ULTRASTRUCTURE OF THE SODIUM PUMP

Comparison of Thin Sectioning, Negative Staining, and Freeze-Fracture of Purified, Membrane-Bound (Na⁺,K⁺)-ATPase

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ABSTRACT

Purified (Na⁺,K⁺)-ATPase was studied by electron microscopy after thin sectioning, negative staining, and freeze-fracturing, particular emphasis being paid to the dimensions and frequencies of substructures in the membranes. Ultrathin sections show exclusively flat or cup-shaped membrane fragments which are triple-layered along much of their length and have diameters of 0.1-0.6 μm. Negative staining revealed a distinct substructure of particles with diameters between 30 and 50 Å and with a frequency of 12,500 ± 2,400 (SD) per μm². Comparisons with sizes of the protein components suggest that each surface particle contains as its major component one large catalytic chain with mol wt close to 100,000 and that two surface particles unite to form the unit of (Na⁺,K⁺)-ATPase which binds one molecule of ATP or ouabain. The further observations that the surface particles protrude from the membrane surface and are observed on both membrane surfaces in different patterns and degrees of clustering suggest that the protein units span the membrane and are capable of lateral mobility. Freeze-fracturing shows intramembranous particles with diameters of 90-110 Å and distributed on both concave and convex fracture faces with a frequency of 3,410 ± 370 per μm² and 390 ± 170 per μm², respectively. The larger diameters and three to fourfold smaller frequency of the intramembranous particles as compared to the surface particles seen after negative staining may reflect technical differences between the methods, but it is more likely that the intramembranous particle is an oligomer composed of two or even more of the protein units which form the surface particles.

KEY WORDS (Na⁺,K⁺)-ATPase · sodium pump · electron microscopy · membrane substructures · membrane proteins · freeze-fracture

Several electron microscope studies have demonstrated substructures in membranes after thin sectioning (30, 31), negative staining (32), and freeze-fracturing (3). The problem of ascribing functions to the observed substructures is evident when the membranes contain a variety of different protein components. Only in a few cases have correlations between structure and function
been pursued on membranes which are largely monofunctional (7, 10, 20, 21, 25, 28).

(Na\(^+\),K\(^+\))-ATPase can be purified from the outer medulla of rabbit kidney by selective extraction of extraneous protein components from the plasma membranes (14) of the cells in the thick ascending limb of Henle. The purified preparations have specific activities of 32-40 \(\mu\)mol P\(_i\) \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) protein and contain only two proteins, the catalytic subunit with mol wt close to 100,000 and a smaller glycoprotein of unknown function. These protein components are undenatured and remain embedded in the lipid bilayer throughout the purification procedure (15). Immunologically, the purified (Na\(^+\),K\(^+\))-ATPase is identical to the sodium pump in human erythrocytes (17) and it catalyzes active sodium transport after incorporation into lipid vesicles (6, 11). The preparation therefore forms an ideal subject for demonstration of the sodium pump by electron microscopy.

The purpose of this study, parts of which have been reported in preliminary form (5, 22, 23), has been to analyze the purified (Na\(^+\),K\(^+\))-ATPase by electron microscopy after thin sectioning, negative staining, and freeze-fracturing, with particular emphasis on the dimensions and frequencies of observed substructures in the membranes. The results show a good correspondence between the enzyme units and the surface particles seen by negative staining and indicate a relationship between the surface particles and the intramembranous particles visualized by freeze-fracturing. The observations suggest that the observed structures represent components of the sodium pump which are membrane intercalated and protrude from the membrane surface.

MATERIAL AND METHODS

Purification of (Na\(^+\),K\(^+\))-ATPase

(Na\(^+\),K\(^+\))-ATPase was purified from the outer medulla of rabbit kidneys by incubation of a microsomal fraction with sodium dodecyl sulfate (SDS) and ATP followed by an isopycnic-zonal centrifugation as described previously (14).

The specific activity of (Na\(^+\),K\(^+\))-ATPase was 32-40 \(\mu\)mol P\(_i\) \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) protein and ouabain-insensitive ATPase activity was not detectable. Polyacrylamide gel electrophoresis in SDS showed that the purified preparation contained only the two major protein components of the (Na\(^+\),K\(^+\))-ATPase (Fig. 1).

Preparation for Electron Microscopy

Samples for electron microscopy were prepared by dilution of aliquots containing 300 \(\mu\)g of enzyme protein to 4 ml with 25 mM imidazole - 1 mM EDTA at pH 7.5. Pellets were prepared by centrifugation for 100 min at 40,000 rpm in a Beckman type 65 rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.).

Some pellets were fixed with 2% glutaraldehyde, other pellets were resuspended and fixed by mixing with an equal amount of 4% glutaraldehyde buffered in 0.1 M sodium cacodylate at pH 7.2. After 2 h, membranes fixed in suspension were rinsed by sedimentation and resuspended in 25 mM imidazole, pH 7.5.

Ultrathin Sectioning

Glutaraldehyde-fixed pellets were postfixed with 1% osmium tetroxide in 0.29 M sodium Veronal buffer at pH 7.2 for 1 h, dehydrated in acetone and embedded in Vestopal. Ultrathin sections were stained with uranyl acetate and lead citrate. Some pellets were stained en bloc with 0.5% uranyl acetate in sodium Veronal buffer at pH 5.2 before dehydration. The ultrathin sections were stained with lead citrate.

Negative Staining

The samples were diluted with imidazole buffer to a concentration of 0.05-0.2 mg of enzyme protein per ml. Aliquots of 2-10 \(\mu\)l were mixed on the grid with
5-10 μl of imidazole buffer or 5-10 μl of a solution of bacitracin (150 μg/ml) which was used as a surfactant (8). After mixing, excess liquid was removed and 10–20 μl of 2% phosphotungstic acid, pH 7.2 with KOH (KPT), was added. After 10–60 s, the droplet was drained off with filter paper and the grid dried.

Freeze-Fracture and Freeze-Etching

Both unfixed and fixed pellets were resuspended in about 20 μl of buffer to provide highly concentrated samples. Droplets were placed directly on specimen support disks and quickly frozen in Freon 22 cooled with liquid nitrogen. Some pellets were resuspended in 30% (vol/vol) buffered glycerol for 1–2 h before freezing. The frozen specimens were fractured in a Balzers freeze-fracture apparatus (BAF 300, Balzers AG, Balzers, Liechtenstein) at -100°C. Shadowing was performed with platinum-carbon either within seconds after fracture or after etching for up to 10 min at 1–2 × 10^-6 mm Hg. The thickness of the shadowing material was 20–30 Å as estimated by a quartz-crystal thin-film monitor. During etching, the edge of the cutting knife was placed above the fractured surface of the specimen to minimize contamination. Replication was completed by evaporation of carbon. The replicas were cleaned with sodium hypochlorite and distilled water and mounted on carbon-coated Formvar films.

The preparations were analyzed in a JEOL JEM 100B electron microscope at an accelerating voltage of 80 kV.

Measurements

Measurements of particle diameters, distances between center to center of particles, and particle frequencies were made on electron micrographs with a final magnification of 200,000 (negative staining preparations) and 150,000 (freeze-fracture preparations). Particle diameters and center-to-center distances were measured to the nearest 0.1 mm through a magnifying ocular. The diameter of a particle in freeze-fracture preparations was determined as the width of the metal deposit at right angles to the direction of shadowing. Areas were measured by point counting, using a square grid.

RESULTS

Ultrathin Sectioning

The sectioned membrane pellets contained exclusively membrane profiles with a length of 0.1–0.6 μm (Fig. 2). Most membranes appeared flattened or cup-shaped. Some vesicle-like profiles were seen, but comparison of adjacent serial sections showed that they represented equatorially cut, cup-shaped membrane fragments.

At high magnifications, the membrane fragments were triple-layered along most of their course when cut at right angles (Fig. 3). The total membrane thickness was 80–100 Å and the distance between the centers of the dense lines of the triple-layered membrane was 40–60 Å. Particulate structures were not observed in thin sections of the membranes. However, in certain places, only a single electron-dense line was seen (Fig. 3, arrows) although the membrane appeared to be cut at a right angle. The total thickness of the line was 50–30 Å and the length of the thin regions varied from 100 to 800 Å.

Negative Staining

After negative staining with KPT, the unfixed membrane fragments appeared as rounded disks with diameters of 0.1–0.6 μm (Figs. 4–7). The membrane fragments were studied with a uniform population of particles (Fig. 5). Direct measurements of the diameter of a large number of particles gave an average value of 27 Å (Table I). However, this value may only reflect the size of that part of the particles which protrudes from the membrane surface (Fig. 9). We therefore also measured the minimum distance between the centers of adjacent particles since it might represent an estimate of the diameters of adjacent particles at closest apposition. The histogram in Fig. 10 shows that the frequency of center-to-center distances rose sharply between 30 and 50 Å, suggesting that the particle diameter within the membrane was larger than that of the part visualized by negative staining. The overall frequency of particles as determined by point counting on a test grid was 12,500 per μm² (Table I). The particles were not uniformly distributed over the membrane surfaces, but formed irregular clusters or strands separated by empty areas (Figs. 4 and 5). The particles never occurred alone within the clusters, but the images did not provide evidence for a definite oligomeric arrangement. The empty areas varied in size, and point counting showed that they formed 34 ± 9 (SD)% of the membrane area in 70 fields. Some membrane fragments were folded and exposed simultaneously both membrane surfaces (Figs. 4 and 6). These folded fragments were easily distinguished from unfolded, stacked fragments. The edge representing the fold of the membrane was distinctly different from the edge of the unfolded membrane, appearing thicker and smoother. We did not observe differences with respect to size or distribution of the particles on the exposed surfaces of the folded membranes.

During the course of this investigation, varia-
Figure 2 Ultrathin section of pellet of purified (Na⁺,K⁺)-ATPase from the outer renal medulla. The pellet contains exclusively small membrane fragments which are flat or cup-shaped. Bar, 0.5 μm. × 46,000.

Figure 3 (Na⁺ + K⁺)-ATPase membranes appear in sections as triple-layered along most of their course. In certain places they appear thinner and show only one electron-dense line (arrows). Bar, 0.1 μm. × 140,000.
**Figure 4** The isolated membranes appear as rounded disks and are covered with small particles. Bar, 0.1 μm. x 130,000.

**Figure 5** The particles on the membranes are arranged in clusters and strands which are separated by empty areas. There are no single particles within the empty areas. Bar, 0.1 μm. x 240,000.
Figure 6 Part of this membrane fragment is folded and both surfaces of the membrane are exposed simultaneously. There is no difference in the distribution of particles between the two sides of the membrane. Bar, 0.1 μm. × 215,000.

Figure 7 Membrane fragment showing peripheral clustering of particles with central empty area. Bar, 0.1 μm. × 225,000.

Figure 8 Negative staining of glutaraldehyde-fixed (Na⁺,K⁺)-ATPase membranes. The particles form larger clusters and strands than in most unfixed membranes. Bar, 0.1 μm. × 145,000.

Freeze-Fracture and Freeze-Etching

Freeze-fracture showed curved membrane fragments at different angles (Fig. 11). Membranes fractured at right angles usually appeared as semi-
TABLE I
Diameters and Frequencies of Particles of Purified (Na\(^+\),K\(^+\))-ATPase Membranes from the Outer Renal Medulla

|                           | Diameter (Å ± SD (n)) | Frequency (Particles/µm\(^2\) ± SD (N)) |
|---------------------------|-----------------------|----------------------------------------|
| Negative staining         |                       |                                        |
| Unfixed                   | 27 ± 6 (351)          | 12,500 ± 2,400 (70)                    |
| Glutaraldehyde-fixed      | 26 ± 6 (222)          | 9,880 ± 1,580 (15)                     |
| Freeze-fracture           |                       |                                        |
| Unfixed                   |                       |                                        |
| A face                    | 102 ± 20 (674)        | 3,410 ± 370 (13)                       |
| B face                    | 97 ± 24 (112)         | 390 ± 170 (7)                          |
| Glutaraldehyde-fixed      |                       |                                        |
| A face                    | 94 ± 24 (209)         | 3,310 ± 350 (22)                       |
| B face                    | 96 ± 26 (191)         | 490 ± 100 (10)                         |

\(n\) = number of particles.
\(N\) = number of fields.

FIGURE 9 Schematic drawing showing protein units (\(P_1\), \(P_2\), and \(P_3\)) in lipid bilayer. After negative staining, only parts of the protein units are delimited by the stain (S). The directly measured diameter \(a\) is therefore smaller than the diameter of the particles inside the membrane \(b\). The right part of the diagram shows two apposed particles. The center-to-center distance between these particles \(b'\) is identical to the diameter of one particle, assuming that the particles are symmetrical. These lines often revealed a substructure consisting of rows of particles.

Tangentially fractured membrane fragments were of two types (Fig. 11). One type appeared concave and was studded with particles (Figs. 11–17). This face is termed the A face. In the recommended nomenclature (2), it might correspond to the P face, but we have not identified the membrane surfaces in the purified preparation. The other type was convex and showed few circular lines indicating a cup-shaped structure. These lines often revealed a substructure consisting of rows of particles.

Regardless of the face, the particles in the unfixed preparations appeared round and symmetry planes were not identified. In unfixed preparations, frozen without cryoprotectants, the diameter of the particles averaged 102 Å on the A face and 97 Å on the B face (Table I and Fig. 10). The particle diameters were similar after glycerol treatment of unfixed membranes, but were slightly smaller after glutaraldehyde fixation.

As shown in Fig. 10 d, the center-to-center distances between adjacent particles in unfixed preparations show a sharp increase in the range from 90 to 100 Å. This measurement is in agreement with the direct measurements of diameters (Fig. 10 c) and thus provides independent evidence for the presence of particles with diameters \(\sim\)100 Å. The same observations were made after fixation with glutaraldehyde, as indicated by the dotted lines in Figs. 10 c and d.

The density of particles was determined by making counts on a large number of membrane profiles from different, unfixed preparations. Table I shows that the average particle frequency was 3,410 per µm\(^2\) on the A face and 390 per µm\(^2\) on the B face. The frequency was almost the same for unfixed and fixed membranes.

The fractured surfaces of membranes prepared...
with or without etching were searched for evidence of pits or depressions. Although irregularities were sometimes noted, regular pits corresponding in frequency to the particles on the opposite surface were not observed.

To examine the particle distribution more closely, the particles on photographs of A faces of unfixed membranes were covered with india ink. Fig. 13 shows that the ink spots were unevenly distributed and formed strands and clusters. Point counting of six unfixed membrane fragments from three preparations after marking with ink as in Fig. 13 showed that the average fraction of empty area was 32 ± 5 (SD)%.

Glutaraldehyde fixation causes extensive clustering of the surface particles seen after negative
Figure 11  Purified (Na⁺,K⁺)-ATPase membranes freeze-fractured without cryoprotectants. Two different membrane faces are seen. The concave face (A face) has a high surface density of particles (A). The other (B face) is convex with fewer particles (B). Some membrane fragments, which are fractured at right angles, appear as curved lines with a particulate substructure. Direction of shadowing is from bottom to top. Bar, 0.5 μm. × 90,000.
staining (Fig. 8). Similarly, cross-linking with glutaraldehyde also caused aggregation of the intramembranous particles (Fig. 15).

Etching for 1–5 min exposed the unfractured membrane surfaces which were separated by small steps from typical A faces (Figs. 16 and 17) or B faces (Fig. 19). Distinct particles were not seen on the etched surfaces, but shallow, granular regions were present. The distribution of these regions was similar to that of the strands and clusters formed by the surface particles seen after negative staining. Along the fracture step, clusters of intramembranous particles were often located directly opposite the shallow, granular regions on the etched surfaces. This observation is consistent with a continuity between the surface particles and the intramembranous particles.

DISCUSSION

Electron microscope analysis using three different preparatory procedures demonstrates that purified (Na+, K+)-ATPase exclusively appears in membrane form. The membranes appear as disks rather than vesicles in ultrathin sections and freeze-fracture preparations, and this shape is consistent with their appearance after negative staining. Negative staining and freeze-fracture show that the membranes possess distinct surface particles and intramembranous particles; the measurements of dimensions and frequencies of these substructures demonstrate that the two techniques disclose different images of the enzyme proteins. We shall discuss the evidence indicating that the surface particles represent a protein unit which contains one large catalytic chain with a mol wt close to 100,000. We shall furthermore discuss the relationship between the surface particles and the intramembranous particles.

Surface Particles

The similarity in the size and distribution of the surface particles on the exposed surfaces of unfolded and folded fragments suggests that the particles represent a protein complex which spans the membrane. This interpretation is in agreement with the transport functions of this protein. It is also known that an intracellular aspect of the sodium pump is accessible to binding of antibody (17) and that ouabain binds to an extracellular aspect of the catalytic protein (19, 27). The data in Table II are in agreement with the notion that the empty area represents lipid bilayer without protein particles. Since different particle distributions as well as different degrees of clustering are observed, it can therefore be concluded that the particles are able to move in the plane of the membrane bilayer. The observation that the surface particles always form clusters or strands and never appear as single particles is consistent with a dimeric arrangement since trimers or tetramers are not observed. However, trimer or tetramer configurations are not excluded since the associa-

FIGURE 12 A face from membrane preparation freeze-fractured without cryoprotectants and showing a high frequency of particles. Bar, 0.1 μm × 120,000.

FIGURE 13 Same micrograph as in Fig. 12, but the particles in part of the field have been covered with ink to illustrate their tendency to form clusters and strands. This figure also allows a better appreciation of the size of the empty area between the particles. Bar, 0.1 μm × 120,000.

FIGURE 14 Membranes treated with 25% glycerol for 1 h before freeze-fracture. The A face shows a similar frequency of particles as on untreated membranes, but the heights of the particles appear lower. Bar, 0.1 μm × 140,000.

FIGURE 15 (Na+ K+)-ATPase membrane fixed in glutaraldehyde before freeze-fracture. The particles are more clustered than in unfixed preparations. Bar, 0.1 μm × 115,000.

FIGURE 16 Unfixed (Na+,K+)-ATPase membrane fractured without cryoprotectants and etched for 5 min. A fracture step separated the A face (upper left) from the exposed membrane surface which shows elevated granular regions but no distinct particles. Bar, 0.1 μm × 180,000.

FIGURE 17 Part of membrane fragment fractured without cryoprotectants and etched for 5 min. Adjacent to the A face (lower half of figure) is the outer membrane surface which shows irregular granular regions. At the step between the A face and the membrane surface, some of these granular regions are located immediately adjacent to, and in apparent continuity with, clusters of intramembranous particles. Bar, 0.1 μm × 175,000.
tion between the subunits may not be sufficiently strong to resist the preparative procedure.

The directly measured diameters of the particles may represent only those parts of the protein complex which protrude from the membrane surface, i.e., "the top of an iceberg." The measurements of the distances between the centers of adjacent particles may provide a more meaningful estimate of the dimensions of the intramembranous part of the particle in a situation of closest apposition of adjacent particles. The observations that center-to-center distances below 30 Å were few and that the frequency rose steeply between 30 and 50 Å suggest that the real particle diameter is close to 50 Å. The observations are therefore compatible with a protein particle of ellipsoid shape, with the parts of the particle protruding from the membrane surface being thinner (20-30 Å) than the intramembranous part (30-50 Å).

The directly measured surface particle diameter (27 Å) is close to the resolving power of the negative staining technique (13). Although the observations are reproducible, this value can therefore be taken only as an approximation of the diameter. It is essential to point out that the particles are observed in high resolution electron micrographs taken close to or in focus. This demonstrates that they are real structures on the membrane surfaces and not related to out-of-focus granularity, which may be misinterpreted as subunits (9, 34). The reality of the particles is further supported by their characteristic distribution in clusters or strands and by the change in distribution after cross-linking of the proteins. Surface particles similar to those presented here have not previously been observed in preparations of (Na⁺, K⁺)-ATPase. Recently, a subunit structure consisting of particles was observed on (Na⁺, K⁺)-ATPase membranes purified from dog renal medulla by repeated solubilization with sodium deoxycholate (DOC) and ammonium sulfate precipitation (33). However, it is unlikely that these particles are related to those observed by us since the measured diameters are much larger (45-70 Å) than those reported here (27 Å). In addition, the particles described here are arranged in clusters, whereas the membranes of the dog renal medulla show a rather uniform granularity. A granularity with a suggestion of periodicity with particles having a diameter of about 45 Å has been observed after negative staining of (Na⁺, K⁺)-ATPase purified after solubilization of membranes from the salt gland of sharks (12).

In an attempt to relate the surface particles to the protein components of the purified (Na⁺, K⁺)-ATPase, we calculated the dimensions and
TABLE II

Comparison of Observed and Calculated Frequencies and Diameters of the Protein Particles in the Purified (Na⁺,K⁺)-ATPase

| Diameter | Frequency |
|----------|-----------|
| Å        | Particles/μm² |

**Observed**
- Surface: 30–50 Å, 12,500
- Intramembranous: 90–110 Å, 3,800

**Calculated**
- Protein unit of:
  - 140,000: 51, 13,700
  - 280,000: 73, 6,800
  - 560,000: 102, 3,400

The observed values are taken from Table I and Fig. 10.

In the calculation of the frequencies, the total membrane area estimated for a fluid mosaic model membrane (29) composed of 1 mg protein, 0.3 mg cholesterol, and 0.73 mg phospholipid (14) was divided by the number of protein units as computed from previous data. The area occupied by the lipid bilayer was $2.3 \times 10^{19}$ Å² when the molecular areas of cholesterol and phospholipid were taken to be 38 and 50 Å² (3). Assuming a protein density of 1.25 g·ml⁻¹ and an arrangement in cylindrical units with a height of 90 Å, the protein area was $8.9 \times 10^{18}$ Å² (total volume of protein divided by 90 Å). The total membrane area was thus $3.2 \times 10^{19}$ Å² per mg enzyme protein.

In the calculations of the number of protein units, the concentration of the unit with mol wt 140,000 was set equal to the concentration of catalytic protein, 7.2 nmol mg⁻¹ protein. The concentration of the unit with mol wt 280,000 was set equal to the concentration of ouabain-binding sites in the purified preparation, 3.6 nmol mg⁻¹ protein (14). The concentration of the hypothetical unit with mol wt 560,000 was assumed to be 1.8 nmol mg⁻¹ protein.

In the calculation of the diameters, the protein units were assumed to have densities of 1.25 g·ml⁻¹ and to be arranged in cylinders with a height of 90 Å.

frequencies of the protein units on the basis of chemical and enzymatic analysis. The catalytic protein with mol wt close to 100,000 accounts for 72% of the total protein in the preparations, and the molar ratio of catalytic protein to glycoprotein with mol wt close to 40,000 is 1:1 (15). Ratios of 2:1 (26) and of 1:2 (18) have been observed in other purified preparations. However, cross-linking experiments suggest that the two proteins are close to one another in the membrane (18) and that the proteins are solubilized by Triton as a unit with mol wt 140,000 (4). As a basis for the calculations in Table II, we have therefore used a protein unit with mol wt 140,000 containing one catalytic chain and presumably one glycoprotein chain. The unit of (Na⁺, K⁺)-ATPase which binds one molecule of ATP or ouabain and incorporates one phosphate molecule from ATP in the presence of Na⁺ and Mg²⁺ has a mol wt close to 280,000 (15, 16) and may thus contain two catalytic proteins with mol wt 100,000. Independent evidence for this subunit structure has been obtained in cross-linking experiments (18). Table II shows that an ellipsoid or cylindrical protein with mol wt 140,000 and a height of 90 Å has a diameter close to 50 Å. With a mol wt of 280,000, the diameter is at least 75 Å. Our measurements suggest that a height of 90 Å and a diameter of 50 Å are upper limits for the dimensions of the surface particles. It seems therefore unlikely that the surface particles contain more than one catalytic protein chain.

The frequency of the protein units can be calculated from the content of phospholipid, cholesterol, and protein, assuming that the proteins are inserted in a lipid bilayer as in the fluid mosaic membrane model (29). The data in Table II show that the estimated frequency of a unit with mol wt 140,000 is in good agreement with that observed for the surface particles after negative staining. The estimated frequency of the protein unit with mol wt 280,000 is about half that observed for the surface particles.

On the basis of comparisons of both the sizes and the frequencies, we therefore propose that one surface particle represents a protein unit which, as its major component, consists of only one catalytic protein chain and that two surface particles associate to form the enzyme unit which binds one molecule of ATP or ouabain.

**Intramembranous Particles**

The surface particles as demonstrated by negative staining and the intramembranous particles observed by freeze-fracture have a similar distribution. Both methods reveal particles arranged in clusters and strands which are larger and more compact in fixed than in unfixed membranes.

This finding, in conjunction with the observations on the etched preparations, shows that the membrane areas with surface particles correspond to the areas containing intramembranous particles.

The differences in dimensions and frequencies between surface and intramembranous particles show that they represent different aspects of the pump proteins. However, an interpretation of the
precise relationship between the surface particles and the intramembranous particles requires consideration of the two different techniques by which they are observed. Thus, negative staining gives a better specimen resolution than freeze-fracture. For this reason, the intramembranous particle may well correspond to an oligomere the individual units of which cannot be demonstrated with the replication technique but would be resolved at the resolution provided by negative staining.

The limitation in the resolution of the freeze-fracture procedure may in part be due to the shadowing material which probably enlarges the diameter of particles or other substructures by 15–25 Å (24) (Fig. 20c–e). There is also evidence that plastic deformation occurs during fracture, and this may obscure existing substructures. Furthermore, it is possible that lipid molecules which surround the protein become associated with the protein during fracture and enlarge the particles observed in the replica. The freeze-fracture technique may also lead to a reduction in the frequency of observed particles on the fractured faces since some particles may fracture within the membrane during membrane cleavage, as proposed before (1) (Fig. 20d).

![Figure 20](image-url)

**Figure 20** Schematic drawing illustrating model interpretations of observations by negative staining and freeze-fracture on membrane-intercalated protein particles. (a) Each elongated particle (P) represents a hypothetical protein unit which spans the lipid bilayer. (b) After negative staining, each protein unit is visualized as a particle on the surface of the membrane. (c–e) During freeze-fracture, different fracture planes are possible. The fracture plane may pass on either side of the particles (c and e) or it may cleave the protein units (b). After metal shadowing, closely associated protein units may not be resolved as individual particles but may appear as one large intramembranous particle due to the thickness of the evaporated metal (c and e). Particles which are split in two halves (d) may not be visualized at all after evaporation of metal. This model would account for the frequency of intramembranous particles being smaller than the frequency of surface particles and for the intramembranous particles being larger than the surface particles.
A comparison of calculated and observed dimensions and frequencies of protein complexes in Table II would suggest that the actual dimensions and frequencies of the intramembranous particles correspond to those calculated for a protein with a mol wt of 560,000. However, as pointed out above, the observed diameter of the intramembranous particles represents a maximum value and the observed frequency a minimum value. The possibility should therefore be considered that one intramembranous particle corresponds to two, three, or even four of the units forming the surface particles. Further studies, possibly involving rotary replication or ultrahigh vacuum freeze-fracture, are necessary to establish this relationship.

We wish to thank Janne Petersen and Marianne Ellegaard for excellent technical assistance.

Received for publication 18 April 1977, and in revised form 18 July 1977.

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