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Reconstitution of *Escherichia coli* Membrane Vesicles with D-Amino Acid Dehydrogenase†

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ABSTRACT: When purified D-amino acid dehydrogenase [Olsiewski, P. J., Kaczorowski, G. J., & Walsh, C. T. (1980) *J. Biol. Chem. 255*, 4487] is incubated with right-side-out membrane vesicles from *Escherichia coli*, the enzyme binds to the membrane in a time- and concentration-dependent manner. As a result, the vesicles acquire the ability to oxidize D-alanine and catalyze D-alanine-dependent active transport. Similarly, incubation of D-amino acid dehydrogenase with inside-out vesicles results in binding of enzyme and D-alanine oxidase activity. Antibody inhibition studies indicate that the enzyme is bound exclusively to the inner cytoplasmic surface of the membrane in native vesicles (i.e., membrane vesicles prepared from cells induced for D-amino acid dehydrogenase). In contrast, similar studies with reconstituted vesicles demonstrate that enzyme binds to the surface exposed to the medium regardless of the orientation of the membrane. Thus, enzyme bound to right-side-out vesicles is located on the opposite side of the membrane from where it is normally found. Remarkably, in the presence of D-alanine, reconstituted right-side-out and inside-out vesicles generate electrochemical proton gradients of similar magnitude but opposite polarity, indicating that enzyme bound to either surface of the membrane is physiologically functional. The results suggest that vectorial proton translocation via the respiratory chain occurs at a point distal to the site where electrons enter the respiratory chain from the primary dehydrogenase, a conclusion that is inconsistent with the notion that the dehydrogenase forms part of a proton-translocating loop.

Cytoplasmic membrane vesicles prepared from *Escherichia coli* by osmotic lysis have become increasingly useful for studying active transport (Kaback, 1970, 1974a, 1980). These vesicles exhibit the same polarity and configuration as the membrane in the intact cell, as shown by many criteria (Kaback, 1971, 1974a; Owen & Kaback, 1978, 1979a,b). Furthermore, the vesicles retain the ability to couple respiration and, as demonstrated recently (Hugenholtz et al., 1981), ATP hydrolysis to the active transport of many different solutes by mechanisms in which chemiosmotic forces play a central, obligatory role (Kaback, 1976; Harold, 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977; Kaczorowski et al., 1980). Thus, as postulated by Mitchell (1961, 1968, 1979a,b), the immediate driving force for active transport in this system is a transmembrane electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$)[1] composed of an electrical potential ($\Delta\psi$, interior negative) and a chemical gradient of hydrogen ions (ΔpH, interior alkaline).

An important achievement in these studies was the development of techniques that allow quantitation of the electrical and chemical components of $\Delta\bar{\mu}_{H^+}$ (Schuldiner & Kaback, 1975; Ramos et al., 1976, 1979; Ramos & Kaback, 1977a–c; Felle et al., 1980). Recently, these techniques were applied to vesicles in which the membrane is inverted relative to the intact cell (Reenstra et al., 1980). In this case, substrate oxidation or ATP hydrolysis leads to the generation of a $\Delta\bar{\mu}_{H^+}$

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\psi$, electrical potential; ΔpH, chemical gradient of hydrogen ions; NEM, *N*-ethylmaleimide; D-ADH, D-amino acid dehydrogenase; D-LDH, D-lactate dehydrogenase; PMS, phenazine methosulfate; DCIP, dichloroindophenol; Q_1, ubiquinone 1; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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of approximately equal magnitude but opposite polarity (i.e., interior positive and acid) to that generated by right-side-out vesicles and intact cells.

Although the energetics of active transport are largely resolved and certain insights have been made with regard to the mechanism of H^+:substrate symport (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Padan et al., 1979; LeBlanc et al., 1980; Cohn & Kaback, 1980; Robertson et al., 1980), very little is known about the mechanism of respiration-linked vectorial proton translocation. According to Mitchell's classic concept of "loops" (Mitchell, 1966, 1968), the proton and/or electron carriers comprising the respiratory chain (or a part of it) are disposed asymmetrically across the membrane in such a manner that, in the first arm of the loop, two protons and two electrons pass vectorially from one carrier on the inner surface of the membrane to the next carrier on the outer surface (e.g., reduction of ubiquinone by reduced flavin adenine dinucleotide). The second arm of the loop involves the vectorial transfer of electrons (but not protons) from the carrier on the outer surface of the membrane to a third carrier on the inner surface (e.g., oxidation of reduced ubiquinone by a cytochrome or non-heme iron protein), resulting in the appearance of two protons in the external medium. The sum of the two processes (i.e., extrusion of protons into the external medium and vectorial flow of electrons from the outer to the inner surface of the membrane) results in the generation of a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline). Despite the elegant simplicity of this concept, it has been difficult to test in *E. coli*, since relatively little is known about the molecular architecture of the respiratory chain (Haddock & Jones, 1977).

Several recent studies have focused on electron transfer pathways (Wallace & Young, 1977a,b; Haddock & Jones, 1977; Downie & Cox, 1978; Brookman et al., 1979) and the localization of the energy-coupling site(s) (Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975, 1979; Kaback & Patel, 1978) in *E. coli*. Furthermore, certain membranous dehydrogenases such as D-lactate dehydrogenase (D-LDH; Reeves et al., 1973; Kohn & Kaback, 1973; Futai, 1973, 1974; Short et al., 1974, 1975b; Kaczorowski et al., 1978), α-glycerol-P dehydrogenase (Schryvers et al., 1978), and NADH dehydrogenase (Dancey et al., 1976; Jaworowski et al., 1981) have been solubilized, purified, and shown to bind to the vesicle membrane. In this context, it is noteworthy that some of these dehydrogenases—D-LDH, in particular—have been shown to drive active transport even though the reconstituted enzyme is bound to the external surface of the membrane (Short et al., 1975a,b).

D-Amino acid dehydrogenase (D-ADH) is an inducible enzyme found in *E. coli* B after growth on DL-alanine as a carbon source (Raunio & Jenkins, 1973). The enzyme is membrane bound where it is coupled to the respiratory chain and drives active transport (Kaczorowski et al., 1975a,b, 1977). D-ADH has been solubilized, purified to about 65% homogeneity, and characterized as an iron–sulfur flavoprotein that exhibits, unlike other simple flavoproteins, ubiquinone reductase activity (Olsiewski et al., 1980).

In this paper, we report the reconstitution of right-side-out and inside-out vesicles with D-ADH and demonstrate that the enzyme is able to generate $\Delta\bar{\mu}_{H^+}$ even when it is bound to the "wrong" surface of the membrane. Thus, it seems unlikely that the primary dehydrogenase plays a direct role in vectorial proton translocation.

Experimental Procedures

Materials

[^{3}H]Tetraphenylphosphonium (2.5 Ci/mmol; bromide salt) was prepared by the Isotope Synthesis Group at Hoffmann-La Roche, Inc. under the direction of Dr. Arnold Liebman. L-[U-^{14}C]Proline (248 mCi/mmol), [1-^{14}C]lactose (60 mCi/mmol), [U-^{14}C]acetic acid (54 mCi/mmol), [^{14}C]thiocyanate (27 mCi/mmol; potassium salt), and [^{14}C]methylamine hydrochloride (48 mCi/mmol) were obtained from either Amersham/Searle or New England Nuclear. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Calbiochem. Nitrocellulose membrane filters (0.45-μm pore size) were purchased from Amicon. All other chemicals were reagent grade obtained from commercial sources.

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., 1975b). *E. coli* B was grown on a salt medium supplemented with 0.5% DL-alanine and 2.0% glycerol, and vesicles were prepared as described (Kaczorowski et al., 1975a).[2] 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 a 50-fold excess of buffer at the desired pH. After incubation at room temperature for 20 min, the vesicles were collected by centrifugation: 30 min at 40000*g* for vesicles prepared by osmotic lysis; 1 h at 120000*g* for inverted vesicles. Pellets were then resuspended in a similar volume of buffer and washed again by centrifugation before final resuspension in 0.1 M potassium phosphate at the required pH. Reconstituted vesicles were subjected to the same procedures.

Transport Assays. Uptake of radioactive solutes by membrane vesicles in the presence of reduced phenazine methosulfate (PMS) or D-alanine was determined by filtration as described (Kaback, 1971, 1974b). In all cases, the energy source was added to the vesicles for at least 30 s prior to addition of transport solute to ensure complete energization. Steady-state levels of accumulation were determined from the transport profiles and were usually attained within 10 min with D-alanine as electron donor.

Measurement of Respiration. Rates of oxygen consumption were measured with a Clark-type electrode (YSI Model 53 oxygen monitor; Yellow Spring Instrument Co., Yellow Springs, OH) (Kaczorowski et al., 1975a).

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 (TPP^+) or 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.,

[2] These vesicles contained about 0.09 unit of D-ADH activity per mg of membrane protein, as determined by D-alanine-dependent reduction of dichloroindophenol in the presence of ubiquinone 1 (Olsiewski et al., 1980).

1980) were used for calculations with right-side-out and inside-out vesicles, respectively.

Preparation and Assay of D-*ADH.* D-ADH was prepared as described (Olsiewski et al., 1980) with the following modifications: Whole cells were suspended to approximately 3 times wet weight in 0.1 M potassium phosphate (pH 7.5) containing 10 mM magnesium sulfate, 10 μg/mL DNase, and 10 μg/mL RNase. The cells were broken by two passages through a French pressure cell at 13000 psi. Large debris was removed by centrifugation at 40000*g* for 10 min, and the membrane fraction was collected by centrifugation at 120000*g* for 1 h. All subsequent steps in the purification were performed as described (Olsiewski et al., 1980). The final enzyme preparation was at least 65% homogeneous as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and no single contaminant accounted for more than 5% of the total protein. Enzyme activity was monitored by following D-alanine-dependent reduction of dichloroindophenol (DCIP) spectrophotometrically at 600 nm in the presence of ubiquinone 1 (Q_1; Olsiewski et al., 1980). One unit of activity is defined as 1 μmol of dye reduction per min.

Preparation of Antibodies to D-*ADH.* Antibodies against purified D-ADH were prepared as described by Short et al. (1975a). Briefly, rabbits were immunized with approximately 50 μg of D-ADH in complete and incomplete Freund's adjuvant, followed by booster injections of enzyme in phosphate-buffered saline every 2 weeks. After a high antibody titer was achieved, the animals were bled at regular intervals between booster injections. The serum was pooled, and IgG was purified (Kabat & Mayer, 1971). Rabbits were injected with the enzyme storage buffer for preparation of control IgG. The final protein concentrations of purified IgG were 50 mg of protein/mL (anti-DADH) and 16 mg/mL (control).

Reconstitution of Membrane Vesicles with D-*ADH.* Enzyme [from a stock solution of 3–6 units/mL in 0.1 M potassium phosphate (pH 7.5) containing 0.02% Triton X-100] was diluted into 0.7 mL of 0.1 M potassium phosphate (pH 6.6) at 25 °C. After approximately 1 min, 0.3 mL of a vesicle suspension (ca. 2 mg of membrane protein) was added with immediate mixing. The samples were incubated at 25 °C for 1 h, and the vesicles collected by centrifugation (40000*g* for 20 min with right-side-out vesicles; 120000*g* for 1 h with inside-out vesicles) were resuspended to a final protein concentration of 4 mg/mL in 0.1 M potassium phosphate (pH 6.6).

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

Difference Spectra. Difference spectra were obtained as described by Barnes & Kaback (1971) except that an Aminco DW-2 recording spectrophotometer (American Instrument Co., Silver Spring, MD) was used.

Results

Functional Reconstitution of Membrane Vesicles with D-*ADH.* Right-side-out vesicles prepared from *E. coli* ML 308-225*dld*-3 catalyze neither D-alanine oxidation nor D-alanine-dependent active transport. When the vesicles are treated with D-ADH, however, they exhibit D-alanine-dependent lactose transport (Figure 1). As shown, in the absence of electron donors, lactose transport is minimal. Similarly, addition of D-alanine to untreated vesicles does not induce transport of the disaccharide. On the other hand, when D-alanine is added to vesicles that have been treated with D-ADH, marked stimulation of lactose transport is observed, and within 5–6 min, a steady-state level of accumulation of about

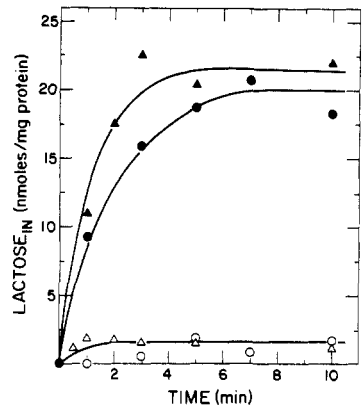

FIGURE 1: Reconstitution of D-alanine-dependent lactose transport in *E. coli* membrane vesicles. *E. coli* ML 308-225*dld*-3 vesicles were reconstituted with D-ADH (0.14 unit/mg of membrane protein) as described under Methods. Vesicles were then diluted in a final volume of 50 μL containing 50 mM potassium phosphate, pH 6.6, 10 mM magnesium sulfate, and 40 μg of membrane protein at 25 °C. [^{14}C]Lactose (0.6 mM, 20 mCi/mmol) was then added, and transport was monitored by filtration in the absence of an exogenous energy source (△), with 10 mM D-alanine (●), or with 20 mM potassium ascorbate and 100 μM phenazine methosulfate under an oxygen atmosphere (▲). The experiment was also performed with 10 mM D-alanine and *E. coli* ML 308-225*dld*-3 vesicles which had been treated with the reconstitution buffer lacking D-ADH (○).

20 nmol/mg of protein is achieved. This value is to be compared to a steady-state level of 22 nmol/mg of protein observed with reduced PMS as electron donor in either reconstituted or untreated vesicles. Although not shown, D-alanine-dependent lactose transport is abolished by potassium cyanide or hydroxyquinoline *N*-oxide, indicating that the reconstituted enzyme functions via the membrane-bound respiratory chain.

Various parameters were investigated in order to optimize the reconstitution procedure with respect to lactose transport. The optimal pH for reconstitution is 6.6; after reconstitution at pH 5.5 and 7.5, lactose transport activity is only about 60% of that observed at pH 6.6. Similarly, 25 °C is the optimal temperature for reconstitution, and experiments performed at 0 and 37 °C yield vesicles with only 30% of maximal activity. When vesicles are incubated with increasing amounts of D-ADH at 25 °C, pH 6.6, for 2 h, collected by centrifugation, and assayed for lactose transport, the results presented in Figure 2A are obtained. D-Alanine-dependent lactose accumulation increases sharply as the concentration of D-ADH is increased from 0 to about 0.10 unit of enzyme activity/mg of membrane protein and remains essentially constant at levels of D-ADH exceeding 0.14 unit/mg of protein. Although not shown, binding of D-ADH to the vesicles continues to increase despite saturation of transport activity. Furthermore, vesicles reconstituted with D-ADH concentrations that yield maximum transport activity (i.e., 0.14 unit/mg of protein) exhibit levels of bound enzyme approximating those of native *E. coli* B vesicles, as judged by dye reduction assays.[3] Reconstituted transport activity is stable since neither repeated washing of the vesicles by centrifugation (at least 4 times) nor incubation at 25 °C for several hours results in significant loss of D-alanine-dependent transport activity. Association of D-ADH with the vesicle membrane is also time dependent (Figure 2B). Thus, at saturating concentrations of D-ADH during recon-

[3] As shown below (cf. Figure 3), approximately equal quantities of anti-D-ADH IgG are required to inhibit D-ADH activity in reconstituted *dld*-3 vesicles and inverted *E. coli* B vesicles, providing further evidence that approximately equal amounts of D-ADH are bound to the membrane under the conditions utilized.

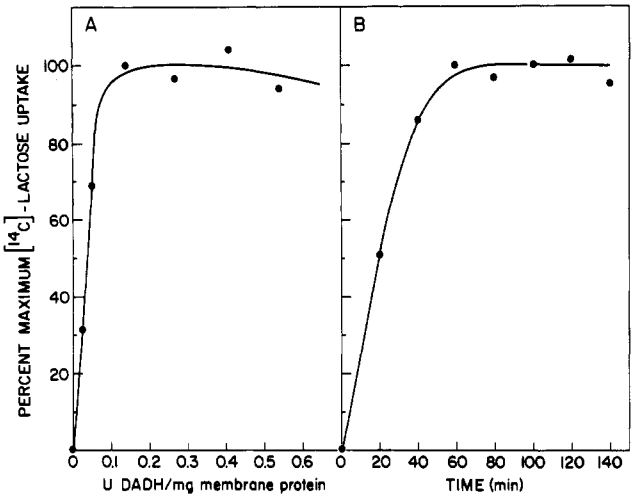

FIGURE 2: Reconstitution of D-alanine-dependent transport activity in *E. coli* ML 308-225*dld*-3 membrane vesicles. (A) Concentration dependence: Vesicles were treated with increasing amounts of D-amino acid dehydrogenase (0–0.6 unit/mg of membrane protein) for 1 h as described under Methods. After the vesicles were washed and resuspended in 0.1 M potassium phosphate, pH 6.6, they were assayed for [^{14}C]lactose transport in the presence of 10 mM D-alanine as described in Figure 1. Steady-state solute accumulation was determined for each enzyme concentration from the transport profile and compared to the maximum level achieved in the reconstituted system (20 nmol of lactose/mg of vesicle protein). Although not shown, a similar dependence for the initial rate of lactose transport vs. enzyme concentration during reconstitution was found. (B) Time dependence: Vesicles were treated with D-ADH (0.14 unit/mg of membrane protein) for various times at 25 °C. The vesicles were then collected and washed by centrifugation, resuspended to ca. 4 mg of protein/mL in 0.1 M potassium phosphate, pH 6.6, and assayed for D-alanine-dependent lactose uptake as described in Figure 1. The maximum steady-state accumulation of lactose in this experiment was 19.5 nmol/mg of protein, and all determinations were normalized to that value for representation.

stitution, D-alanine-dependent transport activity increases essentially linearly up to about 40 min and is maximal at 1 h and thereafter. A number of other factors were found to have no effect on reconstitution: bovine serum albumin (1 mg/mL), magnesium sulfate (10 mM), D-alanine (10 mM), and generation of $\Delta\bar{\mu}_{H^+}$ by means of L-lactate oxidation.

When optimal conditions for reconstitution are used, approximately 30% of the added enzymatic activity (0.045 unit of 0.14 unit/mg of membrane protein) adheres stably to the vesicles as determined by the DCIP/Q_1 dye-coupled assay, and the remaining activity can be detected in the supernatant after centrifugation of the vesicles. In order to determine whether the unbound enzymatic activity is functional for reconstitution, vesicles were added to the supernatant recovered after reconstitution. By repeated treatment of the soluble enzyme with membrane vesicles, essentially all D-ADH activity became membrane bound, indicating that all of the purified enzyme is competent for reconstitution.

Although results will not be presented in detail, reconstitution data similar to those presented for lactose transport were obtained by monitoring D-alanine oxidation. Thus, D-alanine oxidase activity is saturable with respect to both D-ADH concentration and time (cf. Figure 2A,B), and the maximum rate of oxygen consumption in reconstituted *dld*-3 vesicles is 80 nmol (min)$^{-1}$ (mg of protein)$^{-1}$ (Table I). This value is similar to that observed in *E. coli* B vesicles prepared from cells maximally induced for D-ADH (Table I; Kaczorowski et al., 1975a). As reported with D-LDH (Short et al., 1974), although D-alanine oxidation is saturable with respect to D-ADH concentration during reconstitution, membrane-bound

Table I: D-Alanine Oxidase Activity in *E. coli* B and Reconstituted *E. coli* ML 308-225*dld*-3 Membrane Vesicles[a]

	oxygen consumption [nmol (min)$^{-1}$ (mg of protein)$^{-1}$]		
	E. coli B	*E. coli* ML 308-225-*dld*-3	reconstituted *E. coli* ML 308-225*dld*-3
right-side-out vesicles	80	0	80
inverted vesicles	80	0	160

[a] *E. coli* B vesicles were diluted to a final concentration of 0.25 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. At this time, D-alanine was added (10 mM final concentration) and the rate of oxygen consumption measured. Identical experiments were performed with inverted *E. coli* B vesicles (0.25 mg of protein/mL final concentration) and *E. coli* ML 308-225*dld*-3 right-side-out (0.25 mg of protein/mL) and inverted (0.25 mg of protein/mL) vesicles. Samples of both *E. coli* ML 308-225*dld*-3 vesicle preparations were then reconstituted with a saturating amount of D-ADH (specific activity 1.8 unit/mg of protein) as described under Methods and then monitored for oxygen consumption in the presence of 10 mM D-alanine as before.

DCIP/Q_1 reductase activity does not exhibit saturation behavior and continues to increase with higher enzyme concentrations during reconstitution (data not shown). It seems unlikely, therefore, that there is a specific interaction between D-ADH and the membrane with respect to binding.

Using inside-out vesicles prepared from *E. coli* ML 308-225*dld*-3 and the conditions described above for reconstitution, we determined the association of D-ADH with the membrane to be both concentration and time dependent as judged by D-alanine oxidation (data not shown), and a maximum rate of 160 nmol of oxygen consumed (min)$^{-1}$ (mg of protein)$^{-1}$ is observed with a saturating D-ADH concentration (0.12 unit/mg of membrane protein) during reconstitution (Table I). Moreover, as observed with right-side-out vesicles, only 30% of the total D-ADH activity binds to the membranes as measured by dye reduction. Interestingly, however, although roughly the same amount of DCIP/Q_1 reductase activity is bound per milligram of protein with right-side-out and inside-out vesicles, the rate of D-alanine oxidation is clearly 2-fold higher in inverted vesicles. Whether this represents more efficient coupling of D-ADH to the respiratory chain when the enzyme is bound to the "right" side of the membrane is not known, but the observation gives a preliminary indication that paths of electron flow from D-ADH on opposite sides of the membrane may not be identical (cf. below). In any event, inverted vesicles prepared from *E. coli* B maximally induced for D-ADH exhibit essentially the same rate of D-alanine oxidation as their right-side-out counterparts [i.e., 80 nmol (min)$^{-1}$ (mg of protein)$^{-1}$; Table I].

Localization of Reconstituted D-ADH. When purified D-ADH is incubated with increasing amounts of antibody prepared against the partially purified enzyme, DCIP/Q_1 reductase activity is progressively inhibited. Typically, treatment of enzyme with excess anti-D-ADH at 25 °C for 30 min results in 50% inhibition compared to 5% inhibition with the same concentration of control IgG, and incubation at 4 °C overnight causes 80% and 20% inhibition, respectively. As shown in Figure 3A, when right-side-out vesicles prepared from *E. coli* B induced for D-ADH are treated with increasing

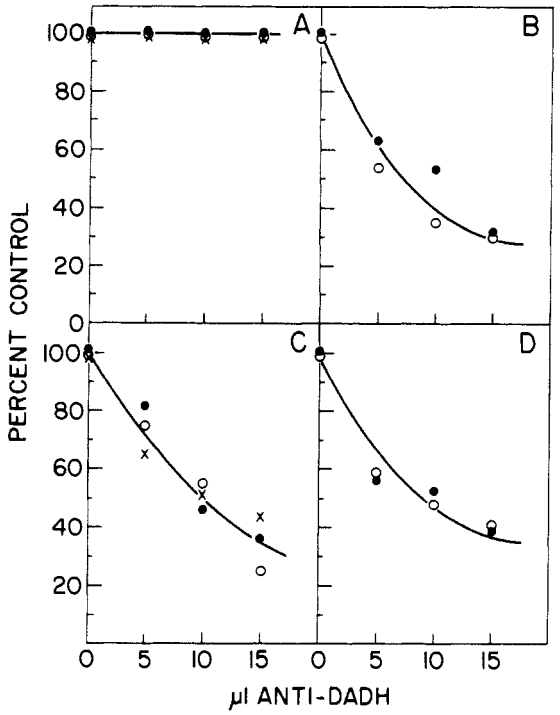

FIGURE 3: Inhibition of D-alanine-dependent oxidase, transport, and dye reductase activities in *E. coli* membrane vesicles by anti-D-ADH. The IgG antibody fraction was purified from antisera prepared against D-amino acid dehydrogenase. Membrane vesicles were treated with 0–15 μL of anti-D-ADH IgG (50 mg of protein/mL) for 30 min at 25 °C followed by an overnight incubation at 4 °C. The vesicles were then assayed for several D-alanine-dependent activities. Vesicles were placed in the chamber of a Clark-type electrode and assayed for oxygen consumption as described in Table I (○). Another preparation was diluted to a final concentration of 2 mg of vesicle protein/mL in 50-μL final volume of 50 mM potassium phosphate, pH 6.6, containing 10 mM magnesium sulfate. D-Alanine (10 mM) and L-[^{14}C]proline (2.0 μM, 248 mCi/mmol) were then added, and proline uptake was monitored for filtration (×). Finally, a third preparation of vesicles was assayed for D-alanine-dependent DCIP/Q_1 reductase activity spectrophotometrically as described under Methods (●). The results of these experiments are presented as a percentage of the activity in vesicles carried through the same procedure but untreated with antibody. In all cases, serum prepared from control animals had minimal effect on these activities. However, oxidase activities diminished during the overnight incubation, even in control vesicles; hence, these values are lower than those reported in Table I. (A) *E. coli* B vesicles prepared by osmotic lysis. Control levels of activities were 60 nmol of oxygen consumed $(min)^{-1}$ $(mg\ of\ protein)^{-1}$ 0.09 unit of a dye reduction/mg of protein, and 0.8 nmol of proline accumulated per mg of protein at the steady state. (B) Inverted *E. coli* B vesicles prepared by French pressure cell techniques. Control oxidase activity was 60 nmol of oxygen consumed $(min)^{-1}$ $(mg\ of\ protein)^{-1}$, and dye reduction was 0.09 unit/mg of protein. (C) Right-side-out *E. coli* ML 308-225*dld*-3 vesicles reconstituted with 0.14 unit of D-ADH per mg of vesicle protein. Control oxidase activity was 30 nmol of oxygen consumed $(min)^{-1}$ $(mg\ of\ protein)^{-1}$, DCIP/Q_1 reduction was 0.045 unit/mg of protein, and steady-state proline accumulation was 0.2 nmol/mg of protein. (D) Inverted *E. coli* ML 308-225*dld*-3 vesicles reconstituted with 0.12 unit of D-ADH/mg of vesicle protein. The control preparations exhibited oxidase activity of 60 nmol of oxygen consumed $(min)^{-1}$ $(mg\ of\ protein)^{-1}$ and dye reductase levels of 0.043 unit/mg of protein.

concentrations of anti-D-ADH, there is no significant inhibition of D-alanine-dependent proline transport, D-alanine oxidation, or D-alanine/DCIP/Q_1 reductase activity. In contrast, when similar experiments are performed with inside-out *E. coli* B vesicles, D-alanine oxidase and dye reductase activities are progressively inhibited by anti-D-ADH, and the profile is almost identical with that observed with the soluble enzyme (Figure 3B). It is apparent, therefore, that D-ADH, like several other membranous enzymes in *E. coli* (Owen & Kaback, 1978, 1979a,b), is normally located on the cytoplasmic face of the membrane.

Table II: Membrane Potential ($\Delta\psi$) and pH Gradient (ΔpH) in Right-Side-Out *E. coli* Membrane Vesicles[a]

		E. coli B		reconstituted *E. coli* ML 308-225*dld*-3	
pH	parameter	D-alanine	ascorbate/PMS	D-alanine	ascorbate/PMS
7.5	$\Delta\psi$ (mV)	−109	−109	−86	−93
5.5	$\Delta\psi$ (mV)	−93	−103	−76	−75
5.5	ΔpH (mV)	−89	−86	−77	−83

[a] *E. coli* B vesicles were suspended in 0.1 M potassium phosphate at the desired pH and 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 or 20 mM potassium ascorbate and 100 μM phenazine methosulfate were added and changes in radioactive solute concentration monitored in the dialysate. The use of ascorbate and phenazine methosulfate necessitated keeping the vesicle suspension under a water-saturated oxygen atmosphere. CCCP (10 μM) was then added to dissipate the gradients formed, and the magnitudes of $\Delta\psi$ and ΔpH were calculated from the extent of cation and weak acid accumulation. Similar determinations were made with *E. coli* ML 308-225*dld*-3 vesicles reconstituted with a saturating amount of D-ADH (0.14 unit/mg of vesicle protein). In both cases, each value presented in the table represents the average of at least three determinations.

Similar titration studies performed with right-side-out and inside-out ML 308-225*dld*-3 vesicles reconstituted with D-ADH are shown in parts C and D of Figure 3, respectively. In both preparations, increasing concentrations of anti-D-ADH cause parallel inhibition of D-ADH activity as judged by either D-alanine oxidation or D-alanine/DCIP/Q_1 reductase activity, and with right-side-out vesicles, inhibition of D-alanine-driven proline transport exhibits the same profile (Figure 3C). Taken as a whole, the results provide a strong indication that during reconstitution, D-ADH associates exclusively with the surface of the membrane exposed to the external medium regardless of the polarity of the vesicles.

$\Delta\bar{\mu}_{H^+}$ in Reconstituted Vesicles. As expected (Ramos et al., 1976; Ramos & Kaback, 1977a), D-alanine oxidation in right-side-out vesicles from *E. coli* B leads to the generation of $\Delta\bar{\mu}_{H^+}$, the components of which vary with the external pH. Thus ΔpH (interior alkaline) is present at pH 5.5 and absent at pH 7.5 (not shown), while $\Delta\psi$ (interior negative) is present at both extremes of pH (Table II). Moreover, the magnitude of ΔpH and $\Delta\psi$ is similar to that observed during reduced PMS oxidation. Intriguingly, when right-side-out *dld*-3 vesicles are reconstituted with D-ADH under conditions where the enzyme is bound to the wrong surface of the membrane, D-alanine oxidation still leads to the generation of a $\Delta\bar{\mu}_{H^+}$ with the same polarity (i.e., interior negative and alkaline), and the magnitude of the ΔpH and/or $\Delta\psi$ generated at pH 5.5 and 7.5 is similar to that observed with reduced PMS (Table II).

Similar determinations with inverted *E. coli* B vesicles and inverted *dld*-3 vesicles reconstituted with D-ADH are presented in Table III. Clearly, in both instances, D-alanine or reduced PMS oxidation gives rise to a $\Delta\bar{\mu}_{H^+}$ of comparable magnitude but opposite polarity to that observed in right-side-out vesicles. Importantly, therefore, the direction of $\Delta\bar{\mu}_{H^+}$ is determined by the polarity of the membrane and not by the path of

Table III: Membrane Potential ($\Delta\psi$) and pH Gradient (ΔpH) in Inverted *E. coli* Membrane Vesicles[a]

		E. coli B		reconstituted *E. coli* ML 308-225*dld*-3	
pH	parameter	D-alanine	ascorbate/PMS	D-alanine	ascorbate/PMS
5.5	$\Delta\psi$ (mV)	+76	+77	+84	+93
7.5	$\Delta\psi$ (mV)	+78	+70	+102	+110
7.5	ΔpH (mV)	+90	+81	+93	+70

[a] Inverted *E. coli* vesicles were suspended at 4 mg of protein/mL in 0.1 M potassium phosphate at the desired pH. Energy-dependent generation of $\Delta\psi$ (interior positive) or ΔpH (interior acid) was then monitored by flow dialysis using 50 μM potassium [^{14}C]thiocyanate (27 mCi/mmol) and 8 μM [^{14}C]methylamine (48 mCi/mmol), respectively. D-Alanine (10 mM) or potassium ascorbate (20 mM) and phenazine methosulfate (100 μM), in the presence of an oxygen atmosphere, were used as energy sources, and gradient formation was detected by measuring changes in the levels of radioactivity in the dialysate. Gradients were dissipated with 10 μM CCCP, and the magnitude of the potentials formed was calculated from anion and weak base accumulation. Similar experiments were performed with inverted *E. coli* ML 308-225*dld*-3 vesicles which had been reconstituted with a saturating amount of D-ADH (0.12 unit/mg of membrane protein). All values presented in the table represent the average of at least three determinations.

Table IV: Effect of *N*-Ethylmaleimide Treatment on Membrane Potential ($\Delta\psi$) in *E. coli* ML 308-225*dld*-3 Vesicles Reconstituted with D-ADH[a]

	$\Delta\psi$ (mV)	
	control	NEM treated
right-side-out vesicles	−73	−73
inverted vesicles	+75	+39

[a] *E. coli* ML 308-225*dld*-3 vesicles were suspended in 0.1 M potassium phosphate, pH 6.6 (2 mg of protein/mL for right-side-out vesicles and 4 mg of protein/mL for inverted vesicles), and treated with 1 mM NEM for 10 min at 25 °C. After this time, 10 mM dithiothreitol was added to quench the reaction, and vesicles were collected and washed by centrifugation as described under Methods. Both vesicle preparations were then reconstituted with a saturating amount of D-ADH and collected once again by centrifugation. Control vesicles which were not treated with NEM were carried through an identical procedure. Finally, all vesicles were resuspended in 0.1 M potassium phosphate, pH 5.5, and assayed for D-alanine-dependent generation of membrane potential by flow dialysis techniques as described in Tables II and III. Each value listed in the table represents the average of two determinations. DCIP/Q_1 dye reduction studies indicate that the same amount of D-ADH binds to NEM-treated and untreated control vesicles.

electron flow from the primary dehydrogenase. Moreover, as reported by Reenstra et al. (1980), ΔpH (interior acid) is observed at pH 7.5 (Table III) but not at pH 5.5 (not shown), a situation opposite to that obtained in right-side-out vesicles.

Electron Transfer Pathways from D-*ADH Bound to Opposite Sides of the Membrane Are Not Identical.* Although spectra are not presented, with reconstituted right-side-out *dld*-3 vesicles, the anaerobic steady-state level of cytochrome reduction in the presence of D-alanine is only 50% of that observed in the presence of dithionite. In contrast, with reconstituted inside-out *dld*-3 vesicles, D-alanine oxidation leads to complete reduction of the cytochrome chain (i.e., addition of D-alanine leads to the same degree of reduction as dithionite, and addition of dithionite after D-alanine reduction causes no further spectra change). Thus, the interaction of D-ADH with the respiratory chain differs, depending upon the location of the enzyme.

Treatment of right-side-out vesicles with sulfhydryl reagents such as *N*-ethylmaleimide (NEM) inhibits oxidation of several substrates (Barnes & Kaback, 1971; Kaback & Hong, 1973), and electron transfer is blocked prior to the site of proton translocation since neither the oxidation of reduced PMS nor the generation of $\Delta\bar{\mu}_{H^+}$ in the presence of the artificial electron carrier is altered (Kaback & Patel, 1978). In the case of D-lactate oxidation, NEM inhibits both oxygen consumption and the generation of $\Delta\bar{\mu}_{H^+}$, although it does not affect the activity of solubilized D-LDH (Kaczorowski et al., 1978). Furthermore, when right-side-out *dld*-3 vesicles are treated with NEM and then reconstituted with D-LDH, D-lactate is oxidized effectively and induces the formation of $\Delta\bar{\mu}_{H^+}$ (G. J. Kaczorowski and H. R. Kaback, unpublished experiments).

Since solubilized D-ADH is inactivated by NEM (unpublished experiments), right-side-out and inverted *dld*-3 vesicles were treated first with NEM, washed, and then reconstituted with a saturating concentration of D-ADH (Table IV). With control vesicles that were not treated with NEM, $\Delta\psi$ values of −73 and +75 mV are observed for right-side-out and inside-out vesicles, respectively, in the presence of D-alanine. Similarly, a value of −73 mV is observed in right-side-out *dld*-3 vesicles treated with NEM prior to reconstitution. On the other hand, pretreatment of inside-out vesicles causes inhibition of D-alanine-dependent proton translocation, and $\Delta\psi$ is reduced to +39 mV. Therefore, as suggested for D-LDH (Kaback & Patel, 1978), the electron transfer pathway from D-ADH on the inner surface of the membrane appears to involve a sulfhydryl-sensitive component that is proximal to the site of proton translocation, and this component is bypassed by electrons coming from enzyme bound to the external surface of the vesicles.

Discussion

The experiments presented in this paper demonstrate that right-side-out and inside-out cytoplasmic membrane vesicles from *E. coli* can be functionally reconstituted with D-ADH. That is, exposure of vesicles of either polarity to D-ADH under the conditions described leads to the binding of enzyme in such a manner that electrons abstracted from the substrate are passed into the membrane-bound respiratory chain and result in the generation of $\Delta\bar{\mu}_{H^+}$. Moreover, it is evident from antibody inhibition studies that D-ADH is associated exclusively with the outer surface of the vesicles regardless of their polarity, a conclusion that is not surprising considering the size of D-ADH and the permeability of the vesicle membrane. What is remarkable, however, is that D-alanine oxidation in right-side-out vesicles reconstituted with D-ADH leads not only to the generation of $\Delta\bar{\mu}_{H^+}$ but also to a $\Delta\bar{\mu}_{H^+}$ of polarity and magnitude indistinguishable from those of the native system. Clearly, therefore, the polarity of vectorial proton translocation is dependent on the orientation of the vesicle membrane and not on the pathway of electron flow from the primary dehydrogenase.

Despite the functional identity of D-ADH bound to either side of the vesicle membrane, it is apparent that the pathways of electron flow from the enzyme bound to each surface are not the same. Thus, D-ADH bound to the cytoplasmic surface of the vesicles quantitatively reduces the respiratory chain, while enzyme bound to the external surface reduces only about 50% of the cytochromes reduced by dithionite. Consistently, inverted vesicles reconstituted with D-ADH exhibit twice the maximal rate of D-alanine oxidation as reconstituted right-side-out vesicles. Finally, studies with NEM suggest that

D-alanine oxidation catalyzed by D-ADH bound to the cytoplasmic surface of the membrane involves a sulfhydryl-containing respiratory intermediate located between the primary dehydrogenase and the site of vectorial proton translocation. In contrast, this intermediate does not appear to be involved in the path of electron flow from D-ADH bound to the external surface of the membrane.

Despite a lack of precise information regarding the chemical nature, number, order, and topographical arrangement of the respiratory intermediates within the matrix of the *E. coli* membrane, certain noteworthy points bear reiteration: (i) Oxidation of D-alanine and D-lactate or reduced PMS (Short et al., 1975b; S. Ramos and H. R. Kaback, unpublished experiments) from either side of the membrane is able to generate a $\Delta\bar{\mu}_{H^+}$ of the same magnitude and polarity (interior negative and alkaline), and (ii) the site at which $\Delta\bar{\mu}_{H^+}$ is generated in vesicles prepared from aerobically-grown *E. coli* is probably located prior to the cytochromes (Barnes & Kaback, 1971; Kaback & Barnes, 1971; Schuldiner & Kaback, 1975; Stroobant & Kaback, 1975, 1979). Given these conclusions, it seems very unlikely that D-ADH or D-LDH constitutes part of a proton-translocating loop in the respiratory chain. On the other hand, it must be emphasized that this contention says nothing about the mechanism of proton translocation per se. That is, the argument that these primary dehydrogenases do not participate in a loop does not preclude the presence of such structures in the respiratory chain at a step distal to the primary dehydrogenases. Furthermore, it is interesting that this situation is not peculiar to bacterial membrane vesicles. Thus, turnover of α-glycerol-P dehydrogenase or succinate dehydrogenase energizes mitochondria despite the presence of these enzymes on the outer and inner surface, respectively, of the inner mitochondrial membrane (Alexandre et al., 1980).

Ubiquinone is a lipid-soluble respiratory intermediate that has been postulated to play a direct role in proton translocation via a "protonmotive ubiquinone cycle" (Mitchell, 1975a,b, 1976, 1980). In addition, solubilized D-ADH reduces ubiquinone directly, making it a likely candidate for the initial electron acceptor from D-ADH on the external surface of the membrane. For these reasons, preliminary reconstitution experiments (unpublished) were carried out with vesicles from the ubiquinone–menaquinone double mutant isolated by Wallace & Young (1977b). Vesicles prepared from the mutant and reconstituted with D-ADH exhibit neither D-alanine oxidation nor D-alanine-dependent transport activity, although dye reduction studies demonstrate that enzyme binds normally. When the vesicles are treated with ubiquinone 8 (Stroobant & Kaback, 1979), however, and then reconstituted with D-ADH, the vesicles are completely functional for these D-alanine-dependent activities. Although the results are provocative, they do not allow resolution of a role for ubiquinone in electron transfer per se, as opposed to dual roles in both electron transfer and vectorial proton translocation.

Although respiration and transport activity exhibit saturation as a function of enzyme concentration, binding of D-ADH continues to increase as judged by dye reaction [for analogous data with D-LDH, see Short et al. (1974)]. Thus, binding of dehydrogenase to the outer surface of the vesicle membrane does not seem to involve specific receptor sites. This conclusion is supported by preliminary experiments (K. Haldar, P. J. Olsiewski, G. J. Kaczorowski, C. T. Walsh, and H. R. Kaback, unpublished experiments) in which vesicles were reconstituted sequentially with D-LDH and D-ADH. These results suggest that the presence of one dehydrogenase does not alter the binding of the other.

There are a few clear differences in the properties of D-ADH and D-LDH with respect to reconstitution that are interesting. D-ADH appears to have relatively less affinity for the membrane as evidenced by the observation that it is solubilized by lower concentrations of Triton X-100 [compare Olsiewski et al. (1980) to Kohn & Kaback (1973)]. Furthermore, as opposed to D-LDH, which partitions essentially quantitatively into the membrane during reconstitution (Short et al., 1974), only 30% of the D-ADH binds on a single exposure to vesicles even though all of the enzyme seems to be competent for reconstitution. Finally, with D-LDH, chaotropic agents such as guanidine are important for maximal reconstitution (Reeves et al., 1973; Short et al., 1974), while no such requirement is observed for D-ADH.

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