Heterogeneity of Membrane Vesicles from *Escherichia coli* and Their Subfractionation with Antibody to ATPase

(membrane transport/bioenergetics/NADH oxidase/D-lactate dehydrogenase)

JAMES F. HARE, KENNETH OLDEN*, AND EUGENE P. KENNEDY

Departments of Biological Chemistry and Physiology, Harvard Medical School, Boston, Massachusetts 02115

Contributed by Eugene P. Kennedy, September 23, 1974

**ABSTRACT**  The energy-transducing, Mg-Ca activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) of *E. coli* is located on the inner surface of the cytoplasmic membrane. Antibody to purified ATPase has now been used to demonstrate that membrane vesicles as ordinarily prepared by the lysozyme-EDTA method consist of two distinct populations. About half the vesicles are everted, and thus readily agglutinated by antibody to ATPase, while half are right-side out. NADH oxidase (reduced NADH oxidoreductase EC 1.6.99.3) activity is associated almost entirely with everted vesicles, while the ability to concentrate proline is a property of the right-side out vesicles. The results explain the failure of previous workers to observe the energization of membrane vesicles by oxidation of NADH.

The study of membrane transport processes in cell-free membrane vesicles from *Escherichia coli* was introduced by Kaback and his collaborators and has found wide-spread application in other laboratories (1).

It has been claimed (1) largely on the basis of their morphology that vesicles prepared by the procedures of Kaback (2) are essentially homogeneous, and that a significant number do not become everted during lysis of cells in the course of their preparation. However, there is growing evidence from studies of the biochemistry and function of these vesicles that they are heterogeneous. Investigations of van Thienen and Postma (3) and of Futai (4) show that the Mg-activated ATPase is localized on the inner face of the cytoplasmic membrane of *E. coli*. ATP is essentially impermeable to the membrane. The ATPase activity of vesicles prepared by procedures based on those of Kaback is consistent with the view that about half of these vesicles are inside-out; that is, with the ATPase exposed to the medium. Similarly, studies of glycerophosphate dehydrogenase, using ferricyanide as an electron acceptor that cannot penetrate the membrane, reveal that about half of these enzyme sites are exposed to the medium in isolated vesicle preparations (5). In intact cells of *E. coli*, these enzymes, like the ATPase, are localized on the inner face of the membrane.

In previous work in this laboratory (6), the Mg-activated ATPase from *E. coli* membranes has been purified to homogeneity. We now find that antibodies to the purified enzyme can be used to agglutinate everted vesicles in which the ATPase is exposed. Such agglutinated vesicles can easily be separated from right-side-out vesicles by low-speed centrifugation. We find that osmotic shock of spheroplasts prepared by treatment with lysozyme-EDTA produces two distinctly different populations of vesicles. About half are everted and thus agglutinated by antibody, whereas half are right-side out.

We have also found that the NADH oxidase activity of *E. coli* is localized on the inner face of the cytoplasmic membrane. When vesicles were agglutinated by antibody to the ATPase, 85% of the NADH oxidase activity was recovered in the agglutinated vesicles. In contrast, the uptake of proline takes place in right-side out, unagglutinated vesicles. This demonstration of the functional heterogeneity of the vesicles explains the observation of Kaback and Barnes (7) that oxidation of NADH does not provide energy for the uptake of proline in isolated vesicles.

During the preparation of this manuscript, Hampton and Fyfece (8) published the results of studies showing that NADH oxidase is localized on the inner surface of membrane vesicles from *Bacillus subtilis*.

**MATERIALS AND METHODS**

**Preparation of Membranes.** Cultures of strains AN 180, a derivative of K12, and of ML 308-225 were generous gifts of Drs. G. B. Cox and T. H. Wilson, respectively. Cells were grown at 37°C on minimal medium 63 (9) supplemented with 0.1% casamino acids and thiamine, with 1% glycerol as a carbon source. After growth to about 7 × 10^6 cells/ml, the cells were harvested by centrifugation and washed twice in 0.01 M Tris·HCl at pH 8.0. The procedure of Kaback (2) was used to convert these cells to spheroplasts. Cells were resuspended (1 g of wet weight per 80 ml) in 0.03 M Tris·HCl, 20% sucrose (pH 8.0) and stirred at 25°C. Potassium EDTA (pH 7.0) and lysozyme were added to final concentrations of 0.01 M and 0.5 mg/ml, respectively. After a 30-min incubation at 25°C, the EDTA-lysozyme treated cells were isolated by centrifugation at 16,000 × g for 15 min.

For preparation of membrane vesicles, spheroplasts from 4 g (wet weight) of cells were resuspended in 4 ml of 0.1 M potassium phosphate at pH 6.6, containing 20% sucrose, 20 mM MgSO4, and RNAse and DNase (each 3 mg/ml) and gently homogenized. This suspension was poured directly into 300 volumes of 0.05 M potassium phosphate, (pH 6.6) warmed to 37°C. The suspension was vigorously shaken for 15 min. The lysate was then made 0.015 M in MgSO4 and shaken for 15 min at 37°C as before. Membranes were isolated from the lysate by centrifugation for 30 min at 45,000 × g and resuspended to 5 ml for each original 300 ml of lysate. Unbroken cells and spheroplasts were removed by centrifugation at 3000 × g for 10 min. Since some membranes were also sedimented at this low speed, the pellet was resuspended in 0.1
M potassium phosphate, (pH 6.6) containing 10 mM EDTA, 30 mM MgSO₄, and RNase and DNase (each 100 μg/ml) and incubated at 37° for 30 min with shaking. Membranes were again removed by centrifugation at 45,000 × g for 30 min and cleared of unbroken cells and spheroplasts as above by resuspension and centrifugation at 3000 × g. Membrane-containing supernatants from low-speed centrifugations were combined and centrifuged at 800 × g for 30 min to remove remaining nonmembrane material and finally isolated by sedimentation at 45,000 × g for 30 min. Membrane vesicles were resuspended in 0.1 M potassium phosphate, pH 6.6, containing 10 mM MgSO₄ and used immediately or kept at 0° for no more than 36 hr until used. These procedures closely follow those used by Kaback (2).

For preparation of sonicated membranes, spheroplasts were suspended in 10 ml of 0.05 M Tris·SO₄, 5 mM MgSO₄, 0.1 mM EDTA, and 10 mM mercaptoethanol (pH 7.8) for each gram wet weight of cells used and sonicated in 5-ml portions with an MSE sonicator and 0.9-cm probe for two 30-sec bursts at an amplitude setting of 7–8 μm with a 1-min cooling period between bursts. Membrane fragments were cleared of debris by centrifugation for 10 min at 3000 × g for 10 min and isolated by sedimentation at 100,000 × g for 30 min. These membranes were resuspended and homogenized thoroughly before use.

Fractionation of Vesicles with Antibody to ATPase. Rabbit antisera to purified Mg-Ca activated ATPase (6) from E. coli was generously provided by Dr. Ronald Hanson. The antibody-containing γ-globulin fraction was obtained from 5 ml of the antisera by batch DEAE treatment as described by Stanworth (10), and dialyzed against two changes of 0.1 M potassium phosphate containing 10 mM MgSO₄, pH 6.6. After concentration of 2.5 ml over an Amicon PM 10 filter, the protein concentration of the final γ-globulin fraction was usually 8–10 mg/ml. Serum from unimmunized rabbits was processed similarly to yield the control γ-globulin fraction.

Membrane preparations were suspended in 1.0 ml of 0.1 M potassium phosphate of pH 6.6 containing 10 mM MgSO₄ at a protein concentration of 0.5 mg/ml. Various amounts of antibody to ATPase or of control γ-globulin were added, the volume made to 2.0 ml, and the suspensions incubated for 1 hr at 30°. The tubes were then held for 5 hr at 0°, and centrifuged at low speed (approximately 1000 × g) on an International clinical centrifuge for 2 min. The pellet contained the agglutinated membrane fraction. Unagglutinated membranes were collected by further centrifugation at 45,000 × g for 30 min. Both membrane fractions were then resuspended in 0.5 ml of 0.1 M potassium phosphate of pH 6.6 containing 10 mM MgSO₄.

Preparation of [3H]Leucine-Labeled Membranes. Cells were grown to a density of approximately 2 × 10⁶/ml, 0.01% arginine replacing the casamino acid supplement. After the addition of 0.02 μmol of L-[3H]leucine (0.5 × 10⁶ cpm/μmol), incubation was continued until a cell density of 7 × 10⁶ was reached. Cells were then harvested and membranes prepared as described above. Membrane specific radioactivity ranged from 500 to 1000 cpm/mg of membrane protein.

Other Assays. NADH oxidase was assayed at 30° by following the initial decrease in absorbance at 340 nm in cells of 1-cm path length. The assay system contained 0.5 mM NADP (P and L Biochemicals), 0.05 M Tris·HCl (pH 8.0), 0.01 M MgSO₄, and 10–100 μg of membrane protein in a total volume of 1 ml.

ATPase activity was assayed by measuring the 32P-labeled orthophosphate released from [γ-32P]ATP at 30° as described by Hanson and Kennedy (6). The reaction mixture (1 ml) contained 0.4 mM ATP (20,000 cpm 32P/nmol), 0.2 mM MgSO₄, 50 mM Tris·SO₄, and 0.25 mg of membrane protein. The reaction was stopped after 5 min by the addition of 1 ml of 10% trichloroacetic acid and cooling to 0°. Inorganic phosphate was extracted by the method of Lindberg and Ernst (11) and an aliquot of the organic layer was taken for 32P radioactivity analysis.

Proline transport was measured at 25° in 0.2 ml of 0.05 M potassium phosphate (pH 6.6) containing 0.01 M MgSO₄ and 100–250 μg of membrane protein. The reaction was started by the addition of either 10 mM lithium lactate (Calbiochem) (or 20 mM sodium ascorbate and 0.1 mM phenazine methosulfate), and 10 μM 1-[3H]proline (10⁶ cpm/nmol). The reaction was stopped after 5 min by the addition of 5 ml of 0.05 M potassium phosphate buffer containing 0.01 M MgSO₄, pH 6.6, at 25°, filtered on prewashed 0.45 μm nitro-

### Table 1. Oxidation of NADH by cells and preparations derived from cells of ML 308-225

<table>
<thead>
<tr>
<th>Preparation</th>
<th>NADH oxidase (nmol of NADH per min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>2</td>
</tr>
<tr>
<td>Sonicated cells</td>
<td>136</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>26</td>
</tr>
<tr>
<td>Sonicated spheroplasts</td>
<td>163</td>
</tr>
</tbody>
</table>

Assay of NADH oxidase activity is described under Materials and Methods.
cellulose filters (Matheson and Higgins), and washed with another 5 ml of carrier free buffer. The filters were dried and counted in 10 ml of Triton-toluene counting solution (12) in a Packard liquid scintillation spectrometer. Protein was estimated by the method of Lowry et al. (13).

RESULTS

Distribution of total protein in vesicles fractionated with antibody to ATPase

When membrane vesicle preparations from cells of strains AN 120, AN 180, or ML 308-225 were subjected to low-speed centrifugation without treatment with antibody, about 85% of the total protein remained in the supernatant fraction. This result was not altered by the addition of control gamma-globulin that did not contain antibody to ATPase. However, addition of gamma-globulin fractions containing antibody to ATPase lead to the agglutination of about half of the vesicles that would otherwise remain in the slow-speed supernatant (Fig. 1). When the membrane vesicle preparation was subjected to sonic irradiation prior to addition of antibody, nearly all of the protein was recovered in the fraction agglutinated by antibody, indicating that the membrane fragments so obtained were almost entirely inside-out.

Localization of NADH oxidase

Intact cells of E. coli oxidize NADH added to the medium at a very slow rate (Table 1). The activity of spheroplasts, the membranes of which have the same sidedness as intact cells, is somewhat higher, but is increased more than 6-fold when the spheroplasts are sonically disrupted. The sites of NADH oxidase thus appear to be largely or entirely on the inner aspect of the cytoplasmic membrane.

NADH oxidase in vesicles agglutinated by antibody to ATPase

If sites for the oxidation of NADH are available only on inside-out vesicles, then this activity should be largely concentrated in the fraction of vesicles agglutinated by antibody to ATPase. Fig. 2 shows the results of an experiment to test this hypothesis. More than 80% of the NADH oxidase activity was recovered in the fraction agglutinated by the antibody. Clearly, fractionation with antibody has separated the vesicles into two distinct populations, one of which (inside-out fraction) contains nearly all of the NADH oxidase activity. When the supernatant fraction containing vesicles not agglutinated by antibody, was subjected to sonic disruption, the NADH oxidase activity increased more than 6-fold, indicating that the right-side-out vesicles do indeed contain NADH oxidase sites that can be exposed when the membranes are everted during sonic disruption.

Uptake of proline in vesicles fractionated with antibody to ATPase

The active uptake of L-proline, energized by the oxidation of lactate or ascorbate plus phenazine methosulfate, takes place principally in right-side-out vesicles, that cannot be agglutinated by antibody to ATPase (Table 2).

DISCUSSION

The proportion of everted membrane vesicles in a given preparation will obviously depend upon the exact conditions employed during their isolation, and these may be expected to vary somewhat from laboratory to laboratory, even when the same general method is employed. In our hands, about half of the vesicles prepared by the lysozyme-EDTA method are everted, and this result is not significantly different if the vesicles are derived from strain ML 308-225 or from the K12 derivative AN 180. This result is in good agreement with the reports of Futai (4) and Weiner (5), who have provided evidence that D-lactate dehydrogenase and sn-glycero-3-P dehydrogenase, as well as the Mg-Ca activated ATPase, are localized on the inner surface of the cytoplasmic membrane. Using impermeable electron acceptors, such as ferricyanide, these workers showed that vesicles prepared by the proce-

![Fig. 2. Association of NADH oxidase activity with vesicles agglutinated by antibody to ATPase. Membrane vesicles from ML 308-225 were treated with antibody and agglutinated essentially as in the experiment of Fig. 1, and assayed for NADH oxidase activity as described under Materials and Methods.](image-url)

TABLE 2. Uptake of proline in vesicles fractionated with antibody to ATPase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>None</th>
<th>Lactate</th>
<th>Ascorbate phenazine methosulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated vesicles</td>
<td>0.15</td>
<td>1.07</td>
<td>2.80</td>
</tr>
<tr>
<td>Agglutinated by antibody</td>
<td>0.02 ± 0.01</td>
<td>0.11 ± 0.05</td>
<td>0.61 ± 0.19</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.14 ± 0.05</td>
<td>1.08 ± 0.58</td>
<td>2.97 ± 1.32</td>
</tr>
</tbody>
</table>

Procedures for fractionation of vesicles with antibody and for measuring uptake of proline are described under Materials and Methods. The variations indicated are the average deviations from the mean for four separate fractionations.
dyes based on those of Kaback (2) had activity corresponding to about half-everted membranes.

Since the present work has shown that NADH oxidase activity is also largely or entirely due to everted membranes, the preparations of Barnes and Kaback (14) must also have had a high proportion of everted vesicles, as shown by their high rate of oxidation of NADH.

Since the oxidation of NADH was relatively ineffective in supplying energy for membrane transport in comparison to the oxidation of D-lactate, Kaback and Barnes (14) were lead to postulate a special role of D-lactate dehydrogenase in energizing membrane transport. The present results indicate that in fact the oxidation of NADH and active transport of substrates such as proline are processes taking place in different populations of vesicles. The isolation by Simoni and Stahlecker (15) of mutants with defective D-lactate dehydrogenase, but with unimpaired transport in vivo had already indicated that D-lactate dehydrogenase does not play a unique or special role in membrane transport.

Altendorf and Staehelin (16) concluded from studies with the electron microscope that employed the freeze-cleavage technique, that their membrane vesicle preparations contained almost entirely right-side-out vesicles. To explain the contrast between their findings and those of Futai (4) and Weiner (5), Altendorf and Staehelin (16) suggested that molecules of ATPase and other enzymes may migrate from the internal surface of the membrane to the external surface during lysis of the spheroplasts, giving rise to “hybrid” vesicles, largely right-side out, but with some protein molecules translocated from the inside. Our results do not support this model, but rather suggest that the vesicles consist of two distinct types, largely (or entirely) everted, and largely (or entirely) right-sided.

This research was supported by grants from the National Institute of General Medical Sciences, NIH GM-13952-09 and GM-18731-03.