REGULAR STRUCTURES IN UNIT MEMBRANES

III. Further Observations on the Particulate Component of the Suckling Rat Ileum Endocytic Membrane Complex

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ABSTRACT

Further morphological observations on the particulate components decorating the lumenal surfaces of membranes of the endocytic complex of the epithelial cells of the suckling rat ileum are presented. The particles each measure ~7.5 nm across and give the appearance of the capital letter H in frontal view. They consist of the enzyme n-acetyl-β-glucosaminidase (NAG). They are arranged in rows called "decorated strips" with the symmetrical lateral bars in register and spaced ~14.5 nm apart. Decorated strips lie side-by-side in the external (lumenal) surface of the membrane. They are parallel and sometimes spaced ~14.5 nm apart making an orthogonal lattice. The lateral spacing between the decorated strips under certain conditions is reduced and sometimes there is shear between the adjacent ones. Occasionally, shear is present within the decorated strips themselves, with slight displacement of the two sides of each H-shaped particle.

A purified preparation of these membranes has been studied by electron microscopy using thin sectioning, negative stain, Markham translation and optical diffraction computer image reconstruction methods. The individual particles comprising the array can be seen in the membrane surface in profile view when dried in a pool of negative stain. They appear either triangular or diamond-shaped in such views. If triangular, they appear to consist of three domains at the corners of an equilateral triangle. One side of each triangular figure is parallel to the membrane surface but separated from it by a dense band of negative stain ~2 nm thick that runs along the surface of the membrane. Sometimes a fourth symmetrical domain is visible within this dense band, giving a diamond-shaped figure. This fourth domain connects the particle to the membrane. Thus, each H-shaped particle is a double structure, with each half in profile view appearing as a diamond figure of four symmetrical domains. Each H-shaped particle is believed to consist of either two or four molecules of NAG.
In a recent article (14) we described certain patterns of organization of a specialized unit membrane system, the endocytic complex, in the apical portions of the epithelial cells of the suckling rat ileum. During the period of suckling, arrays of H-shaped particles in a square lattice are present in the external (lumenal) surface of this membrane, as earlier described by Wissig and Graney (26).

The particles have recently been isolated in this laboratory and identified as the lysosomal enzyme \( n\)-acetyl-\( \beta \)-glucosaminidase (NAG) (10-12). Their attachment to the membrane is by means of a newly described filamentous protein as reported in the accompanying second paper of this series (12). The first paper of this series dealt with the appearance of the particles in frontal views in negatively stained preparations, as well as in thin sections and freeze-fracture-etch (FFE) preparations. The second dealt mainly with biochemical studies of these membranes and the particles. The present paper deals with the appearance of the particles in profile views in negatively stained preparations.

MATERIALS AND METHODS

Some of the present observations were based on studies of a highly purified preparation of endocytic complex membranes isolated from suckling rat ileums as described in the accompanying paper (12), and some were made on crude preparations.

The purified membrane fractions were suspended in 10 mM HEPES buffer (Sigma Chemical Co., St. Louis, Mo.) at pH 7.6. In two instances the membranes were further treated: (a) Fig. 4 and the related enlargements show a preparation treated briefly with 0.5% deoxycholate. (b) Fig. 15 shows a preparation exposed to 0.1% Triton X-100. Treatment (a) seemed to exaggerate the tendency for the vesicles to become elongated, and the particles at the edges of the elongated cylindrical forms seemed to stand out more clearly. Treatment (b) facilitated finding single membrane sheets and increased the clarity of the surface particles.

The 1% phosphotungstic acid (PTA) solution was prepared as described in the previous paper (12). The negative-stain spray technique utilized a Freon spray device marketed by Ernest F. Fullam, Inc., Schenectady, N.Y. The spray droplets consisting of equal mixtures of the membrane suspension with the negative stain solution were deposited on thin carbon films containing small holes. The grids were usually made hydrophilic by exposure to a 30-s glow discharge in a Balzers 120 shadow-casting unit at about 0.5 Torr (Balzers High Vacuum Corp., Santa Ana, Calif.). Electron micrographs were taken with a Philips 301 or Siemens Elmiskop 102 electron microscope.

The electron micrographs were examined in an optical diffractometer to select the areas with the best diffraction patterns, i.e., the highest resolution. These diffraction patterns were photographed and the same area was then scanned with a microdensitometer for computer processing. Images were reconstructed in which periodic details were averaged and nonperiodic details were filtered out. These computer-filtered images were obtained in collaboration with Dr. Harold Erickson of Duke University, using the computer system described elsewhere (3, 5).

Calculations of approximate spherical molecular volumes were made as follows (22): \( \frac{1}{2} \text{g molecular weight} \) was divided by Avogadro's number and this was multiplied by 0.77 cc/g, the approximate partial specific volume of protein neglecting hydration.

RESULTS

The lattice of particles is recognizable in negatively stained preparations in Figs. 1, 3-4, 9, 12, and 18-19 but is not so clear as in Fig. 15 which is a part of Fig. 6 of our previous paper (14) in which we succeeded in getting some membrane fragments deposited as single sheets. Most of the fragments studied consist of completely or partially collapsed vesicles. Thus, there are always at least two superimposed lattices. This leads to confusion of the images. In favorable cases the two superimposed lattices can be separated and analyzed by optical diffraction methods. The diffracted spots can then be indexed on two identical lattices (Fig. 20).

Whenever vesicles are embedded in the dried PTA extending across holes in a carbon film as in Figs. 1-3 or simply flattened out on the carbon film as in Figs. 1, 4-14 and 18-19, it is possible to observe the H-shaped \( \sim 7.5 \text{nm} \) particles in profile view. The particles often appear triangular as in Figs. 2-8. This triangular appearance is accentuated by superimposing multiple images using the technique of Markham et al. (16). This technique delineates three spherical domains at the corners of the triangles as in Figs. 3-8. The inset to the upper right in Fig. 3 is an enlargement of the area marked as a dotted rectangle in which the image has been enhanced by several translations. The upper right inset in Fig. 4 is a similarly enlarged and enhanced image of the upper marked area in Fig. 4. Fig. 7 is an enlargement of the lower enclosed area in Fig. 4, and Fig. 8 is the corresponding translated and enhanced image. Figs. 5-6 show enlargements and the corresponding enhanced image of the middle rectangular area indicated in Fig. 4. All of these enhanced images...
FIGURE 1 PTA-bacitracin (PTA-B) negatively stained preparation of purified membrane fraction. A and B designate relatively cylindrical vesicles. Some vesicles are included within negative stain condensed over holes in the carbon film. The two arrows to the upper right point to detached strips of membrane particles ("decorated strips"). × 88,000.

FIGURE 2 High magnification of the edge of a vesicular form displaying particles having a roughly triangular shape. PTA-B. × 736,000.

resemble closely Fig. 2, which shows the edge of a vesicle in a direct unenhanced image. The triangular appearance of the image in Fig. 2 is consistent with the presence of three domains, each ~3 nm in diameter, as suggested by the Markham translation images even though they do not always show up individually.

There is a thin dense band of negative stain about 2-nm thick that can be seen clearly in many places, separating the profiles of the particles from the edges of the membranes. This is particularly clear along the bottom edge of the vesicle in Fig. 3, between the opposed arrows. It is also particularly clear in the lower half of Fig. 12. Occasionally, some light structural elements may be seen in this dense band as indicated by the arrows in the enlarged area from Fig. 9 shown in Figs. 10-11 and by the large arrows in Figs. 12-14. In such instances, the ~7.5 nm particles appear to be diamond shaped rather than triangular. This shape change occurs whenever the fourth domain in the particles is revealed. This fourth domain is directly connected to the edge of the membrane. Fig. 15 shows deposits of PTA (arrows) between the rows of particles that measure ~7.5 nm in width. We believe that it is one or more of these deposits of PTA seen superimposed edge-on in the profile views that produces the ~2-nm thick bands separating the rows of particles from the edges of the membranes. It seems clear that some structure is embedded in these bands to connect the particles to the membranes. However, if this structure is of a size comparable to that of the three domains making up the triangular particles, it might well be obscured by the PTA in most places. Furthermore, it seems reasonable to expect the radius of curvature to be smaller in the case of vesicles dried down on a carbon film than embedded in dried PTA in a hole in a carbon film as in Fig. 3. It is only in the former cases that we have ever seen
structural details within these dense bands. It seems clear, then, that the PTA adjacent to the rows of particles tends to obscure a fourth domain of the particle that connects it to the membrane. These domains are revealed in Figs. 9-14 as a result of fortuitous local thinning of the bands of PTA.

The Markham translation technique accentuates the diamond-shaped appearance of the particles seen in Figs. 9-10, 12-13 as shown in Figs. 11 and 14. These diamond-shaped particles appear to have four domains, with one of these domains apparently attaching the particle to the surface of the membrane. The triangular appearance is produced when this fourth domain at the membrane surface is obscured by PTA. It is interesting that PTA sometimes appears to penetrate or accumulate in the center of the particles, between the domains in profile views. This shows up most clearly in Fig. 4 (upper marked area and the inset enlargement) and in Figs. 13-14.

We have attempted to gain further insight into the structure of the particles in the lattice by using a computer system to filter out nonperiodic noise. Fig. 15 is a small segment of Fig. 6 in our previous paper (14) which we chose for analysis because of its unusual clarity. It is a single membrane layer showing particles in a square lattice. Fig. 16 is an optical diffraction pattern obtained from this area, and Fig. 17 is the corresponding computer-reconstructed image. Fig. 17 shows that there is a faint fibrillar structure connecting the lateral vertical bars of the H figure and that there is an inward curvature to the bars. In Fig. 17 the pair of arrows designates a “decorated strip” as defined in the accompanying paper (12). Each decorated strip consists of a row of dense (bright in this image) particles connected by dense strands. In Fig. 15 the particles are almost in perfect lateral register as pairs giving the H figure and are arranged in an almost perfect orthogonal lattice. There is, however, a slight shear between the individual decorated strips.

We have obtained diffraction patterns from the confused images of the two superimposed lattices of collapsed vesicles and reconstructed images by the computer method showing each of the superimposed lattices with interesting results. The area marked in Fig. 18 and enlarged in Fig. 19 was analyzed in this way. Fig. 20 is the optical diffraction pattern obtained from the area in Fig. 19. All the diffraction spots may be assigned to one of two reciprocal lattices, whose unit cells are drawn in Fig. 20. The lattices have slightly different dimensions, and one is inverted with respect to the other (as would be expected for the top and bottom surfaces of a closed vesicle) but they are essentially identical. Since the diffraction spots from the two lattices are separated, filtered images of each surface could be reconstructed using the computer system. These are shown in Figs. 21 and 22. The image in Fig. 21 corresponds to the lattice on the left in Fig. 20, and that in Fig. 22 to the one on the right. In Figs. 21 and 22 the image again consists of decorated strips as designated by the pairs of arrows, but here the shear between the strips is greater and the strips are closer together than in Fig. 17. They resemble in this respect the appearance of the 10.5-nm square lattice reported in Fig. 7 of the first paper (14) in this series. The shear is such that particles on adjacent strips are displaced approximately half a unit cell. In this case, the shear appears to extend within each decorated strip as well as between adjacent strips. These observations show that the individual decorated strips are free to move both longitudinally and laterally in the membrane surface. They show further that the individual particles are fixed relative to one another in the longitudinal direction in each strip by the dense strands connecting them. In addition, each half-particle is fixed to the corresponding half-particle within the strip though it can move slightly as indicated by the internal shear. This is supported by the fact that the particles are released in decorated strips which are double structures. We believe that the two separate rows of mass along the arrows connecting the half-particles are ligatin (12). There is no evidence of intermolecular binding between the ligatin strands which could serve to stabilize the decorated strips as double structures; it appears that this function is attributable, instead, to the H-shaped particles which are physically connected.

Fig. 23 is a schematic diagram that shows the particles as they appear in the lattice from above (face view) and from the side (profile view). The dotted lines indicate that the adjacent particles in the rows are attached end-to-end (Fig. 23, face view). As indicated in the accompanying paper (12), this attachment structure consists of filaments of ligatin that bind the particles to the membrane, and this is supported further by the computer images. The computer images also reveal the crossing of the H and the inward bend in the lateral bars. In the profile view, each particle appears as a tetrad with internal domains approxi-
FIGURE 12  Edge of flattened vesicle on a carbon film prepared by the same method as that used in Fig. 9. Note the diamond forms designated by the large arrows. Thin dense band of PTA separating the particles from the membrane is seen in the lower part of the picture. In the upper part of the picture, this band is apparently thinner because particles show up in it and appear to touch the membrane. The interruption of this dense band by particles shows up most clearly in the translated image in Fig. 14. $\times 400,000$.

FIGURE 13  Enlargement of area designated by the arrows in Fig. 12 to show the diamond forms. $\times 1,040,000$.

FIGURE 14  Markham translation enhanced image of Fig. 13. $\times 1,040,000$.

FIGURE 3  Vesicle from the purified membrane fraction entirely embedded in PTA-B negative stain in a hole in a carbon film. The area designated by the rectangle was enlarged and enhanced by the Markham translation technique. The resultant image is printed in the inset enlargement. The aligned arrows below point to a dense band $\sim 2$ nm thick of PTA-B separating the particles from the membrane. $\times 310,000$. Inset, $\times 552,000$.

FIGURE 4-8  Elongated cylindrical form from a preparation that was exposed briefly to deoxycholate. The lower area designated by a rectangle enlarged in Fig. 7. Fig. 8 represents the enhanced image resulting from translation of this region. The middle rectangle is enlarged to the left in Fig. 5, and Fig. 6 represents the corresponding translation image. The upper rectangle was enlarged and subjected to translation. The resultant enhanced image is inserted to the upper right. Fig. 4, $\times 207,000$. Inset, $\times 477,000$. Figs. 5-8, $\times 560,000$.

FIGURE 9  Edge of a vesicle from a crude homogenate of ileum prepared by PTA negative staining without the use of surface active agents. The indicated rectangle is enlarged in Fig. 10 to show two regions in which particles are seen as diamond forms. $\times 84,000$.

FIGURE 10  Enlargement of area designated by the dotted rectangle in Fig. 9. The arrows designate particles appearing as diamond-shaped four-domain forms. $\times 576,000$.

FIGURE 11  Translation image of Fig. 10. $\times 576,000$. 

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approximately 3 nm in diameter but this measurement is, of course, only an approximation as is made clear by Finch and Klug (7, 8), Unwin (23), and Unwin and Klug (24).

DISCUSSION

The particles are NAG (12). NAG seems to cover the cisternal surfaces of the endocytic complex membranes rather completely. This enzyme cleaves the β-linked n-acetyl-glucosamine groups from glycoproteins, mucopolysaccharides and glycolipids (25). Casein, the major milk protein, is a complex family of phospho-proteins containing glycosyl residues (25). Often, n-acetyl-glucosamine is the first sugar moiety in a large glycosyl residue and serves to attach the whole residue to the polypeptide chain by linkage to asparagine (25). NAG thus acts to cleave whole glycosyl residues containing many monosaccharides from casein and thus plays a key role in the process of digestion of maternal milk. Its immobilization might enhance its activity and thus facilitate its key role. For example, Bernath and Vieth (2) showed a 70% increase in activity of lysozyme when the enzyme was immobilized on the surfaces of detergent micelles. In addition to a potential enhancement of activity, conservation of the enzyme (19, 29) may also play a role in the immobilization of NAG in the endocytic complex since this prevents its displacement into the intestinal lumen or the large apical lysosomal vacuole.

As reported in the accompanying paper, NAG isolated from the endocytic complex migrates in sodium dodecyl sulfate (SDS) polyacrylamide gels as two bands with apparent mol wt of ~100,000 daltons and ~110,000 daltons (12). Both are PAS positive, indicating glycosylation, and therefore their true molecular weights may be expected to be smaller. It is not clear whether these are two forms of the active enzyme or two subunits of the enzyme. As stated in the accompanying paper (12), negative-stain studies of the enzyme in solution have so far failed to yield reproducible images in which we can identify the monomeric forms. Thus, we so far have no direct electron microscope evidence about its size or shape in solution. We can, however, relate the images of the particles in the membrane to the enzyme molecule by comparing our findings with those of others on negatively stained enzyme molecules. The papers by Wrigley et al. (27) and Haschemeyer and de Harven (9) illustrate the power and limitations of this method in studying enzyme molecules. Racker et al. (20) found that mitochondrial adenosine triphosphatase (ATPase), with a mol wt of 284,000 daltons, appears as a particle 8.5 nm in diameter. A protein molecule of this molecular weight, if globular, would fill a sphere of 9.6 nm diameter. Similarly, negatively stained preparations of argininosuccinase, which has a mol wt of 200,000 daltons, appear as roughly spherical particles ~7.5 nm in diameter, according to our measurements from micrographs in Haschemeyer and de Harven (9). The corresponding calculated sphere for a globular protein of this molecular weight would be 7.3 nm in diameter. Glutamine synthetase molecules each consist of 12 spherical subunits, each of 50,000 daltons (15). The whole molecule in negative stain in one projection is 14.0 nm in diameter and in the other, 8.2 nm. Each spherical subunit of 50,000 daltons is ~4.5 nm in diameter. The corresponding calculated
sphere for each subunit would be 4.6 nm in diameter. Thus, the volumes of protein molecules as seen in negatively stained preparations closely approximate the actual volumes of the molecules. We know that each of the two forms of NAG described in the previous paper is not globular but may be dumbbell shaped. The whole particle comprised of eight ~3 nm domains in our preparations presumably represents at least one whole molecule of NAG. We believe that the whole particle in fact contains either two or four molecules of NAG. If we consider the whole H-shaped particle to be a cube 7.5 nm on a side, the calculated volume would be 422 nm³. This volume could contain a cube of protein of ~330,000 daltons. If the true mol wt of the enzyme molecule is ~330,000/4 or ~83,000, which seems a reasonable guess on the basis of the SDS gel analysis, then
the particle contains four enzyme molecules. On the other hand, if the active form of this enzyme has two subunits, each of approx. 100,000 daltons, then the particle contains two molecules of NAG. We shall be able to choose between these possibilities as soon as we have determined the true molecular weights of the two forms and the minimum molecular weight of the active oligomeric monomer. Visualization of the known monomeric or dimeric form in negatively stained preparations might also settle this problem.

From a morphological point of view, we think that our observations are unusual. It has been realized for a long time that many enzymes are attached to membranes, and most modern biochemistry texts discuss the importance of this in facilitating the action of chains of enzymes like those acting in mitochondria. However, such presumed arrays of enzymes in membrane surfaces by-and-large have not been directly visualized or have been seen only as vague knobs without clearly definable internal structure. Our studies not only deal with an identified enzyme visualized in situ as a part of a unit membrane but also allow the definition of some distinctive structural features of the enzyme by negative-stain methods. Furthermore, the observations have been directly correlated with parallel studies of the particles in the same membrane by freeze-fracture and thin-sectioning electron microscopy. Mitochondrial ATPase is, to our knowledge, the only other example of a membrane-bound enzyme in which any such close correlations of structure and function have been made and here, even though the molecule was first visualized in negatively stained preparations in 1962 by Fernández-Morán (6), only recently has it been convincingly demonstrated in thin sections by Zaar (28). It has not yet been identified in freeze-fracture-etch preparations, and no distinctive internal features that would allow this molecule to be identified in isolation have been defined. To be sure, other enzymes may have been observed in situ attached to membranes but none of these have been definitely identified functionally and defined structurally to an extent to allow them to be recognized and differentiated from other enzymes. For example, a kind of “coated” vesicle in which the coat might represent enzyme molecules has been observed in Hydra by Slautterback (21). Similarly, McKanna (17) has made related observations in “coated” vesicles in peritrich protozoans. Further, ~6 nm particles attached to intestinal microvillar membranes were thought to be a disaccharidase by Eichholz and Crane (5), Overton et al. (18) and Johnson (13), but this has been disputed by Benson et al. (1). Of course, many soluble enzymes have been studied by negative stain methods with definition of distinctive internal features as reviewed by Haschemeyer and de Harven (9) but, as far as we are aware, none of these have been studied in situ attached to a membrane. Many enzymes are known to be located in membranes but so far have eluded direct visualization and characterization of the kind reported here.

NAG is, on the basis of our findings, now sufficiently well characterized to allow it to be singled out and identified in negatively stained preparations even if it were located in a group of different enzymes in a membrane surface. If distinctive internal features can be defined for other membrane enzymes, it may become possible to single out and identify them in functional arrays of other enzymes directly in electron micrographs. Improvements in technique may make this possible in the near future, and this could lead to the solution of many problems.

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