Molecular structure of membrane vesicles from *Escherichia coli*

(crossed immunoelectrophoresis/zymograms/antibody adsorption/membrane topology)

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ABSTRACT The molecular architecture of membrane vesicles prepared from *Escherichia coli* ML 308-225 has been studied by using crossed immunoelectrophoresis, and a reference pattern of 52 discrete immunoprecipitates has been established. Progressive immunoabsorption experiments conducted with untreated control vesicles and with physically disrupted vesicles demonstrate that the membrane-associated immunogens fall into two categories: (i) those immunogens typified by ATPase (ATP phosphohydrolase, EC 3.6.1.3) and NADH dehydrogenase [NADH:(acceptor) oxidoreductase, EC 1.6.99.3] whose expression is minimal unless the vesicles are disrupted; and (ii) immunogens such as Braun's lipoprotein that are expressed to similar extents in untreated and in disrupted vesicles. A mathematical relationship between the peak area subtended by an immunoprecipitate in the crossed immunoelectrophoresis system and the quantity of vesicles used in the adsorption process has been derived. This relationship allows quantitation of the degree to which specific membrane immunogens partition between exposed and unexposed surfaces of the vesicle membrane. The results demonstrate conclusively that >95% of the membrane in the vesicle preparations is in the form of sealed sacculi with the same polarity as the intact cell. Moreover, the findings provide a strong indication that dislocation of immunogens from the inner to the outer surface of the membrane during vesicle preparation does not occur to an extent exceeding 11%.

Topologically sealed cytoplasmic membrane vesicles have provided a useful model system for the study of certain transport phenomena in bacteria (1-3). These vesicles are essentially devoid of cytoplasmic constituents and catalyze active transport by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate compounds. In addition, recent studies with this system (3-8) provide strong confirmation of the hypothesis that chemosmotic phenomena (9-12) play a central, obligatory role in this process.

Despite a considerable body of evidence (see ref. 13 for a review) demonstrating that bacterial membrane vesicles prepared by osmotic lysis (14, 15) retain the same polarity as the intact cell, doubts apparently exist regarding the molecular structure of these vesicles. It has been argued, for example, that a significant percentage of these vesicles are everted (i.e., inside-out) and thus have a polarity that is opposite to that of the intact cell (16, 17). Alternatively, it has been suggested that, during vesicle preparation, certain membrane proteins become detached from the inner surface of the membrane and are readsorbed onto the outer surface (18-22). This dislocation process, it is proposed, results in vesicles that are "mosaics" in the sense that some membrane proteins are located on both sides of the membrane in a nonrandom fashion (21, 22). Much of the data on which these conclusions are based are derived from the use of two broad experimental approaches: (i) comparison of

the specific activities of membrane marker enzymes in intact vesicles with vesicles made permeable to substrate (16, 18, 19); and (ii) the use of "monospecific" antisera either to inhibit selectively those enzyme activities expressed on the exposed surface of the vesicles or to agglutinate vesicles that possess a certain topology (17, 20-22). Both of these approaches have inherent limitations, not the least of which is the propensity for the activities of certain membrane-associated enzymes to be radically affected by reagents or techniques that perturb membrane structure (23). Moreover, simple agglutination experiments conducted on mixed populations of membrane vesicles may be biased by entrapment of unagglutinated vesicles in the loose three-dimensional precipitin lattice that is formed.

In light of this controversy, a comprehensive immunological investigation of the topology of membrane vesicles isolated from *Escherichia coli* ML 308-225 has been initiated. The preliminary results presented here are based on the use of a novel, high-resolution crossed immunoelectrophoresis (CIE) technique that has none of the inherent limitations of the techniques mentioned above. The applicability of this method to the study of microbial membrane architecture was demonstrated first by Johansson and Hjertén (24) in studies of *Acholeplasma laidlawii* and the technique was utilized subsequently by Owen and Salton (25, 26) to establish the asymmetric nature of the plasma membrane of *Micrococcus lysodeikticus*. In this communication, CIE is used to study the topology of isolated membrane vesicles. In addition, techniques are described (see ref. 27 in addition) that allow quantitation of the distribution of specific membrane immunogens between the two faces of the membrane. The results demonstrate that >95% of the vesicles prepared by osmotic lysis (14, 15) retain the same polarity as the intact cell and that dislocation of immunogens occurs to a minimal extent.

METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($t^{-}z^{-}y^{+}a^{+}$) was grown on minimal medium A (28) containing 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared as described (14, 15). Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6), frozen in liquid nitrogen, and maintained at or below -70° prior to use.

For adsorption experiments with intact or disrupted vesicles, membrane suspensions (3.85 mg of protein per ml) were concentrated to one-quarter of their original volume by centrifugation ($30,000 \times g$ for 30 min at 4°) and resuspension in 0.1 M potassium phosphate (pH 6.6). Aliquots of this suspension (12 mg of protein per ml) were removed for physical disruption or

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Abbreviations: CIE, crossed immunoelectrophoresis; NaDodSO₄, sodium dodecyl sulfate.

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detergent solubilization, and untreated control samples were held at 0°.

Disruption of Membrane Vesicles. Sonic disruption of vesicles was performed at 0° using 30-sec or 1-min pulses from an MSE 100W ultrasonic disintegrator fitted with a 3/8-inch (9.5 mm) probe (29). Alternatively, disruption was achieved by three passages through an Aminco French pressure cell at 6500 psi (44.8 mPu). Dispersal of membrane vesicles in detergent was accomplished by addition of 20% (wt/vol) sodium dodecyl sulfate (NaDodSO₄) to a final concentration of 0.3%. At this protein/NaDodSO₄ ratio (i.e., 4:1), most of the membrane proteins are "solubilized" but retain their native antigenic properties (unpublished data).

Preparation of Triton X-100 Extracts for CIE Analysis. Vesicles were pelleted by centrifugation (48,000 × *g* for 1 hr at 4°) and resuspended in 50 mM Tris-HCl (pH 8.6) containing 4% (vol/vol) Triton X-100 and 5 mM EDTA to a final protein concentration of about 15 mg/ml. After incubation at 25° for 1 hr, the suspension was centrifuged (17,500 × *g* for 45 min at 25°) and the supernatant fraction (about 11 mg of protein per ml) was divided into 50-μl portions that were frozen and stored at -70° until used.

Preparation of Anti-Vesicle Immunoglobulins. Four rabbits were immunized with membrane vesicles (5.4 mg of protein) given initially both intradermally and subcutaneously at multiple sites with Freund's complete adjuvant. Two booster injections (5.4 mg of protein) were administered subsequently at fortnightly intervals by subcutaneous or intramuscular injection with Freund's incomplete adjuvant. Further intramuscular injections (2.7 mg of protein) were made in Freund's incomplete adjuvant at monthly intervals. Sera from 10 consecutive bleedings were pooled (780 ml), and the immunoglobulins were fractionated and concentrated 10-fold with respect to the original serum as described (25). Prior to adsorption, concentrated immunoglobulins (205 mg of protein per ml) that were stored routinely at 4° in 0.1 M NaCl/15 mM NaN₃ were extensively dialyzed against 0.1 M potassium phosphate (pH 6.6).

Adsorption of Anti-Vesicle Immunoglobulins. In adsorption experiments designed to assess the effect of washing on vesicle integrity, concentrated immunoglobulins (0.54 ml containing 85 mg of protein) were incubated with frequent shaking for 1 hr at 20° with 2.0 ml of control vesicles or with 2.0 ml of vesicles that had been washed a given number of times. Vesicles were then removed by centrifugation (30,000 × *g* for 30 min at 4°) and the supernatant fractions containing the unadsorbed immunoglobulins were dialyzed against 0.1 M NaCl/15 mM NaN₃ and used for CIE. In adsorption experiments designed to assess the molecular structure of membrane vesicles, 0.5-ml aliquots of immunoglobulins (89 mg of protein) were incubated in a similar manner with 0-1.5 ml of intact control vesicles or disrupted vesicles (containing 0-18 mg of membrane protein) and sufficient buffer (0.1 M potassium phosphate, pH 6.6) to give a final volume of 2.0 ml. After removal of vesicles by centrifugation, the supernatant fractions were treated as described above and used for CIE.

Crossed Immunoelectrophoresis. The methods for performing CIE in the presence of Triton X-100 and for staining the immunoplates with Coomassie brilliant blue or for specific enzymes have been described (25, 26, 30, 31). Electrophoresis in the first dimension was routinely performed in a Holm-Nielsen water-cooled immunoelectrophoresis chamber at 5.4 V/cm for 75 min. Electrophoresis in the second dimension was performed at 2 V/cm for 18-24 hr. Peak areas were estimated by photocopying dried immunoplates and subsequently excising and weighing 10 copies of each peak. The average weight

was then computed. Photocopy paper routinely weighed 7.89 mg/cm².

Protein Determinations. These were carried out by using a modification (32) of the method of Lowry *et al.* (33) that eliminates interference by Triton X-100. Bovine serum albumin was used as standard.

Chemicals. Agarose was obtained from Miles Labs and Triton X-100 from Research Products International. All other materials were of reagent grade obtained from commercial sources.

RESULTS

A complex CIE reference immunoprecipitin pattern with 52 discrete immunoprecipitates has been established for *E. coli* ML 308-225 membrane vesicles by manipulating the concentrations of antigen and antibody used during immunoelectrophoresis. A schematic diagram of this pattern with assigned numbers is presented in Fig. 1. Thirteen of these antigens, including no. 12 (ATPase; ATP phosphohydrolase, EC 3.6.1.3), no. 15, 19, and 27 [three NADH dehydrogenases; NADH:(acceptor):oxidoreductase, EC 1.6.99.3], and no. 49 [Braun's lipoprotein (34)] have been identified by zymograms and by the use of monospecific antibodies or purified antigens. Details of these experiments and other features of the reference pattern will be presented elsewhere.

In order to assess the extent to which different specific immunogens are expressed on the vesicle surface, anti-vesicle immunoglobulins were adsorbed with increasing quantities of

FIG. 1. Schematic representation of the CIE pattern for plasma membrane vesicles of *E. coli* ML 308-225. Immunoprecipitates are numbered in order of decreasing electrophoretic mobility of the peak maxima (anode to the left and top). The line width is intended to indicate the intensities observed for the immunoprecipitates. The dotted lines (....) denote precipitates that are detected only at relatively high concentrations of immunoglobulin (>5.4 mg of protein per ml). The lower portions of immunoprecipitates 20, 25, and 35 and the upper portions of immunoprecipitate 36 were difficult to visualize. Antigens 19 and 27 showed lines of partial identity. Some numbers (i.e., 29 and 31) and the bottoms of some immunoprecipitates (i.e., 23, 24, 30, 33, and 40) are omitted for clarity. Many of the weaker immunoprecipitates shown here are not visualized on photographic reproduction (cf. Fig. 2).

control vesicles or with identical quantities of vesicles disrupted by sonication or passage through a French pressure cell or, alternatively, solubilized by treatment with NaDodSO₄. The relative efficiency with which antibodies to a specific membrane antigen are removed in these progressive adsorption studies reflects the relative degree to which that antigen is expressed in the different vesicle preparations. Thus, if the vesicles are asymmetric with respect to the distribution of proteins, antibodies directed against a membrane immunogen located on the inner surface of the vesicle membrane should be decreased much less effectively by the untreated control vesicles than by the disrupted vesicles. Furthermore, antibodies directed against a membrane immunogen present on the outer surface of the vesicle membrane should be adsorbed equally as effectively by untreated and disrupted vesicles.

The immunoplates presented in Fig. 2 represent series of typical adsorption experiments in which the final immunoplates were stained either with Coomassie blue or by a zymogen technique that highlights NADH dehydrogenase. There are at least two, and probably three, distinct immunoprecipitates (corresponding to no. 15, 19, and 27 in Fig. 1) that exhibit NADH dehydrogenase activity (Fig. 2 C and D). Moreover, it is visually obvious that the peak areas subtended by these

immunoprecipitates do not increase significantly (i.e., antibody is not adsorbed) when control vesicles are used for immuno-adsorption (Fig. 2C₁₋₄), whereas a dramatic increase in peak area is observed (i.e., antibody is adsorbed) when disrupted vesicles are used (Fig. 2D₁₋₄). Thus, it may be concluded that these NADH dehydrogenases are essentially inaccessible to antibodies in untreated control vesicles but are readily accessible in the disrupted preparations. Although the difference between intact (Fig. 2A₁₋₄) and disrupted (Fig. 2B₁₋₄) vesicles is less pronounced with respect to ATPase (antigen no. 12), it is apparent that antibodies against this immunogen are also adsorbed much less readily by intact vesicles relative to disrupted vesicles. In contradistinction to the results for NADH dehydrogenase and ATPase, the areas subtended by immunoprecipitates no. 47 and 49 (Braun's lipoprotein) increase to similar extents after immuno-adsorption with either intact (Fig. 2A₁₋₄) or disrupted (Fig. 2B₁₋₄) vesicles, indicating that these immunogens are equally accessible to antibodies in both preparations.

Data from these and similar experiments are represented graphically in Fig. 3 *insets*, where the peak area subtended by precipitates no. 12 (Fig. 3A, ATPase), 15 (Fig. 3B, NADH dehydrogenase), 47 (Fig. 3C), and 49 (Fig. 3D, Braun's lipoprotein) are plotted as a function of the quantity of vesicles used

FIG. 2. Effect of adsorption of anti-vesicle immunoglobulin with control and disrupted vesicles. Anti-vesicle immunoglobulin was adsorbed with 0 ml (A₁, B₁, C₁, and D₁), 0.1 ml (A₂, B₂, C₂, and D₂), 0.5 ml (A₃, B₃, C₃, and D₃), or 1.0 ml (A₄, B₄, C₄, and D₄) of untreated control vesicles (A and C) or vesicles that had been disrupted by sonication (B) or treatment with NaDodSO₄ (D). After removal of vesicles by centrifugation, the immunoglobulins were incorporated into agarose gels (75 μ l/ml). In each instance, 28 μ g of membrane protein dissolved in Triton X-100/EDTA was added to the well in the lower right-hand corner of the immunoplates. The plates were electrophoresed and stained for protein with Coomassie brilliant blue (A and B) or for NADH dehydrogenase (C and D). Although data will not be presented, with regard to B, similar results were obtained with vesicles that were disrupted by sonication, passage through a French pressure cell, or solubilization with Triton X-100; with regard to D, similar results were obtained with vesicles that were disrupted by passage through a French pressure cell or solubilization with NaDodSO₄ or Triton X-100. Although antigen 28 stained for NADH dehydrogenase activity (C and D), this is probably an artifact caused by entrapment of small amounts of antigen 27 (data not shown). The anode is to the left and top of all gels.

FIG. 3. Effect of progressive immunoadsorption on the peak areas (A) of selected immunoprecipitates: (A) antigen no. 12, ATPase; (B) antigen no. 15, NADH dehydrogenase; (C), antigen 47; (D) antigen no. 49, Braun's lipoprotein. Anti-vesicle immunoglobulin was adsorbed as with different volumes (v) of untreated vesicles containing given amounts of protein ($\bullet$). Alternatively, the immunoglobulin was adsorbed with identical aliquots of vesicles that were disrupted by sonication for 30 sec ($\blacktriangle$) or 5 min ($\circ$), passage through a French pressure cell ($\blacksquare$), or dispersion in NaDodSO₄ ($\square$). The adsorbed immunoglobulin was incorporated into agarose gels (75 μ l/ml) and analyzed against a Triton X-100/EDTA extract of membrane vesicles (28 μ g of protein) by CIE. The peak areas were estimated from immunoplates that were stained either with Coomassie brilliant blue or by zymogram techniques. In large part, day-to-day variations in the temperature of the cooling bath during CIE account for the fluctuations in the absolute values of $1/A_0$ between different adsorption series.

during immunoadsorption (expressed as volume). Several features of these and other experiments (unpublished data) are noteworthy. First, it is clear that the vesicle immunogens fall into two categories: (i) those typified by ATPase and NADH dehydrogenase (Fig. 3 A and B insets), antibodies to which are adsorbed much less effectively by control vesicles relative to disrupted vesicles; and (ii) those typified by antigen no. 47 and Braun's lipoprotein (Fig. 3 C and D insets), antibodies to which are removed equally effectively by control and disrupted vesicles. Second, to a first approximation, the efficiency with which antibodies to a particular immunogen are removed during immunoadsorption with disrupted vesicles is largely independent of the disruption method utilized. Finally, the area subtended by the immunoprecipitates is not linearly related to the quantity of vesicles used for immunoadsorption. In this context, the following considerations are important.

The quantitative aspects of CIE (27) imply that, for a particular immunogen i ,

$$A_v = \frac{k_1 C}{B_1} \quad [1]$$

in which A_v is the peak area subtended by immunoprecipitate i after adsorption with v ml of vesicles, C is the amount of i analyzed, B_1 is the amount of anti- i immunoglobulin remaining after adsorption, and k_1 is the area-loading constant for i .

Clearly,

$$B_1 = B_0 - B_v \quad [2]$$

in which B_0 is the amount of anti- i immunoglobulin present in unadsorbed serum and B_v is the quantity of immunoglobulin

removed after adsorption with v ml of vesicles suspension. If it is assumed that, under conditions of antibody excess, B_v is proportional to the amount of adsorbing antigen available (an assumption that appears to be valid to a first degree of approximation for fractionated immunoglobulins),

$$B_v = k_2 xyv \quad [3]$$

in which y is the concentration of i in the membrane, x is the degree to which i is expressed (i.e., its availability to antibody), and k_2 is an agglutination constant which incorporates, among other parameters, the valency of i .

Substituting Eqs. 2 and 3 in Eq. 1 and inverting gives

$$\frac{1}{A_v} = \frac{B_0}{k_1 C} - \left[\frac{k_2 y}{k_1 C} \right] xv \quad [4]$$

or

$$\frac{1}{A_v} = \frac{1}{A_0} - K xv \quad [5]$$

in which $K = k_2 y/k_1 C$ and is constant for i under the conditions of the adsorption experiments, and A_0 is the peak area prior to adsorption.

According to Eq. 5, $1/A$ should be a linear function of v , a prediction that is borne out clearly in practice (Fig. 3). It is also apparent from Eq. 5 that the ratio of the slopes for i obtained for disrupted and control vesicles (i.e., $x_{\text{disrupted}}/x_{\text{control}}$) gives a direct estimate of the extent by which disruption increases the availability of i to antibodies. Values computed in this fashion for antigens no. 12 (ATPase), 15 (NADH dehydrogenase), 47, and 49 (Braun's lipoprotein) are given in Table 1. It is evident that less than 11% and 2%, respectively, of the ATPase and NADH dehydrogenase are accessible to antibodies in intact membrane vesicles. On the other hand, antigen no. 47 and Braun's lipoprotein are expressed to essentially the same extent in control, sonicated, and French-pressed vesicles and to a slightly increased extent after treatment with NaDodSO₄.

DISCUSSION

The results presented in this communication demonstrate dramatically that the ability of three NADH dehydrogenases and ATPase, immunogens associated with membrane vesicles prepared from *E. coli* ML 308-225, to adsorb antibodies is minimal unless the vesicles are disrupted. Similar results have also been obtained for polynucleotide phosphorylase (EC 2.3.7.8), dihydroorotate dehydrogenase (EC 1.3.3.1), D-lactate dehydrogenase (EC 1.1.1.27), and a number of other as yet unidentified antigens such as no. 8 and 28 (Fig. 1) (unpublished data). Each of these immunogens is expressed at least 9 times

Table 1. Effect of disruption of *E. coli* ML 308-225 membrane vesicles on expression of membrane immunogens

Antigen	Ratio		
	$x_{\text{son.}}/x_{\text{con.}}$	$x_{\text{FP.}}/x_{\text{con.}}$	$x_{\text{NaDodSO}_4}/x_{\text{con.}}$
ATPase (antigen no. 12)	9.7	9.0	9.7
NADH dehydrogenase (antigen no. 15)	87.0	55.0	89.0
Antigen no. 47	1.0	1.1	1.7
Braun's lipoprotein (antigen no. 49)	1.1	0.9	2.6

$x_{\text{con.}}$, $x_{\text{son.}}$, $x_{\text{FP.}}$, and x_{NaDodSO_4} represent the extent to which a given immunogen is expressed in control, sonicated (5 min), French-pressed, and NaDodSO₄-treated membrane vesicles, respectively. Data were computed from Fig. 3 and other similar experiments.

more effectively in disrupted vesicles relative to the untreated control preparations. It is significant, of course, that these enzymes are presumably associated with the inner face of the plasma membrane in the intact cell. Consequently, because only 2% or less of the NADH dehydrogenase and only 11% of the ATPase are accessible to antibody (Table 1), it seems reasonable to conclude that at least 98% of the membrane in the control preparations is in the form of topologically closed vesicles with the same orientation as the intact cell and that dislocation of membrane immunogens occurs to an extent that cannot exceed about 11%. It is also noteworthy that dislocation of immunogens appears to vary from about 2% (at most) for NADH dehydrogenase to about 11% (at most) for ATPase. Possibly, this variation may result from differences in the extent to which specific membrane components associate with the lipid bilayer (23). It is also possible that a small percentage (no more than about 2%) of the total membrane may be in the form of everted vesicles or open sheets of membrane.

The quantitative data presented here rest on two main assumptions, both of which are justifiable. The primary assumption is that the amount of immunoglobulin adsorbed (B_0) is proportional to the amount of adsorbing antigen available. This contention appears to be valid below the equivalence point (i.e., in regions of moderate antibody excess; cf. ref. 35) and is supported by the linear relationship observed between $1/A_0$ and v (Fig. 3). The second assumption is that the increased effectiveness with which antibodies to some immunogens are removed after vesicle disruption results entirely from relaxation of physical constraints imposed by a topologically closed, asymmetric membrane. As an alternative, it could be argued that this phenomenon results from increased expression of antigenic determinants in perturbed, open sheets of membrane; however, this argument seems untenable for the following reasons: (i) All of the disruption methods utilized increase antigen expression to a similar extent; in addition, essentially no difference is observed when the vesicles are dispersed in 0.5% Triton X-100 prior to immunoadsorption (data not shown). (ii) There are a group of immunogens typified by no. 47 and 49 whose expression is virtually unaffected by membrane disruption. Antigen no. 49 has been identified as the most abundant protein in the outer membrane of *E. coli* [Braun's lipoprotein (34)]. Small amounts of this component should be expressed on the outer surface of vesicles that manifest the same orientation as the intact cell if, as suggested (36), regions of fusion or "junctions" occur between the inner and outer membranes of the intact cell. Moreover, the antigenicity of membrane-associated lipoprotein is known to depend upon the structure of the outer membrane (37) and this property may account for the increased expression of this immunogen after treatment of vesicles with NaDodSO₄ as opposed to sonication or passage through the French press (Fig. 3D).

It is clear from these results that high-resolution CIE is the immunological method of choice for assessing vesicle structure and integrity. Not only does the method allow simultaneous localization of a large number of membrane immunogens, but also it can be quantitated in a manner that does not rely on intrinsic catalytic or other biological activities (38). Finally, it should be emphasized that these results confirm and extend various other experiments (cf. ref. 13) which demonstrate that membrane vesicles prepared from *E. coli* ML 308-225 by osmotic lysis (14, 15) retain the topology of the membrane in the intact cell.

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