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Functional Mosaicism of Membrane Proteins in Vesicles of *Escherichia coli*

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Membrane vesicles of *Escherichia coli* prepared by osmotic lysis of lysozyme ethylenediaminetetracetate (EDTA) spheroplasts have approximately 60% of the total membrane-bound reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (EC 1.6.99.3) and Mg^{2+}-adenosine triphosphatase (ATPase) (EC 3.6.1.3) activities exposed on the outer surface of the inner membrane. Absorption of these vesicles with antiserum prepared against the purified soluble Mg^{2+}-ATPase resulted in agglutination of approximately 95% of the inner membrane vesicles, as determined by dehydrogenase activity, and about 50% of the total membrane protein. The unagglutinated vesicles lacked all dehydrogenase activity and may consist of outer membrane. Lysozyme-EDTA vesicles actively transported calcium ion, using either NADH or adenosine 5'-triphosphate (ATP) as energy source. However, neither D-lactate nor reduced phenazine methosulfate energized calcium uptake, suggesting that the observed calcium uptake was not due to a small population of everted vesicles. Transport of calcium driven by either NADH or ATP was inhibited by simultaneous addition of D-lactate or reduced phenazine methosulfate. Proline transport driven by D-lactate oxidation was inhibited by either NADH oxidation or ATP hydrolysis. These results suggest that the portion of the total population of vesicles capable of active transport, i.e., the inner membrane vesicles, are functionally a homogeneous population but cannot be categorized as either right-side-out or everted, since activities normally associated with only one side of the inner membrane can be found on both sides of the membrane of these vesicles. Moreover, the data indicate that oxidation of NADH or hydrolysis of ATP by externally localized NADH dehydrogenase or Mg^{2+}-ATPase establishes a protonmotive force of the opposite polarity from that established through D-lactate oxidation.

Inner membrane (IM) vesicles prepared by osmotic lysis of lysozyme ethylenediaminetetracetate (EDTA) spheroplasts according to the method of Kaback (7) have proved useful in the study of membrane phenomena (5, 7). Controversy persists, however, regarding the orientation of the membrane of the vesicles relative to the orientation of the same membrane in vivo. Various laboratories have demonstrated that certain enzyme activities cryptic in spheroplasts are in part exposed in lysozyme-EDTA vesicles (2, 21). Those results suggested one of two possibilities: (i) the vesicle preparation contained nearly equal amounts of right-side-out and everted membranes or (ii) certain membrane proteins were translocated from the inner surface of the IM to the outer surface of that membrane, creating a mosaic (2, 21). Visualization of the vesicles by electron microscopy suggested a homogeneous population (1, 7). Moreover, essentially all of the vesicles were capable of active transport when reduced phenazine methosulfate (PMS) was used as an energy donor (7).

Hare et al. (5) reported the separation of two distinct populations of vesicles by absorption with antibody to soluble Mg^{2+}-adenosine triphosphatase (ATPase) (BF_1). They found that approximately 50% of the membrane protein was agglutinated but that the unagglutinated vesicles retained nearly all of the active transport activity, leading to the conclusion that lysozyme-EDTA vesicles consist of a mixture of equal amounts of right-side-out and everted vesicles.

We have likewise fractionated lysozyme-EDTA vesicles with anti-BF_1 serum and have found that approximately 50% of the membrane protein is agglutinated. Using control serum, no agglutination occurred. Also, no agglutination occurred when vesicles prepared from a strain lacking the BF_1 were used. Although only 50% of the protein agglutinated, nearly all of the reduced nicotinamide adenine dinucleo-

tide (NADH) and D-lactate dehydrogenase activities were found in the agglutinated material. The unagglutinated vesicles lacked dehydrogenase activity. According to Osborne et al. (10), 40 to 60% of membrane protein in similar vesicle preparations can be attributed to outer membrane (OM).

Wickner (23) has recently performed similar experiments using vesicles prepared from cells infected with coliphage M13, which inserts its coat protein into the membrane with the antigenic sites directed outwards. The results of agglutination experiments using antiserum to M13 coat protein were similar to our results. However, the experiments were complicated by the fact that control serum agglutinated the vesicles. Also, the unagglutinated vesicles retained NADH dehydrogenase activity with the same specific activity as the original vesicles, even though that fraction would presumably be enriched with OM.

Short et al. (14) and Futai and Tanaka (3) have reported that the D-lactate dehydrogenase, unlike the BF_1, is not accessible to specific antibody in vesicles. The results, taken together with our results, suggest that (i) lysozyme-EDTA vesicles consist of about equal amounts of IM and OM vesicles, and (ii) essentially all of the IM vesicles have enough BF_1 on the outer surface to agglutinate with anti-BF_1 serum, yet little or no D-lactate dehydrogenase is accessible to antibody. Thus, IM vesicles consist of a relatively homogeneous population of mosaic vesicles in which about 60% of the total membrane-bound BF_1 and NADH dehydrogenase activities are on the outer surface of the vesicles. It is not possible to distinguish between a single, entirely homogeneous population and one in which every vesicle is a mosaic with BF_1 on the inner and outer surfaces but in varying amounts.

We have previously reported that these vesicles do not accumulate Ca^{2+} when either D-lactate or reduced PMS is used as the energy donor (13). Everted vesicles, prepared by lysis of intact cells with a French press, utilize either of those efficiently for the transport of calcium. This observation further supports the idea that there are not significant amounts of everted vesicles in preparations of lysozyme-EDTA vesicles.

The question arose as to whether enzymes translocated to the outer surface of the membrane remain functional components of the larger complexes of which they are part. According to Mitchell (9) and West and Mitchell (22), the BF_1 is a portion of a primary active transport system for protons, the BF_0F_1, which establishes an electrochemical proton gradient during the hydrolysis of adenosine 5'-triphosphate (ATP). Likewise, the NADH dehydrogenase is part of the electron transport chain, itself a system responsible for the electrogenic translocation of protons. The polarity of the protonmotive force would depend on the transmembrane arrangement of the components of each system. This suggested to us that hydrolysis of ATP via the BF_1 located on the outer surface of a mosaic membrane might be capable of establishing a protonmotive force with an orientation opposite to the force set up by oxidation of D-lactate or reduced PMS. The BF_1 on the inner surface would not act to set up a protonmotive force due to impermeability of ATP. The polarity of the protonmotive force established by oxidation of NADH would be similar to that of ATP, since only the external dehydrogenase would have access to exogenously added NADH. We report here that either ATP hydrolysis or NADH oxidation can energize calcium transport in lysozyme-EDTA vesicles. Calcium transport activity driven by ATP or NADH is uncoupled by oxidation of D-lactate or reduced PMS. Moreover, transport of proline driven by D-lactate oxidation is uncoupled by either NADH oxidation or ATP hydrolysis. These results suggest that the enzymes located on the outer surface of a mosaic vesicle establish protonmotive forces that are basic and negative outside, in contrast to the acid and positive polarity established by enzyme systems located on the inner surface of the membrane. Simultaneous functioning of both types of systems results in a completed proton circuit, leading to uncoupling.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* K-12 strain 7 (6) and its neomycin-resistant derivative, strain NR70, which lacks the BF_1 (12), were grown with shaking at 37°C in a basal salts medium (16) supplemented with 68 mM glycerol as carbon source.

Preparation of membrane vesicles. Everted membrane vesicles were prepared by lysis of cells with a French pressure cell, as described previously (17). Lysozyme-EDTA vesicles were prepared according to the method of Kaback (7), with the following modifications. Midexponential-phase cells were washed once with a buffer consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 30 mM NaCl and suspended at a ratio of 40 volumes/g of wet cells in 33 mM Tris-hydrochloride (pH 8.0). An equal volume of the same buffer containing 40% sucrose was added, followed by addition of lysozyme and EDTA to 40 μg/ml and 4 mM, respectively. The solution was swirled slowly for 15 min, followed by centrifugation at 12,000 $\times$ *g* for 30 min. The sediment was suspended in a minimal volume of 0.1 M potassium phosphate buffer (pH 6.6) containing 20% sucrose. All steps to

this point were carried out at 4°C. The spheroplasts were then rapidly diluted into 100 volumes of 50 mM potassium phosphate buffer (pH 6.6) and swirled vigorously for 5 min at 37°C. $MgSO_4$ and deoxyribonuclease were added to 6 mM and 20 μg/ml, respectively, and the suspension was swirled for 5 min at 23°C. The remaining steps were all performed at 4°C. The suspension was centrifuged at 49,000 × g for 30 min. The sediment was suspended in 50 mM potassium phosphate buffer (pH 6.6) and centrifuged at 3,000 × g for 15 min. The supernatant solution was then centrifuged at 104,000 × g for 20 min, and the sediment was washed twice with the same buffer. If the vesicles were to be used for calcium transport assays, the final two washes were performed with a buffer consisting of 10 mM Tris-hydrochloride (pH 7.2), containing 0.14 M KCl, 0.5 mM dithiothreitol, and 10% (vol/vol) glycerol and suspended finally in that buffer. All suspensions were performed by repeated passage through an 18-gauge needle using a 3-ml syringe.

Transport assays. Assays of proline transport were performed as described previously (12). In assays designed to determine the effect of NADH or ATP on D-lactate-driven proline transport, NADH or ATP was added 2.5 min prior to the addition of D-lactate. When the effect of anti-BF_1 serum on the inhibition of D-lactate-driven proline transport by ATP, or the effect of NAD^+ on the inhibition by NADH of D-lactate-driven proline transport, was investigated, the antiserum or NAD^+ was added 2.5 min prior to the addition of ATP or NADH, respectively.

Calcium transport assays were performed as described previously (17). In those experiments in which the effect of D-lactate or reduced PMS on ATP- or NADH-driven calcium transport was measured, D-lactate or reduced PMS was added 2.5 min prior to ATP or NADH.

Enzyme assays. Previously described assays were used to measure Mg^{2+}-ATPase activity (18) and D-lactate dehydrogenase activity (12). In all assays, ATP-hydrolyzing activity not inhibited by NaN_3 was subtracted. This corrects for non-BF_1 activity. NADH dehydrogenase was assayed by measuring the decrease in absorbance at 340 nm in an assay mixture containing 0.1 M Tris-hydrochloride (pH 7.2) and 0.15 mM NADH at 23°C. In assays where spheroplasts or vesicles were rendered permeable to substrate with toluene, the spheroplasts or vesicles were incubated with 1% toluene (vol/vol) for 10 min at 23°C. A portion of the suspension was then diluted into the assay mixture.

Absorption with antiserum. Antiserum to the BF_1 was prepared as described previously (18). The agglutination experiments were performed as follows: to 1.5 mg of vesicles was added sufficient antiserum to inhibit four times the Mg^{2+}-ATPase activity of the vesicles. Vesicles incubated with an equal volume of preimmune serum were used as controls. The mixtures were then diluted to a final volume of 6 ml with 50 mM potassium phosphate buffer (pH 7.4), containing 0.14 M NaCl, and allowed to incubate for 1 h at 23°C, followed by 12 h at 4°C. The suspensions were centrifuged at 150 × g for 5 min, and the supernatant solution was carefully withdrawn. The remaining vesicles were centrifuged at 104,000 × g for 20 min at 4°C. Both agglutinated and nonagglutinated vesicles were washed twice with 50 mM potassium phosphate buffer (pH 6.6) and suspended to their initial volumes for assay of protein content and enzymatic activities.

Protein determinations. Protein concentration was determined by the method of Lowry et al. (8).

Chemicals. $^{45}CaCl_2$ (1.3 to 1.4 Ci/mmol) and L-[^{3}H]proline (4.75 Ci/mmol) were purchased from New England Nuclear Corp. Dicyclohexylcarbodiimide (DCCD) was obtained from Eastman Kodak Co. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was the generous gift of P. G. Heytler of the E. I. Du Pont de Nemours Co. All other compounds used in these studies were reagent grade and purchased from commercial sources.

RESULTS

Localization of Mg^{2+}-ATPase and NADH dehydrogenase activities in spheroplasts, French press vesicles, and lysozyme-EDTA vesicles. The localization of Mg^{2+}-ATPase and NADH dehydrogenase activities in spheroplasts and membrane vesicles of *E. coli* is shown in Table 1. In spheroplasts, both activities appear to be associated with the cytoplasmic side of the IM, since little activity was observed unless the spheroplasts were rendered permeable to NADH or ATP by treatment with toluene. Conversely, no increase in those activities was observed in French press vesicles after treatment with toluene, consistent with such vesicles being everted (1–3, 13). Everted vesicles would have the original cytoplasmic side of the membrane accessible to exogenously added substrate. In lysozyme-EDTA vesicles, about 60% of the total NADH dehydrogenase and Mg^{2+}-ATPase activities were measurable without toluene treatment, suggesting that

TABLE 1. *Crypticity of NADH dehydrogenase and Mg^{2+}-ATPase activities in spheroplasts and membrane vesicles*

Prepn	Mg^{2+}-ATPase activity		NADH dehydrogenase activity	
	Units/mg	%[a]	Units/mg	%[a]
Spheroplasts				
Toluene treated	0.206	100	0.860	100
Untreated	0.034	17	0.045	5
French press vesicles				
Toluene treated	0.980	100	1.94	100
Untreated	1.040	106	2.010	104
Lysozyme-EDTA vesicles				
Toluene treated	2.470	100	1.310	100
Untreated	1.55	63	0.730	56

[a] Untreated/toluene treated × 100.

about 60% of the total enzymes were located on the external surface of the membrane.

Distribution of total protein, NADH, and D-lactate dehydrogenase activities after fractionation with antiserum to the BF_1. With lysozyme-EDTA vesicles prepared from strain 7 (BF_1^+), approximately half of the total membrane protein was agglutinated, whereas greater than 90% of the NADH and D-lactate dehydrogenase activities measured in toluene-treated vesicles was agglutinated (Table 2). This result suggests that nearly all vesicles with dehydrogenase activity have BF_1 located on the outer surface, enough BF_1 at least to allow agglutination. The remaining unagglutinated vesicles had little dehydrogenase activity. The remaining NADH dehydrogenase activity, it should be pointed out, was only about 50% active in the absence of toluene (data not shown), indicating that the distribution of activity was the same in the unagglutinated vesicles. That is, a small portion of vesicles with activity remained unagglutinated, but that portion retained the same orientation as the agglutinated vesicles. The rest of the unagglutinated vesicles presumably were OM. Osborn et al. (10) have found that similar vesicle preparations contain 40 to 60% OM by protein, although this depends on the organism and strain. The agglutination is specific for the BF_1, since no protein or dehydrogenase activity was agglutinated with control serum or when lysozyme-EDTA vesicles prepared from the BF_1-deficient strain NR70 were used (Table 2). Thus, it appears that nearly all of the IM vesicles have Mg^{2+}-ATPase and NADH dehydrogenase activities exposed on the outer surface.

Calcium transport activity in lysozyme-EDTA and everted membrane vesicles. Everted vesicles of strain 7 actively accumulate calcium, utilizing NADH, reduced PMS, D-lactate, or ATP as an energy source (Fig. 1B). Lysozyme-EDTA vesicles transport calcium only using NADH or ATP (Fig. 1A), although with lower specific activity than that found in everted vesicles, perhaps due to large amounts of OM. Neither D-lactate nor reduced PMS was an effective energy donor for calcium uptake in lysozyme-EDTA vesicles (Fig. 1A). This differ-

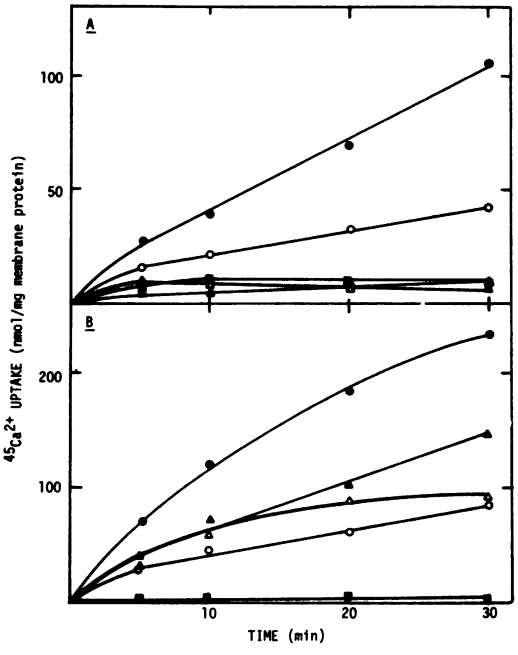

FIG. 1. *Efficiency of various energy donors for calcium transport in membrane vesicles. Membrane vesicles were prepared as described in the text. (A) Assays were performed using 70 μg of membrane protein per ml, with membrane vesicles prepared by osmotic lysis of lysozyme-EDTA spheroplasts. (B) Assays were performed using 50 μg of membrane protein per ml, with membrane vesicles prepared by lysis of intact cells with a French press. Energy sources: (●) 5 mM NADH; (○) 5 mM ATP; (▲) 20 mM lithium D-lactate; (△) 0.1 mM PMS + 20 mM potassium ascorbate; (■) endogenous.*

TABLE 2. *Fractionation of lysozyme-EDTA vesicles with anti-BF_1 serum*

Membrane source	Addition	Membrane protein[a]		NADH dehydrogenase activity[a, b]		D-Lactate dehydrogenase activity[a]	
		mg	% unagglutinated	Units	% Unagglutinated	Units	% Unagglutinated
Strain	None	1.50	100	2.73	100	0.357	100
	Preimmune serum	1.28	85	2.55	93	0 351	98
	Anti-BF_1 serum	0.60	40	0.25	9	0.028	8
Strain NR70	None	1.50	100	3.01	100	0.375	100
	Preimmune serum	1.39	93	3.01	100	0.407	109
	Anti-BF_1 serum	1.38	92	2.95	98	0.388	103

[a] Protein and enzyme activities present in unagglutinated vesicles, as described in the text.
[b] NADH dehydrogenase activity measured in toluene-treated vesicles.

ence in effectiveness of the various energy donors between the two types of membrane vesicle preparations precludes the possibility that the observed calcium transport is occurring in only a small subpopulation of everted vesicles present in the lysozyme-EDTA preparation. Calcium transport in lysozyme-EDTA vesicles requires actual oxidation of NADH via the respiratory chain or hydrolysis of ATP via the BF_0F_1, as shown by the response to various inhibitors (Table 3).

Uncoupling of calcium transport from NADH oxidation or ATP hydrolysis by oxidation of D-lactate or reduced PMS. When lysozyme-EDTA vesicles oxidized D-lactate or reduced PMS at the same time that ATP hydrolysis was occurring, ATP hydrolysis no longer energized calcium transport (Table 4). This inhibition required actual oxidation of the electron transport chain substrate, since KCN prevented it (Table 4). D-Lactate oxidation does not significantly inhibit ATP hydrolysis via the BF_1, nor does ATP hydrolysis inhibit the oxidation of D-lactate (data not shown). This suggests that a true uncoupling of the energy derived from ATP hydrolysis has occurred. Calcium transport energized by NADH oxidation was likewise uncoupled by simultaneous oxidation of D-lactate (Table 4). Since NADH is oxidized directly by PMS, it was not possible to determine the effect of simultaneous oxidation of both substrates. Moreover, it was not possible to specifically inhibit the D-lactate dehydrogenase during the oxidation of NADH, as was done in the above experiment with ATP hydrolysis. KCN inhibits both oxidations, and the specific D-lactate dehydrogenase inhibitors oxalate and oxamate form precipitates with calcium.

TABLE 3. *Effect of inhibitors and uncouplers on calcium transport in lysozyme-EDTA vesicles*

Energy source (5 mM)	Addition	$^{45}Ca^{2+}$ uptake[a]	
		nmol/30 min per mg	%
NADH	None	99.2	100
	10 mM KCN	15.8	16
NADH	1% Ethanol[b]	81.8	100
	10 μM FCCP	6.8	8
	50 μM HOQNO[c]	38.4	47
ATP	1% Ethanol	17.0	100
	10 μM FCCP	0.0	0
	50 μM DCCD	0.0	0

[a] All values corrected for endogenous rates.
[b] FCCP, HOQNO, and DCCD were added as ethanolic solutions.
[c] HOQNO, 2-Heptyl-4-hydroxyquinoline-*N*-oxide.

TABLE 4. *Uncoupling of calcium transport in lysozyme-EDTA vesicles from NADH oxidation or ATP hydrolysis by oxidation of D-lactate or reduced PMS*

Energy source	Addition	$^{45}Ca^{2+}$ uptake[a]	
		nmol/30 min per mg	%
NADH (5 mM)	None	35.7	100
	30 mM D-lactate	14.9	42
ATP (5 mM)	None	18.1	100
	30 mM D-lactate	5.2	29
	30 mM D-lactate + 10 mM KCN	16.1	89
	0.1 mM PMS + 20 mM ascorbate	0.0	0
	0.1 mM PMS + 20 mM ascorbate + 10 mM KCN	15.9	88
	10 mM KCN	18.3	101
D-Lactate (30 mM)	None	4.1	
PMS (0.1 mM) + 20 mM ascorbate	None	4.0	

[a] All values corrected for endogenous rates.

No inhibition of NADH dehydrogenase activity was observed when 7.5 mM D-lactate was added to a reaction mixture containing 1 mM NADH in three different buffer systems: the pH 6.6 buffer system used for proline transport assays, the pH 7.2 buffer system described in Materials and Methods, and the pH 8.0 buffer system used for assay of calcium transport. During an actual calcium transport assay, the presence of 30 mM D-lactate inhibited the utilization of NADH at 5 mM (initial concentration) by only 15%. That inhibition was constant with time up to 30 min. Thus, it seems likely that the effect of D-lactate on NADH-driven calcium uptake does not reflect inhibition of NADH utilization but, rather, an uncoupling.

Uncoupling of proling transport from D-lactate oxidation by hydrolysis of ATP or oxidation of NADH. D-Lactate oxidation energizes proline transport in lysozyme-EDTA vesicles, whereas NADH oxidation or ATP hydrolysis is ineffective (Fig. 2). In the reciprocal experiments to those described above, it was found that simultaneus oxidation of NADH or hydrolysis of ATP with the oxidation of D-lactate prevents proline transport (Fig. 2). Again, since the source of energy, namely, D-lactate oxidation, is not inhibited by ATP hydrolysis, the process may represent an uncoupling. Uncoupling by ATP requires hydrolysis via the BF_0F_1, since it is prevented by anti-BF_1 serum (Fig. 2B). Likewise, the prevention of D-lactate-

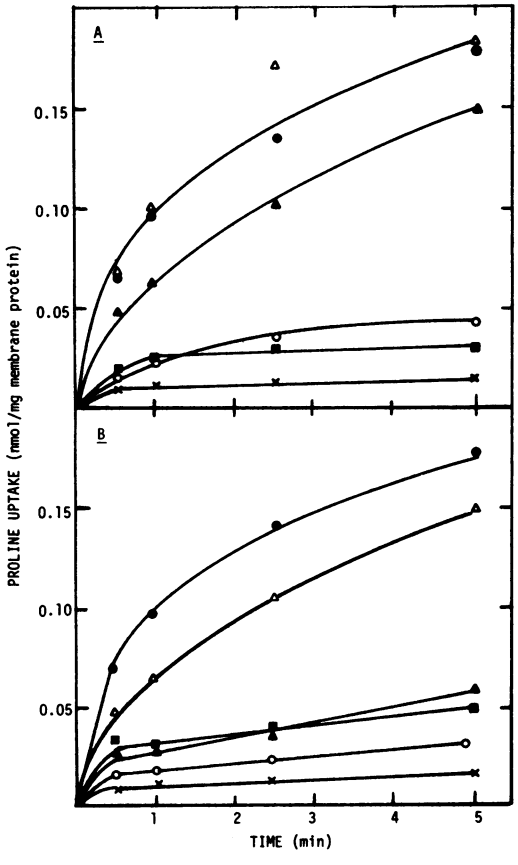

FIG. 2. *Uncoupling of proline transport from* D-*lactate oxidation by NADH oxidation or ATP hydrolysis in lysozyme-EDTA vesicles. (A) Effect of NADH oxidation; additions:* (●) *20 mM lithium* D-*lactate;* (○) *20 mM lithium* D-*lactate + 5 mM NADH;* (△) *20 mM lithium* D-*lactate + 12.5 mM* NAD^+; (▲) *20 mM lithium* D-*lactate + 5 mM NADH + 12.5 mM* NAD^+; (×) *5 mM NADH;* (■) *endogenous. (B) Effect of ATP hydrolysis; additions:* (●) *20 mM lithium* D-*lactate;* (▲) *20 mM lithium* D-*lactate + 5 mM ATP;* (■) *20 mM lithium* D-*lactate + 5 mM ATP + 0.1 ml of preimmune serum;* (△) *20 mM lithium* D-*lactate + 5 mM ATP + 0.1 ml of anti-*BF_1 *serum;* (×) *5 mM ATP;* (○) *endogenous. All assays contained 0.13 mg of membrane protein and were performed at pH 8.0, as described in the text.*

driven proline transport by NADH requires oxidation, since it is reversed by NAD^+, an inhibitor of the NADH dehydrogenase (Fig. 2A). Inhibition of D-lactate oxidation by NADH was not measured, although it apparently does occur (H. R. Kaback, personal communication). Thus, it may be difficult to differentiate between an uncoupling effect and an inhibitory effect of NADH in this situation.

The above experiments were performed at pH 8.0 because of the high pH optimum for ATP hydrolysis via the BF_0F_1 and for calcium transport. When the experiments with proline transport were performed at pH 6.6, higher rates of proline transport were observed, and less inhibition by ATP was found, as would be expected from the low rates of ATP hydrolysis via the BF_0F_1. NADH was equally effective in preventing D-lactate-driven proline transport at either pH (data not shown).

DISCUSSION

Membrane vesicles produced by osmotic lysis of lysozyme-EDTA spheroplasts of *E. coli* have proved to be an invaluable asset for the in vitro study of membrane processes such as active transport (4, 7) and bioenergetics (19). However, their use has generated several controversies. First, there is the question of the orientation of the IM with respect to that of the intact cell. Second, there is the question of the function of several of the enzymes involved in energy transduction.

With respect to the orientation of the vesicles, Kaback (7) has presented evidence based on electron microscopic visualization that the vesicles are morphologically identical, suggesting a homogeneous population of IM vesicles. Those results were later confirmed and extended by Altendorf and Staehelin (1). Those studies utilized an ML strain of *E. coli*, which appears to lose its OM upon preparation of vesicles (7). Vesicles produced from other strains of *E. coli* are not as morphologically homogeneous due to the presence of OM (7).

By functional criteria the vesicles also appear to be homogeneous. All of the IM vesicles transport the D-lactate analogue 2-hydroxy-3-butenoate (7). Everted vesicles transport calcium driven by D-lactate or reduced PMS oxidation; yet no calcium transport is found in EDTA-lysozyme vesicles with those energy sources, suggesting that there are few everted vesicles in those preparations (13). Thus, the evidence suggests that IM vesicles are homogeneous but does not differentiate between right-side-out, hybrid, or mosaic. "Right-side-out" means that the topology of the IM proteins is the same as that found in the IM of intact cells. "Hybrid" implies a membrane in which some enzymes normally found on the inner face of the IM are now found on the outer face due to fusion between right-side-out and everted vesicles. The term "mosaic" in this context is used to mean a membrane in which membrane proteins are localized in a nonrandom manner on both sides of the membrane. Although the mechanism by which a mosaic membrane is produced is not known, such membranes can be differentiated

from right-side-out and hybrid ones. Lysozyme-EDTA vesicles are clearly not right-side-out, since some proteins normally found only on one side of the membrane are found on both sides in those vesicles. The membranes lack an important feature of hybrid membranes. If fusion had occurred between membrane fragments, one would expect that the ratio of enzymes on the inner surface to the outer surface would be the same for all IM enzymes. Yet, the D-lactate dehydrogenase is not accessible to antibody from the outside, suggesting that none of this enzyme is translocated to the outer surface (3, 14). Thus, only certain enzymes and not others appear on the outer surface, a fact consistent with mosaicism.

With respect to the second question, the BF_1 and NADH dehydrogenase are normally components of larger complexes, the ATP synthetase or BF_0F_1 complex and the NADH oxidase. Both of those catalyze the electrogenic translocation of protons across the IM. So, has the whole complex reoriented itself, and, if so, would protons then be translocated inwards, setting up a protonmotive negative and basic outside? It should be pointed out that in the case of these two enzyme complexes, the internally located enzymes would not contribute significantly to the protonmotive force due to the impermeability of the membrane to adenine nucleotides.

Our previous results suggest that calcium is transported via a proton/calcium antiport in *E. coli* (17, 18, 20). Thus, intact cells, which produce a proton gradient acid outside, pump calcium outwards as protons flow inwards via the antiporter. In everted vesicles the reverse is true: the polarity of the protonmotive force is acid and positive inside, so that a flow of protons down an electrochemical gradient via the antiporter couples to the active inward flux of calcium. In lysozyme-EDTA vesicles, NADH oxidation or ATP hydrolysis energizes the active accumulation of calcium, suggesting that those enzymes are still part of their respective complex and are capable of establishing an electrochemical proton gradient with a polarity basic and negative outside. On the other hand, D-lactate oxidation does not energize calcium transport, and the available evidence suggests that the entire D-lactate oxidase is oriented within the membrane such that it produces a gradient acid and positive outside (3, 11, 14). Since proline transport is energized by a protonmotive force acid and positive outside (4), D-lactate oxidation drives proline uptake, whereas neither NADH oxidation nor ATP hydrolysis can do so. The observed uncoupling would, then, most likely reflect the simultaneous extrusion and uptake of protons by two different electrogenic pumps of opposite orientations. However, it may be possible that a reversal of the orientation of a pump would not cause a reversal of the proton gradient. For example, addition of D-lactate dehydrogenase to membranes derived from strains defective in that enzyme results in a situation in which the enzyme is immunologically located on the outer surface, yet can energize proline transport (14). Still, that is not the case in the systems utilized in this report: here D-lactate dehydrogenase is not accessible from the outer surface.

In conclusion, the data presented in this paper suggest that the use of the appropriate transport system allows a determination of the polarity of the protonmotive force. Ramos et al. (11) and Stroobant and Kaback (15) have reported that NADH oxidation does not produce a protonmotive force unless coenzyme Q is added exogenously. This is clearly inconsistent with the data reported above. Moreover, no values of the protonmotive force established by ATP hydrolysis via the BF_0F_1 have been reported. For those reasons, future efforts will be directed toward the measurement of the polarity and magnitude of the forces established through the metabolism of each substrate.

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