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ELECTRON MICROSCOPIC VISUALIZATION OF THE ARRANGEMENT OF THE TWO PROTEIN COMPONENTS OF $(Na^+ + K^+)$-ATPase

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Summary

The information obtained by electron microscopic examination of highly purified membrane preparations of $(Na^+ + K^+)$-ATPase after freeze-fracturing or negative staining suggests the following conclusions. The catalytic 100 000 dalton protein component penetrates with its greater 'globular' mass the plasma membrane and protudes with its smaller mass from the protoplasmic surface by a stalked knob carrying the catalytic centre. The 40 000 dalton glycoprotein component is anchored in the membrane interior by a non-polar 'fibrous' side chain, whereas its major polar mass projects from the outer membrane surface forming a surface coat of ill-definable substructure.

Earlier electron microscopic studies on the ultrastructure of highly purified membrane preparations of $(Na^+ + K^+)$-ATPase done after negative staining [1,2] and after freeze-fracturing [2] revealed, in addition to the gross morphological appearance, the distribution and shape of granules on one of the membrane surfaces and of particles in the membrane core which by analogy were both attributed to protein components of the enzyme. The present paper aims at electron microscopic visualization of the sidedness, arrangement and shape of both the catalytic protein component and the glycoprotein component of $(Na^+ + K^+)$-ATPase.

The $(Na^+ + K^+)$-ATPase preparation was purified from the membrane

Abbreviations: $(Na^+ + K^+)$-ATPase, Na^+- and K^+-transporting, Mg^{2+}-dependent adenosine triphosphatase of plasma membrane; (Ca^{2+})-ATPase, Ca^{2+}-transporting, Mg^{2+}-dependent ATPase of sarcoplasmic reticulum.

fraction of the Henle loop in the outer medulla of pig kidney by dodecyl sulfate treatment in presence of ATP and isopycnic zonal centrifugation exactly as described by Jørgensen [3]. The purified enzyme remaining bound to membrane fragments showed an activity of 1500—2000 μmol $P_i \cdot h^{-1} \cdot$ mg protein^{-1} and contained, besides lipids, the catalytic protein of 100 000 dalton and the glycoprotein of approx. 40 000 dalton, constituting together 80—90% of the total protein amount.

For freeze-fracturing, the enzyme preparations suspended for 30 min in 0.1 M phosphate buffer (pH 7.2) with 30% glycerol (approx. 2 mg protein per ml) were frozen in liquid Freon 12 at approximately —150° C. Specimens were fractured at —100° C in a Balzers BA 360 M. For negative staining, the preparations were suspended in 0.1 M phosphate buffer (pH 7.4) (approx. 0.1 mg protein per ml) and transferred to formvar- and carbon-coated grids. Aqueous solutions of potassium phosphotungstate (2%), potassium silicotungstate (2%) or ammonium molybdate (4%) all adjusted to pH 7.4 with KOH were used for negative staining. The specimens were analyzed after air drying. The results obtained with the different stains were similar. With both techniques, a Jeol JEM-100 B electron microscope with an accelerating voltage of 80 kV was applied. Photographs were taken at magnifications ranging from × 20 000 to × 90 000.

Freeze-fracturing. The enzyme preparations contain, in addition to a few vesicles, predominantly flat or cup-shaped membrane fragments of 0.2—1.0 μm length and occasionally discs also, with diameters of 0.1—0.5 μm (Fig. 1A). Hence, the discs do not appear to represent fracture faces of vesicles as described by van Winkle et al. [2], but they seem to correspond mostly to surface-exposed, cup-shaped membrane fragments. Both the fragments and the discs show a substructure consisting of globular particles with an approximate diameter of 80—120 Å (Fig. 1B). As well-established, the cleavage produced by freeze-fracturing occcurs within the membrane interior and parallel to the membrane plane. The exposed faces are therefore related to either the original protoplasmic leaflet, or the original outer leaflet of the membrane. In our preparations, one part of the fracture faces (probably representing the protoplasmic leaflet) shows a particle density of approx. 3000/μm^2, and the other part (probably representing the outer leaflet) of approx. 1500/μm^2. Although the number of particles per membrane area in both membrane halves cannot be simply added, their real density in the membrane preparation can be assumed to lie between 3000 and 4500/μm^2. Hence, the particles which probably represent mostly the catalytic 100 000 dalton protein component of ($Na^+ + K^+$)-ATPase (see below) appear to penetrate the plasma membrane to a large percentage. This interpretation is supported by the well-established findings that the substrate, ATP, and the negative effector, ouabain, combine with the catalytic ($Na^+ + K^+$)-ATPase protein from the intracellular and extracellular space, respectively. The clustering of the particles, observed in about 1/3 of the membrane faces (Fig. 1B) may be caused by exposure to low temperature and detergent during isolation and purification of the membrane preparation.

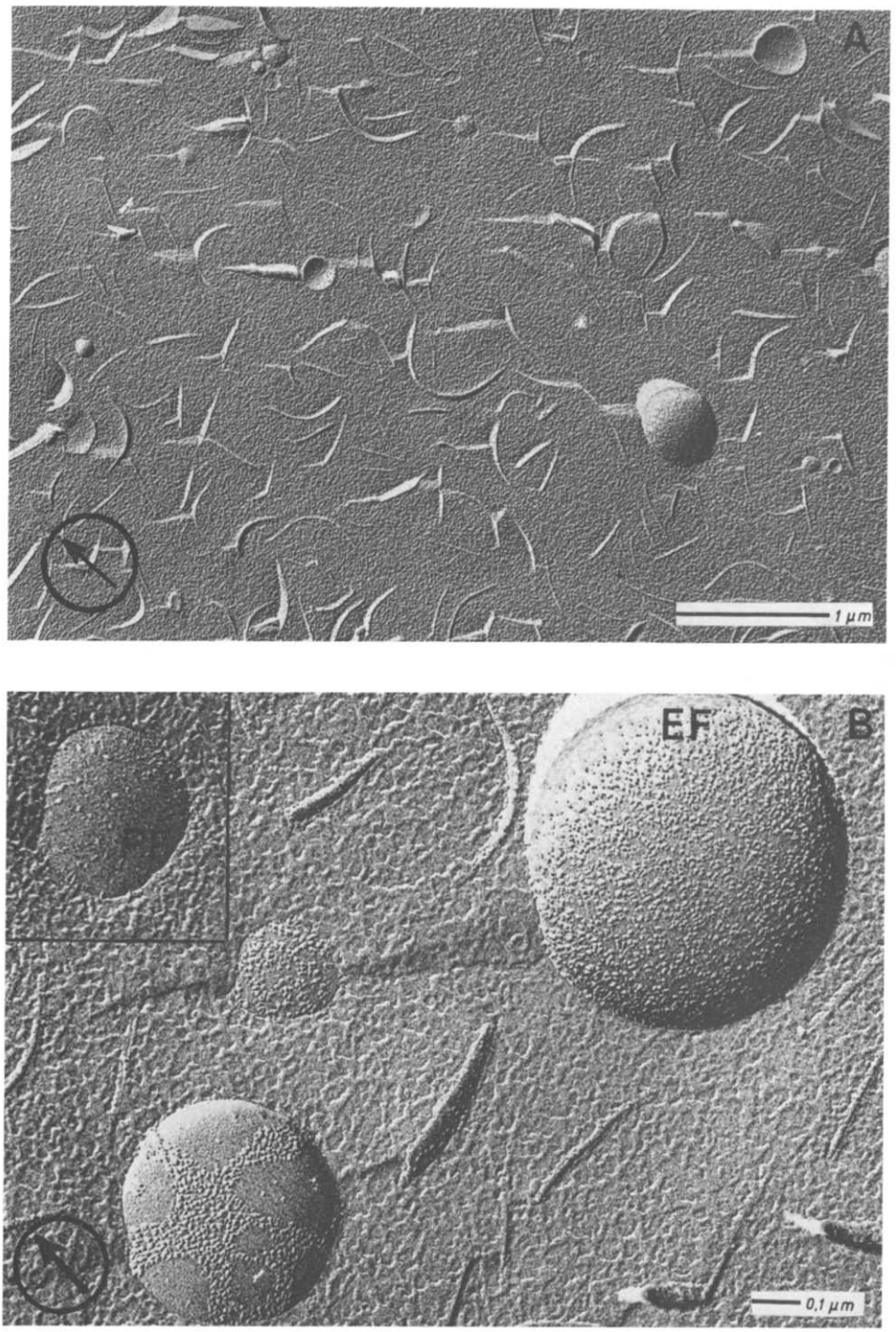

Fig. 1. Freeze-fracture replica of a membrane preparation of highly purified ($Na^+ + K^+$)-ATPase. A. The preparation mainly consists of flat or cupshaped membrane fragments, but also contains a few discs and vesicles. Magnification, X 21 500. B. The fracture faces of discs show a substructure consisting of globular particles whose densities are on the exterior face (EF) higher than on the protoplasmatic face (PF). Magnification, X 66 500.

Negative staining. The two surfaces of the membrane-bound enzyme show different morphological features. The large membrane fragments which are irregularly shaped and partially associated with each other stick with their surfaces parallel to the grid so that the one or the other membrane surface is exposed (Fig. 2A and B). The 'protoplasmic' surface (see below) observed to be about 10% only shows granules of approx. 50 Å transversal diameter projecting from smooth membrane areas between them (Fig. 2A). The granules are, in part, evenly distributed or arranged in clusters and strands. The granule density amounts to 4200/μm^2 and is thus very similar to the particle density found in the freeze-fractured membrane. Even if closely packed, the distance between the granules is sufficient to visualize the granules also in the case of the vertical projection of the membrane surfaces found with small fragments (Fig. 2C—E, single arrows). If the thickness of the screened fragments is thin enough, granules of 50 Å largest diameter are depicted on the 'protoplasmic' surface and appear to be attached to the membrane core by a short stalk. Similar knobs joined to the protoplasmic membrane surface are also found in electron micrographs of ultra-thin sections of unfixed kidney tubular cells obtained by cryo ultramicrotomy [9]. Moreover, similar structures are formed by the catalytic protein of (Ca^{2+})-ATPase likewise projecting from the protoplasmic surface of sarcoplasmic reticulum [4—6]. By analogy, we assume that the stalked granules are formed by the catalytic 100 000 dalton protein component and contain the catalytic centre of ($Na^+ + K^+$)-ATPase known to be exposed to the protoplasmic surface of the plasma membrane.

The other, apparently 'outer', surface shows a much finer granularity than the protoplasmic surface so that definite granules cannot be differentiated (Fig. 2B). By exclusion, this fine granularity corresponds to the broader surface coat of about 50 Å in diameter, which is depicted in the case of the vertical projection of the small membrane fragments (Fig. 2C—E, double arrows). This surface coat shows a globule-like, aerial-like or a fuzzy coat-like substructure. Apparently, the superimposition of background granules ('phase grain') on the detailed membrane surface structure prevents an accurate determination of shape and size of this structural element. Since (Ca^{2+})-ATPase lacks both a similar surface coat [4—6] and a glycoprotein component [7], it appears almost safe to conclude that the additional surface coat found exclusively in the membrane preparation of ($Na^+ + K^+$)-ATPase is formed by its 40 000 dalton glycoprotein component. The thickness of the membrane core and of the total membrane including both surface coats was measured to be 55 Å and 140 Å, respectively.

Tentative integration of informations obtained by both procedures. The catalytic protein component of 100 000 dalton forms stalked knobs projecting from the membrane core to the protoplasma. This arrangement appears to be ideally fitted for the substrate accessibility of catalytic centre. The projecting part observed with negatively stained membranes can, in view of its small size, account for only a part of the catalytic protein. The remainder appears to penetrate the lipid bilayer and to account for the particles seen in the inner and outer half of the freeze-fractured membrane. The glycoprotein component projects from the membrane core to the

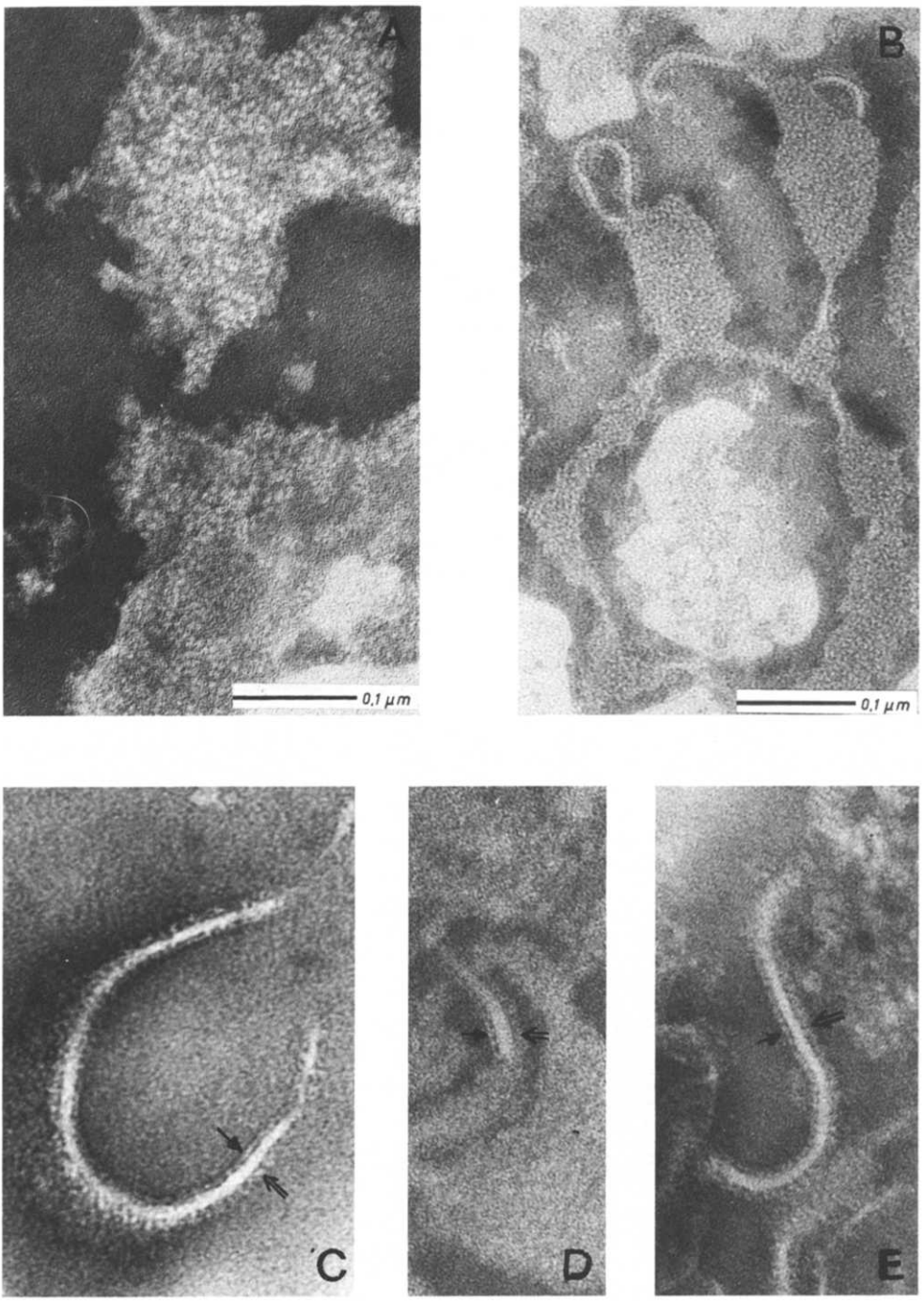

Fig. 2. With phosphotungstate negatively-stained membrane preparation of highly purified ($Na^+ + K^+$)-ATPase. A and B. Surface projection of large membrane fragments revealing the 'protoplasmic' surface with definite granular substructure (A) or the 'exterior' surface with fine granularity (B). Magnification, × 180 000). C,D,E. Vertical projection of small membrane fragments showing stalked knobs on the 'protoplasmic' surface (single arrows) and an ill-definable substructure on the 'exterior' surface (double arrows). Magnification, × 300 000.

extracellular space forming a surface coat of ill-definable substructure under conditions of negative staining. In the membrane core, the glycoprotein could be attached to the catalytic protein by a non-polar, 'fibrous' side chain which, due to its small mass, forms no visible particles in the fracture plane (cf. ref. 8). The nature of the firm non-covalent interaction between the two protein components of $(Na^+ + K^+)$-ATPase preserving copurification during detergent treatment remains an open question. An orienting interaction with the glycoprotein could be important for the correct insertion and arrangement of the catalytic protein in the membrane.

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