with D-ADH and D-LDH, two membrane-bound flavoproteins that have been solubilized and extensively purified (Kohn & Kaback, 1973; Kaczorowski et al., 1978; Olsiewski et al., 1980). We have assayed functional reconstitution by measuring substrate-dependent [^{14}C]lactose accumulation, respiration, reduction of electron-accepting dyes, generation of a $\Delta\bar{\mu}_{H^+}$, and enzymatic product formation. The results indicate that the two dehydrogenases do not compete for common binding sites on either face of the membrane but transfer electrons to a common redox component of the respiratory chain. This redox component appears to be the rate-limiting step in respiration and occurs prior to the site of vectorial proton translocation.

Experimental Procedures

Materials

[^{3}H]Tetraphenylphosphonium bromide (2.5 Ci/mmol) was synthesized by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman. [1-^{14}C]Lactose (60 mCi/mmol) was purchased from Am-

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (K.H., P.J.O., and C.W.), and the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110 (G.J.K., A.B., and H.R.K.). *Received December 17, 1981; revised manuscript received March 8, 1982.*

‡ Present address: Department of Biology, New York University, New York, NY 10003.

§ Fellow of the Helen Hay Whitney Foundation. Present address: Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.

‖ Present address: Division of Biochemistry, Jadavpur University, Calcutta, India.

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\Psi$, electrical potential; ΔpH, chemical gradient of hydrogen ions; D-ADH, D-amino acid dehydrogenase; D-LDH, D-lactate dehydrogenase; PMS, phenazine methosulfate; DCIP, dichlorophenolindophenol; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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ersham/Searle, D-[1-^{14}C]alanine from ICN Chemical and Radioactive Division, and D-[1-^{14}C]lactate from New England Nuclear. Nigericin was supplied by Dr. J. Wesley of Hoffmann-La Roche, Inc.

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225*dld*-3 ($i^-z^-y^+a^+dld^-$; Hong & Kaback, 1972) was grown on minimal salts medium (Davis & Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate), and right-side-out membrane vesicles were prepared by osmotic lysis (Kaback, 1971; Short et al., 1975). Inverted membrane vesicles were prepared by passage of cells through a French pressure cell at low shear forces (Hertzberg & Hinkle, 1974; Rosen & McClees, 1974; Reenstra et al., 1980). All vesicle preparations were stored in 0.1 M potassium phosphate (pH 6.6) and frozen in liquid nitrogen.

For experiments at different pHs, vesicles were thawed rapidly at 46 °C and resuspended in at least a 20-fold excess of buffer at the desired pH. After incubation at room temperature for 20 min, the vesicles were collected by centrifugation: 15 min at 45000*g* for right-side-out vesicles and 2 h at 210000*g* for inverted vesicles. Pellets were then resuspended in 0.1 M potassium phosphate at the desired pH and protein concentration.

Transport Assays. Uptake of [^{14}C]lactose by right-side-out membrane vesicles in the presence of a given electron donor was measured by filtration as described (Kaback, 1971, 1974b). In all cases, the electron donor was added to the vesicles for at least 30 s prior to addition of [^{14}C]lactose to ensure complete energization. Steady-state levels of solute accumulation were determined from the transport profile and by flow dialysis (Ramos et al., 1976, 1979; Ramos & Kaback, 1977a).

Respiration Rates. Rates of oxygen consumption were measured with a Clark-type electrode (YSI Model 53 oxygen monitor, Yellow Springs Instrument Co., Yellow Springs, OH) (Barnes & Kaback, 1971; Kaczorowski et al., 1975; Olsiewski et al., 1981).

Determination of $\Delta\Psi$, ΔpH, and $\Delta\bar{\mu}_{H^+}$. Respiration-dependent generation of $\Delta\Psi$ (interior negative or positive) was determined by monitoring the equilibrium distribution of radioactive tetraphenylphosphonium bromide (TPP^+) or potassium thiocyanate, respectively, by flow dialysis (Ramos et al., 1979; Reenstra et al., 1980). Similarly, ΔpH (interior alkaline or acid) was determined from the steady-state level of accumulation of either radioactive acetic acid or methylamine, respectively, by flow dialysis (Ramos et al., 1979; Reenstra et al., 1980). Intravesicular volumes of 2.2 μL/mg of protein (Barnes & Kaback, 1971) and 1.1 μL/mg of protein (Reenstra et al., 1980) were used for calculations with right-side-out and inside-out vesicles, respectively.

Preparation of D-LDH and D-ADH. D-LDH was purified as described [Kohn & Kaback, 1973; Kaczorowski et al., 1978 (1 nmol of D-LDH = 0.01 mg of 90% pure enzyme = 6.38 units of D-LDH)]. D-ADH was prepared by the modified method of Olsiewski et al. (1980, 1981) (1 nmol of D-ADH = 0.16 mg of 70% pure enzyme = 0.27 units of D-ADH).

Assay for Dehydrogenase Activities. D-LDH activity was assayed spectrophotometrically by monitoring D-lactate-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the presence of phenazine methosulfate (PMS) (Kohn & Kaback, 1973). One unit of activity is defined as 1 μmol of MTT reduced/min. D-ADH activity was monitored by following D-alanine-dependent reduction of dichlorophenolindophenol (DCIP) spectrophotometrically at 600 nm in the presence of ubiquinone-1 (Olsiewski et al., 1980). One unit of activity is defined as 1 μmol of DCIP reduced/min.

Reconstitution of Membrane Vesicles. Right-side-out membrane vesicles were reconstituted with D-LDH in the presence of guanidine hydrochloride as described by Short et al. (1974). Inside-out vesicles were reconstituted similarly, except that vesicle protein and enzyme protein concentrations were 2-fold higher than those for right-side-out vesicles. Right-side-out and inside-out vesicles were reconstituted with D-ADH as described (Olsiewski et al., 1981).

Doubly reconstituted vesicles were prepared by first reconstituting with D-LDH. These vesicles were then washed several times to remove traces of guanidine hydrochloride and reconstituted with D-ADH according to Olsiewski et al., (1981). The optimal conditions for the preparation of doubly reconstituted membrane vesicles were found to be identical with those employed for reconstitution of vesicles with each enzyme individually.

Protein. Protein was estimated according to the method of Lowry et al. (1951) with crystalline bovine serum albumin as the standard.

Separation of Radioactive Pyruvate from D-[1-^{14}C]Alanine and D-[1-^{14}C]Lactate. D-[1-^{14}C]Alanine was separated from [1-^{14}C]pyruvate on Dowex 50 columns (1.5–2.0 mL) equilibrated in 200 mM citrate (sodium salt, pH 2.2). The pyruvate eluted from the column in the first milliliter of eluant. The bound radioactive D-alanine was then eluted with 2 mL of 2 N NH_4OH. The pyruvate was stable to degradation for the length of the assay as determined by the controls described in Silverstein & Boyer (1964). Radioactive D-lactate was separated from pyruvate by adding carrier pyruvate and subsequently precipitating it as the 2,4-dinitrophenylhydrazone complex by the procedure of Silverstein & Boyer (1964).

Preparation of Dilaurylphosphatidylcholine Vesicles. Dilaurylphosphatidylcholine was sonicated in a bath-type sonicator for 30 min and suspended in 100 mM potassium phosphate buffer (pH 7.5) at 3 mg/mL according to the procedure of Strittmatter et al. (1978). Multilamellar liposomes were removed by centrifugation at 100000*g* for 20 min. The supernatant, containing vesicles 200–400 Å in diameter, was used in the reconstitution experiments. The lipid content was measured by phosphate analysis by the method of Duck-Chong (1979) after the phosphate in the buffer was dialyzed away to obtain a low background. Reconstitution of liposomes with D-LDH and D-ADH was carried out under the same conditions as described for inside-out vesicles. The amount of enzyme bound was determined by the D-lactate–MTT or D-alanine–DCIP reductase activity associated with the pellet after reconstitution.

Results

Binding of D-LDH and D-ADH. Membrane vesicles prepared from *E. coli* ML 308-225*dld*-3 exhibit neither D-lactate- nor D-alanine-dependent respiratory activity, and consequently these substrates do not generate a $\Delta\bar{\mu}_{H^+}$ (Olsiewski et al., 1981). Previous studies have shown that *E. coli* ML 308-225*dld*-3 vesicles can be functionally reconstituted with either D-LDH or D-ADH (Short et al., 1974; Olsiewski et al., 1981). Therefore, the present study was initiated to determine the effect of parallel dehydrogenase reconstitution on binding and transport.

Initially, right-side-out vesicles prepared by osmotic lysis were reconstituted with D-LDH (saturating for D-lactate-dependent O_2 uptake and lactose transport) (Short et al., 1974), followed by reconstitution with increasing amounts of D-ADH.

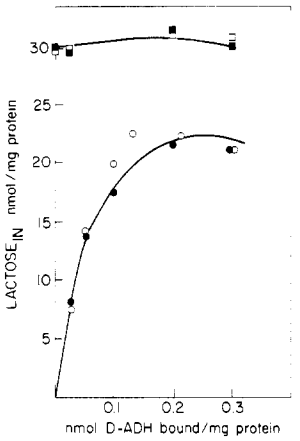

FIGURE 1: Parallel reconstitution of D-alanine- and D-lactate-dependent lactose transport. *E. coli* ML 308-225*dld*-3 vesicles were reconstituted with 0.3 nmol of D-ADH/mg of membrane protein, 0.45 nmol of D-LDH/mg of membrane protein, or both as described under Methods. The vesicles were then diluted to a final volume of 50 μL containing 40–60 μg of protein, 50 mM potassium phosphate (pH 6.6), and 10 mM magnesium sulfate at 25 °C. Uptake of [^{14}C]lactose (0.6 mM; 20 mCi/mmol) at 10 min was measured by filtration in *E. coli* ML 308-225*dld*-3 vesicles reconstituted with (a) 0–0.3 nmol of D-ADH/mg of membrane protein energized by D-alanine (10 mM) (○), (b) 0.45 nmol of D-LDH/mg of membrane protein and 0–0.3 nmol of D-ADH/mg of membrane protein energized by D-alanine (10 mM) (●), (c) 0.45 nmol of D-LDH/mg of membrane protein and 0–0.3 of D-ADH/mg of membrane protein energized by D-lactate (10 mM) (□), and (d) 0.45 nmol of D-LDH/mg of membrane protein and 0–0.3 nmol of D-ADH/mg of protein energized by D-alanine (10 mM) and D-lactate (10 mM) (■). No uptake of radioactive solute was observed in the absence of D-alanine or D-lactate nor in *E. coli* ML 308-225*dld*-3 vesicles treated with reconstitution buffers lacking D-ADH and D-LDH (data not shown).

The vesicles were then assayed for steady-state [^{14}C]lactose accumulation (Figure 1). As shown, the steady-state level of lactose accumulation is a linear function of bound D-ADH from 0 to about 0.05 nmol of D-ADH bound/mg of membrane protein and becomes saturated at D-ADH levels in excess of 0.2 nmol/mg of protein. Vesicles that were not treated with D-LDH but reconstituted with increasing amounts of D-ADH exhibit an identical profile for D-alanine-dependent lactose accumulation. These results suggest that the sites of association of D-ADH with the outer surface of the vesicles are not blocked by D-LDH. Furthermore, the bound D-LDH remains functional, as determined by D-lactate-dependent lactose uptake, and reconstitution with D-ADH does not affect this activity. Consistently, reconstitution with D-ADH does not cause release of D-LDH into the supernatant, as measured by D-lactate-dependent dye reduction (data not shown). Thus, D-ADH does not displace bound D-LDH from the surface.

When the doubly reconstituted vesicles are assayed for lactose accumulation in the presence of both D-alanine and D-lactate, lactose is accumulated to the same levels observed with D-lactate alone, and there is no additive effect (cf. Figure 1 and below). Thus, there does not appear to be a common binding site for the enzymes at the site of their interaction with the respiratory chain on the outer surface of the membrane. A similar relationship has been observed by studying parallel reconstitution with glycerol-3-phosphate dehydrogenase and D-lactate dehydrogenase in the same vesicles (A. Bhaduri and H. R. Kaback, unpublished results).

Conditions found to be optimal for the preparation of doubly reconstituted vesicles are the same as those reported for reconstitution with each enzyme separately (Short et al., 1974, 1975; S. Ramos and H. R. Kaback, unpublished results; Olsiewski et al., 1981). Because reconstitution with D-LDH

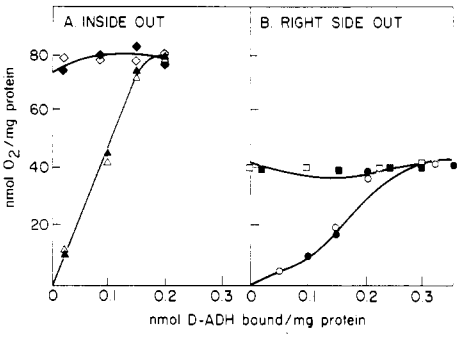

FIGURE 2: Parallel reconstitution of D-alanine- and D-lactate-dependent oxygen uptake in *E. coli* ML 308-225*dld*-3 vesicles. (A) Inside-out vesicles: Inverted vesicles were treated with D-ADH or D-ADH and D-LDH as described under Methods. Vesicles were then diluted to a final concentration of 0.30 mg/mL of protein in 0.1 M potassium phosphate (pH 6.6)–10 mM magnesium sulfate and placed in the chamber of a Clark-type oxygen electrode whose temperature was maintained at 25 °C, and the background rate of oxygen consumption was measured. Given substrates were then added (10 mM final concentration), and oxygen uptake was monitored in vesicles reconstituted with (a) 0–0.2 nmol of D-ADH/mg of membrane protein with D-alanine as substrate (△) and (b) 0.2 nmol of D-LDH/mg of membrane protein and 0–0.2 nmol of D-ADH/mg of membrane protein with (i) D-alanine as substrate (▲), (ii) D-lactate as substrate (◊), and (iii) D-alanine plus D-lactate as substrate (♦). (B) Right-side-out vesicles: Oxygen uptake was measured as described above with *E. coli* ML 308-225*dld*-3 vesicles reconstituted with (a) 0–0.2 nmol of D-ADH/mg of membrane protein with D-alanine as substrate (○) and (b) 0.2 nmol of D-LDH and 0–0.2 nmol of D-ADH/mg of membrane protein with (i) D-alanine as substrate (●), (ii) D-lactate as substrate (□), and (iii) D-alanine plus D-lactate as substrate (■).

requires guanidine hydrochloride, which irreversibly inactivates D-ADH, the membranes were first reconstituted with D-LDH, washed, and then reconstituted with D-ADH. However, neither enzyme, after the double reconstitution, can be displaced by repeatedly washing the vesicles with 100 mM potassium phosphate (pH 6.6), suggesting stable association of the proteins with the membrane. As shown previously for vesicles reconstituted separately with either dehydrogenase (Short et al., 1974; Olsiewski et al., 1981), both D-lactate- and D-alanine-dependent lactose transports in the doubly reconstituted system are inhibited by potassium cyanide and hydroxyquinoline *N*-oxide, confirming the argument that the enzymes function via the membrane-bound respiratory chain (data not shown).

A similar study using inverted membrane vesicles prepared from *E. coli* ML 308-225*dld*-3 was carried out. The effect of D-LDH on reconstitution with D-ADH at the cytoplasmic face of the membrane was investigated by monitoring D-alanine- and D-lactate-dependent respiration. As shown in Figure 2A, when inside-out vesicles are first reconstituted with a saturating amount of D-LDH, followed by reconstitution with increasing amounts of D-ADH, the rate of D-alanine-dependent respiration increases almost linearly from 0 to about 0.15 nmol of D-ADH bound/mg of protein and remains essentially constant at levels above 0.15 nmol of D-ADH/mg of protein. An identical profile is observed for vesicles reconstituted with D-ADH only, indicating that there is no competition between D-ADH and D-LDH for binding at the cytoplasmic face of the membrane. Similarly, rates of D-lactate oxidation are not affected by the presence of D-ADH and the simultaneous oxidation of the substrates does not exceed the maximal rate of respiration observed with either enzyme. (The rate of reduced PMS oxidation is 2.5-fold higher, indicating that the respiratory chain is not saturated during oxidation of D-alanine and/or D-lactate.) Respiration rates with right-side-out doubly reconstituted vesicles are shown in Figure 2B, and similar

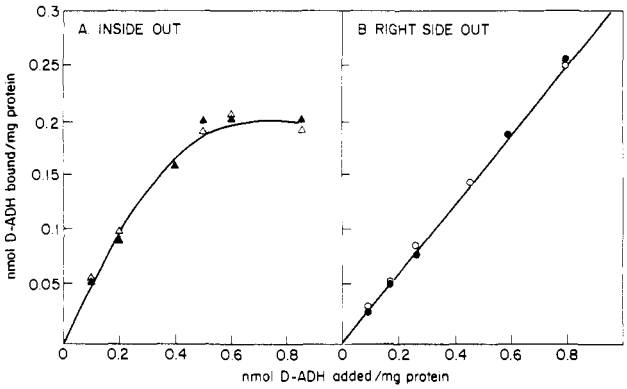

FIGURE 3: Binding of D-ADH and D-LDH to *E. coli* ML 308-225*dld*-3 vesicles. (A) Inside-out vesicles: Bound D-alanine-dependent DCIP reductase activity was monitored in inverted vesicles reconstituted with 0–0.8 nmol of D-ADH/mg of protein (△) and in inverted vesicles reconstituted with 0.2 nmol of D-LDH/mg of protein and 0–0.8 nmol of D-ADH/mg of protein (▲). (B) Right-side-out vesicles: Right-side-out vesicles were incubated with increasing amounts of D-ADH (0–0.8 nmol of D-ADH/mg of protein) (○) as described under Methods. For preparation of doubly reconstituted membranes, the vesicles were first reconstituted with 0.45 nmol of D-LDH/mg of protein and then exposed to D-ADH at concentrations ranging from 0 to 0.8 nmol of D-ADH/mg of protein (●). Vesicles were diluted in 0.1 M potassium phosphate (pH 7.5) to a concentration of 40–60 μg of protein/mL (final volume 1 mL) and assayed for D-alanine-dependent DCIP reductase activity at 25 °C. Units of dye reductase activity were then converted to nanomoles of enzyme as described under Methods. In both (A) and (B), D-LDH was also quantitated by measuring MTT reductase activity of the vesicles before and after reconstitution with D-ADH. No displacement of D-LDH was observed (data not shown).

results are observed. Notably, however, the maximum rate of respiration with right-side-out vesicles is only half of that observed for the inside-out system, as observed previously in reconstitution studies with D-ADH (Olsiewski et al., 1981). Hence, it is evident that the dehydrogenases do not compete with each other for binding on either membrane surface.

The data presented in Figure 3 provide further support for this conclusion. In these studies, dye reductase activities of vesicles reconstituted with D-ADH and D-LDH were used to estimate the number of enzyme molecules bound. With inside-out vesicles (Figure 3A), bound D-alanine-dependent dye reduction is a saturable function of the D-ADH added, both in the presence and absence of previously bound D-LDH. Binding is essentially linear from 0 to 0.5 nmol of D-ADH added/mg of protein and no more than 0.2 nmol of D-ADH/mg of membrane protein is bound at higher concentrations of D-ADH. Similarly, inside-out vesicles incubated with increasing levels of D-LDH exhibit saturable D-lactate-dependent dye reduction activity (data not shown). These data also argue against the presence of a common receptor for the two dehydrogenases.

With right-side-out vesicles (Figure 3B), bound D-alanine-dependent dye reductase activity increases with increasing amounts of D-ADH independent of prior reconstitution with D-LDH and continues to increase linearly far beyond the point where lactose accumulation, respiration, and levels of the $\Delta\bar{\mu}_{H^+}$ show saturation (cf. below). These results are similar to those reported earlier with D-LDH (Short et al., 1974), where 80% of the enzyme added becomes associated with the membrane and the activity continues to increase linearly with the amount of enzyme added. Over the range of concentrations employed with D-ADH, only 30% of the enzyme binds under optimal conditions, and the percentage bound is independent of reconstituted D-LDH. Furthermore, the binding behavior of the dehydrogenases with right-side-out vesicles is almost identical with that observed with pure dilaurylphosphatidylcholine vesicles. With D-LDH, about 80% of the enzyme becomes stably associated with the liposomes, and binding increases linearly with enzyme concentration. Similarly, binding of D-ADH to liposomes is a linear function of enzyme concentration, although the amount of enzyme bound is reduced when compared with right-side-out *E. coli* membrane vesicles (i.e., 13% as compared with 30% of the added enzyme). In any event, there is no indication of saturable binding behavior for either enzyme with dilaurylphosphatidylcholine liposomes. The results, taken together, suggest that the enzymes bind nonspecifically to the surface of the membrane and not to specific protein molecules in the bilayer.

Although the inner face of the membrane can be saturated with either dehydrogenase, these results are insufficient to indicate a specific receptor site. Given the characteristics of D-LDH and D-ADH binding to dilaurylphosphatidylcholine liposomes, it is apparent that the enzymes have different affinities for phospholipids. If specific lipid domains exist in vesicles to which D-LDH and D-ADH exhibit nonequivalent affinities, then apparent saturation behavior may result from differential association with these domains. Moreover, since the cytoplasmic face contains a large amount of protein relative to the external face (Owen & Kaback, 1979a,b), the saturation behavior observed with inside-out vesicles may also be the result of a mass action effect, reflecting a lack of available surface rather than saturation of specific receptor sites.

Proton Translocation Occurs at a Site between Reconstituted Dehydrogenases and Cytochrome Chain. When right-side-out vesicles are reconstituted with amounts of D-ADH and D-LDH that saturate substrate-dependent respiratory activity, addition of either D-alanine or D-lactate results in generation of a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline). As shown in Table I, at pH 5.5, the $\Delta\bar{\mu}_{H^+}$ generated across the membrane is approximately −150 mV with D-alanine, D-lactate, or both substrates as electron donor. The $\Delta\Psi$ component remains essentially constant over a pH range of 5.5–7.5, while the ΔpH component varies from −80 to 0 mV (data not shown), consistent with earlier studies (S. Ramos and H. R. Kaback, unpublished results; Olsiewski et al., 1981). For doubly reconstituted inside-out vesicles, oxidation of D-alanine and/or D-lactate generates a $\Delta\bar{\mu}_{H^+}$ of comparable magnitude but opposite polarity to that observed with right-side-out vesicles. These results confirm previous studies that demonstrate that the sign of the $\Delta\bar{\mu}_{H^+}$ is determined by the polarity of the membrane and not by the direction of electron flow from the primary dehydrogenase (Short et al., 1975; S. Ramos and H. R. Kaback, unpublished results; Olsiewski et al., 1981).

Although higher values of $\Delta\bar{\mu}_{H^+}$ can be supported by these vesicles in the presence of reduced PMS (Table I), oxidation of both substrates by the doubly reconstituted vesicles does not increase the magnitude of the $\Delta\bar{\mu}_{H^+}$ over that obtained with either substrate. This suggests that electrons from the dehydrogenases pass through the same vectorial site of proton translocation, a conclusion that is further elucidated by the data presented in Figure 4. When D-[^{14}C]alanine is oxidized in the presence of unlabeled D-lactate, the V_{max} for [^{14}C]pyruvate production drops to about half of that observed when D-[^{14}C]alanine is added alone. Similarly, the conversion of D-[^{14}C]lactate to [^{14}C]pyruvate also is inhibited by 50% when both enzymes are engaged in catalysis (not shown). Hence, turnover of both enzymes is limited to the same degree by a slow step in the respiratory chain that lies at or before the site of proton expulsion. Furthermore, this step is symmetric with respect to both dehydrogenases in the reconstituted vesicles.

Table I: Membrane Potential ($\Delta\Psi$) and pH Gradient (ΔpH) in Reconstituted Vesicles[a]

	energy source	pH	$\Delta\Psi$ (mV)	ΔpH (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)
right-side-out vesicles reconstituted with					
D-ADH	D-Ala	5.5	−66	−80	−146
D-LDH	D-lac	5.5	−73	−78	−151
D-ADH + D-LDH	D-Ala + D-lac	5.5	−62	−81	−143
D-ADH + D-LDH	reduced PMS	5.5	−88	−95	−183
inside-out vesicles reconstituted with					
D-ADH	D-Ala	7.5	+68	+81	+149
D-LDH	D-lac	7.5	+71	+80	+151
D-ADH + D-LDH	D-Ala + D-lac	7.5	+65	+84	+149
D-ADH + D-LDH	reduced PMS	7.5	+82	+100	+182

[a] Right-side-out *E. coli* ML 308-225*dld*-3 vesicles were reconstituted with D-ADH (0.3 nmol/mg of protein), D-LDH (0.45 nmol/mg of protein), or both enzymes and suspended in 0.1 potassium phosphate (pH 5.5) at a final protein concentration of 4 mg of protein/mL. Samples were placed in the top chamber of a flow dialysis apparatus, and either 20 μM [^{3}H]TPP$^+$ (2.5 Ci/mmol) or 16 μM [^{14}C]acetic acid (54 mCi/mmol) was added for determination of $\Delta\Psi$ (interior negative) or ΔpH (interior alkaline), respectively. After an appropriate time 10 mM D-alanine, 10 mM D-lactate, 10 mM D-alanine plus 10 mM D-lactate, or 20 mM potassium ascorbate and 100 μM PMS were added, and changes in radioactive solute concentration were monitored in the dialyzate. The vesicle suspension was kept under a water-saturated oxygen atmosphere. CCCP (10 μM) was then added to dissipate the gradients formed, and $\Delta\Psi$ and ΔpH were calculated from the extent of cation and weak acid accumulation. Measurements in inverted reconstituted vesicles were carried out at 10 mg/mL final protein concentration in 0.1 M potassium phosphate (pH 7.5) with 8 μM [^{14}C]methylamine (48 mCi/mmol) and 50 μM potassium thio[^{14}C]cyanate (40 mCi/mmol) to measure ΔpH (interior acid) and $\Delta\Psi$ (interior positive), respectively. The experiments were carried out as described for right-side-out vesicles. All values presented in the table represent an average of three determinations.

Table II: Respiration Rate of Reconstituted Vesicles[a]

	substrate	O_2 uptake (nmol min^{-1} mg^{-1})	pyruvate formation (nmol min^{-1} mg^{-1})
right-side-out vesicles reconstituted with			
D-ADH	D-Ala	40	80
D-LDH	D-lac	45	76
D-ADH + D-LDH	D-Ala + D-lac	40	76
D-ADH + D-LDH	reduced PMS	200	
inside-out vesicles reconstituted with			
D-ADH	D-Ala	80	160
D-LDH	D-lac	80	160
D-ADH + D-LDH	D-Ala + D-lac	89	162
D-ADH + D-LDH	reduced PMS	200	

[a] *E. coli* ML 308-225*dld*-3 vesicles reconstituted with saturating amounts of D-ADH, D-LDH, or both enzymes, as described under Methods, were diluted to a final concentration of 0.3 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6) and placed in the chamber of a Clark-type oxygen electrode whose temperature was maintained at 25 °C. Magnesium sulfate was added at 10 mM and the background rate of respiration monitored for 5 min. D-Alanine, D-lactate, or both were then added (each at 10 mM final concentration), and rates of oxygen consumption were measured. Additionally, oxygen consumption was monitored in the presence of 20 mM potassium ascorbate and 100 μM PMS, and the values were corrected for nonenzymatic rates of oxygen consumption. Unreconstituted *E. coli* ML 308-225*dld*-3 vesicles showed no respiratory activity in the presence of one or both substrates. All values presented in the table represent an average of three determinations.

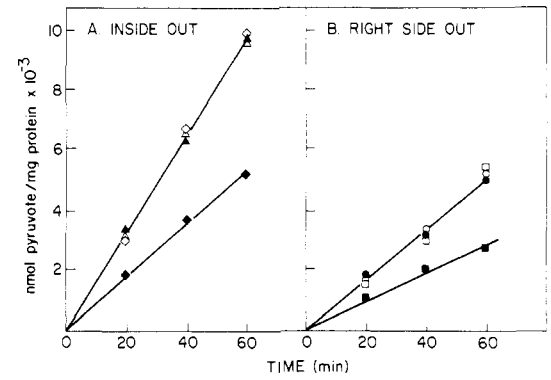

FIGURE 4: Rates of [^{14}C]pyruvate formation in *E. coli* ML 308-225*dld*-3 vesicles reconstituted with D-ADH, D-LDH, or both enzymes. (A) Inside-out vesicles: Inverted vesicles were reconstituted with 0.2 nmol of one or both enzymes/mg of membrane protein as described under Methods. Vesicles (100 μg of protein) were then diluted to a final volume of 50 μL in 0.1 M potassium phosphate (pH 6.6) containing 10 mM magnesium sulfate at 25 °C. D-[^{14}C]Alanine (0.5 mCi/mmol; 10 mM) and/or D-[^{14}C]lactate (0.7 mCi/mmol; 10 mM) were (was) added to the incubation, and [^{14}C]pyruvate was separated from the assay mixture as described under Methods. Where indicated, D-lactate (unlabeled) was added to 10 mM. [^{14}C]Pyruvate formed at given times was monitored in vesicles reconstituted with (a) D-ADH in the presence of D-[^{14}C]alanine (△), (b) D-LDH in the presence of D-[^{14}C]lactate (◇), (c) D-ADH and D-LDH in the presence of D-[^{14}C]alanine (▲), and (d) D-ADH and D-LDH in the presence of D-[^{14}C]alanine and D-lactate (◆). (B) Right-side-out vesicles: Formation of [^{14}C]pyruvate was measured as described in (A) in right-side-out vesicles reconsituted with (a) 0.3 nmol of D-ADH/mg of membrane protein in the presence of D-[^{14}C]alanine (○), (b) 0.45 nmol of D-LDH/mg of membrane protein in the presence of D-[^{14}C]lactate (□), (c) 0.45 nmol of D-LDH/mg of membrane protein and 0.3 nmol of D-ADH/mg of membrane protein in the presence of D-[^{14}C]alanine (●), and (d) 0.45 nmol of D-LDH/mg of membrane protein and 0.3 nmol of D-ADH/mg of protein in the presence of D-[^{14}C]alanine and D-lactate (■). [^{14}C]Pyruvate was not formed in control experiments for (A) and (B) with vesicles treated with reconstitution buffer lacking both enzymes.

Although inhibition of turnover of one enzyme by another is observed in both right-side-out and inside-out vesicles (Figure 4), the rate-determining step is not equivalent for electrons entering from either side of the membrane. As shown previously, the maximum rate of respiration (O_2 consumption) is 2-fold higher for inside-out reconstituted vesicles (80 nmol min^{-1} mg^{-1}) as compared to that for right-side-out reconstituted vesicles (40 nmol min^{-1} mg^{-1}) under the same conditions (Table II), and the rates do not increase upon simultaneous oxidation of D-alanine and D-lactate. The corresponding rates for [^{14}C]pyruvate formation (160 nmol min^{-1} mg^{-1} and 80 nmol min^{-1} mg^{-1}, respectively) are in accord with these expectations, since the reduction of molecular oxygen to water is a four-electron process, while that of substrate oxidation to pyruvate is only a two-electron oxidation. Interestingly, the respiration rates of vesicles in the presence of reduced PMS (200 nmol of O_2 min^{-1} mg^{-1}, Table II) far exceed the V_{max} presented here. Thus, 80 nmol of O_2 min^{-1} mg^{-1} is not the maximal rate of electron flow through the respiratory chain. These results suggest a common rate-limiting step for electron transfer from dehydrogenases to the cytochromes that occurs before the site(s) of proton translocation. Since rates of electron flow are 2-fold higher for reducing equivalents coming from the inner face of the membrane, different rate-determining steps and possibly different routes of electron transfer exist for electrons from each side of the membrane.

Discussion

The results presented in this paper demonstrate that *E. coli* membrane vesicles can be reconstituted simultaneously with

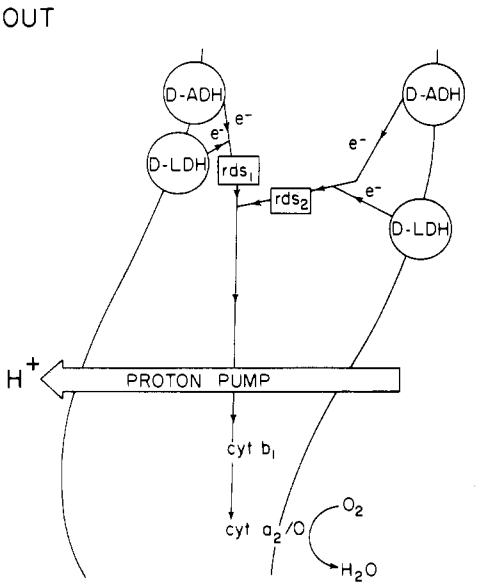

FIGURE 5: Schematic representation of paths of electron flow from D-LDH and D-ADH bound to outer and inner surfaces of membrane: D-ADH, D-amino acid dehydrogenase; D-LDH, D-lactate dehydrogenase; rds_1, rate-determining step for electrons derived from D-ADH and D-LDH on the outer surface; rds_2, rate-determining step for electrons derived from D-ADH and D-LDH on the inner surface; cyt b_1 and cyt a_2/O signify membrane-bound cytochromes.

purified preparations of two *E. coli* membrane flavoenzymes, D-ADH and D-LDH, and that parallel reconstitution can take place on either face of the cytoplasmic membrane. We have examined this phenomenon using five assays: active transport of lactose, respiration, reduction of electron-accepting dyes, enzymatic product formation, and generation of a $\Delta\bar{\mu}_{H^+}$. The results also demonstrate that these enzymes do not compete with each other for binding and suggest independent sites of association on both surfaces of the membrane. In addition, preliminary evidence (A. Bhaduri and H. R. Kaback, unpublished results) demonstrates independent sites of association for yet another primary electron donor, α-glycerol-phosphate dehydrogenase. Taken together, therefore, the data are inconsistent with the presence of a receptor protein(s) in the membrane that acts as a common site of association for primary dehydrogenases in the *E. coli* respiratory chain. Studies on D-ADH and D-LDH binding to liposomes suggest that the affinity of the enzymes for phospholipid can satisfactorily account for their binding to right-side-out vesicles, which also argues against the presence of specific receptor proteins for either dehydrogenase.

Although D-ADH and D-LDH have no distinct sites of association with the membrane, their enzymatic action appears to drive proton translocation at the same site. Previous studies show that oxidation of D-alanine or D-lactate (Olsiewski et al., 1981; S. Ramos and H. R. Kaback, unpublished results) from either side of the membrane in right-side-out vesicles is able to generate a $\Delta\bar{\mu}_{H^+}$ of the same magnitude and polarity (interior negative and alkaline), thus making it unlikely that D-ADH or D-LDH constitutes a part of a proton translocating loop in the respiratory chain. However, this does not exclude the existence of a proton translocating loop distal to both dehydrogenases.

Evidence that the enzymes transfer substrate electrons to a common redox component of the respiratory chain includes the following: (1) the V_{max} rates of respiration are the same for both enzymes on a given membrane surface, indicating a common rate-limiting step; (2) the magnitudes of the $\Delta\bar{\mu}_{H^+}$ generated by D-ADH or D-LDH turnover are equivalent; (3) turnover of one enzyme inhibits turnover of the other. Given these data and the observation that rates of respiration induced by reduced PMS oxidation are higher than the V_{max} rates supported by either enzyme, it is concluded that the rate-limiting step for respiration in the reconstituted system occurs prior to the site of proton translocation. As observed previously (Olsiewski et al., 1981), however, respiration rates for reconstituted inside-out vesicles are 2-fold higher than those observed for similarly reconstituted right-side-out vesicles, indicating a different rate-determining step and possibly distinct routes for electron flow from either surface of the membrane.

The scheme presented in Figure 5 is consistent with these observations. Each enzyme binds to either membrane face due to its affinity for phospholipid, although physiologically dehydrogenase molecules are found on the cytoplasmic face alone (Owen & Kaback, 1978, 1979a,b). Separate paths of electron flow from each dehydrogenase converge to a common arm containing the rate-determining step for electron flux from each side of the membrane and lead to a common site of proton translocation located before the cytochromes. Reduced PMS oxidation drives the same proton translocating mechanism and supports a higher $\Delta\bar{\mu}_{H^+}$, because of a higher rate of electron transfer. Hence, electrons from reduced PMS bypass the earlier rate-determining redox components.

Finally, it seems unlikely that electron transfer from the primary dehydrogenases in the *E. coli* membrane occurs through a static, rigidly ordered sequence of electron-transfer intermediates. Data presented in this paper and elsewhere (Downie & Cox, 1978; Olsiewski et al., 1981) indicate that there are probably several routes of electron transfer from primary dehydrogenases to the site(s) of proton translocation and suggest that dehydrogenases bound to the lipid bilayer may transfer electrons to subsequent redox acceptors via collisions between transversely mobile intermediates rather than more permanently fixed complexes. In other words, the situation here may well be analogous to electron transfer between cytochrome b_5 and cytochrome b_5 reductase (Strittmatter & Rogers, 1975) and within the mitochondrial respiratory chain (Schneider et al., 1980) where it has been proposed that the rate of lateral diffusion of respiratory intermediates is the rate-limiting step for oxidoreduction.

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Influence of Cholesterol on the Structural Preferences of Dioleoylphosphatidylethanolamine–Dioleoylphosphatidylcholine Systems: A Phosphorus-31 and Deuterium Nuclear Magnetic Resonance Study†

C. P. S. Tilcock,* M. B. Bally, S. B. Farren, and P. R. Cullis

ABSTRACT: The polymorphic phase behavior of mixtures of synthetic dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) and the influence of cholesterol on these phase preferences have been investigated by employing nuclear magnetic resonance (NMR) techniques. In particular, ^{31}P NMR procedures are utilized to study the overall phase preferences of these mixed systems, whereas ^{2}H NMR is employed to monitor the structural preferences of individual components of these systems by using versions of DOPE and DOPC which are deuterium (^{2}H) labeled at the C_{11} position of the acyl chains. The results obtained show that DOPE–DOPC systems containing as little as 20 mol % DOPC initially assume lamellar structure at 40 °C, even though DOPE in isolation prefers the hexagonal (H_{II}) organization at this temperature. However, this lamellar organization appears to represent a metastable state, as incubation for extended periods at 40 °C results in formation of a structure, possibly the cubic phase, in which the phospholipids experience isotropic motional averaging. The addition of cholesterol induces hexagonal (H_{II}) phase organization. ^{2}H NMR studies of appropriately labeled versions of these systems indicate that cholesterol does not produce such effects by associating preferentially with either DOPE or DOPC. Further, in situations where bilayer, hexagonal, or "isotropic" phases coexist in the same sample, the phospholipids exhibit apparently ideal mixing behavior.

The functional roles of cholesterol in biological membranes are not well understood. Within the terms of the fluid mosaic model (Singer & Nicholson, 1972), it is generally postulated that cholesterol plays a role in modulating the "fluidity" of the lipid environment, thereby affecting membrane function. However, there is little evidence to support the proposal that modulation of fluidity plays important functional roles in vivo. Reasons for this include the fact that lipids such as gel-state lipids which can inhibit the activity of integral protein in reconstituted protein–lipid systems [see, for example, Warren et al. (1975)] do not appear to exist in most biological systems. This is particularly true of eukaryotic cell membranes. Further, the ability of physiologically relevant factors (such as pH, ionic strength, divalent cation concentration, or even membrane protein) to isothermally modulate membrane fluidity to gain the necessary regulation in appropriate lipid mixtures is far from established.

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