Structure of (Na^+,K^+)-ATPase as Revealed by Electron Microscopy and Image Processing

MANIJEH MOHRAZ and P. R. SMITH
Department of Cell Biology, New York University Medical Center, New York 10016

ABSTRACT (Na^+,K^+)-ATPase was studied by electron microscopy and image processing of negatively stained and freeze-dried and shadowed crystalline sheets induced by a number of inorganic salts. Extensive experiments have identified new conditions for optimum crystal formation. Two crystal forms have been observed, one with a monomer and the other with a dimer, in the unit cell. Both show the same structure for the enzyme monomer. The enzyme can also be crystallized after partial proteolysis of its alpha subunit by trypsin. The proteolysed enzyme crystallizes under the same conditions as the whole enzyme. Comparison of the mass distributions in the images of the intact and proteolysed enzyme has allowed the tentative identification of the location of the alpha subunit within the monomer. The relationship between the structure of the crystallized enzyme and that of the enzyme in its native form is discussed, as is its apparent close structural relationship to the calcium-ATPase.

(Na^+,K^+)-ATPase is a transmembrane protein that utilizes energy from ATP hydrolysis to transport K^+ into and Na^+ out of the cell against concentration gradients for both these ions. The molecule consists of a catalytic subunit (alpha, M_r = 100,000) and a glycoprotein subunit (beta, M_r = 50–60,000). The unglycosylated part of the beta subunit makes up 37–40,000 daltons of its total molecular mass and the balance consists of carbohydrate which is located on the exoplasmic surface of the cell (4). In the enzyme from mammalian kidneys, the mass of the carbohydrate is ~13% of the total molecular weight. It has been suggested that the differences in the molecular weight of the beta subunit from different sources may be due to variations in the carbohydrate composition (4).

The enzyme has been available in purified form for more than a decade (10, 11, 15) and a wealth of information about its reaction mechanism has emerged in the past few years (see recent reviews by Cantley [4] and Jorgensen [14]). Despite progress in other areas, little has been learned about the molecular structure of the enzyme. Information about the amino acid sequences of the two subunits is still incomplete and it is uncertain if the minimum active unit is composed of an alpha-beta monomer or an $(alpha\text{-}beta)_2$ oligomer. The recent discovery of a method whereby two-dimensional crystalline sheets of the membrane-bound enzyme can be formed (21) has opened the way for its study using the methods of high resolution electron microscopy and image processing (1).

In this paper we report an electron microscopic study of two crystalline forms of (Na^+,K^+)-ATPase sheets and of a new crystal made from the tryptic-digested enzyme. The structures of these three different crystalline sheets were analyzed using computer image processing. We demonstrate that the two crystal forms of the intact enzyme possess the same molecular strucure, and that the structure of the partially proteolysed enzyme can be related to it. This observation allows the tentative identification of a region of the molecule as the alpha subunit. Moreover, the induction of crytalline sheets by various inorganic salts, some of them inhibitors of ATP hydrolysis by the enzyme, has provided hints about the relationship of the structure of the molecule in the crystal to that of the active enzyme.

MATERIALS AND METHODS

Materials: Dog kidneys were a gift from the Department of Experimental Surgery of the New York University Medical Center. Fresh pig kidneys were obtained from a slaughterhouse. ATP (disodium salt, special quality) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were of the highest available purity.

Biochemical Techniques: Protein concentrations were measured following the method of Markwell et al. (17). SDS PAGE was performed as described by Laemmli (16). The enzyme activity was measured using the procedure of Jorgensen (12).

Purification: (Na^+,K^+)-ATPase was purified in membrane-bound form from the outer medulla of pig and dog kidneys, following the "fixed angle rotor" procedure of Jorgensen (11). Purified enzyme was collected as a pellet and resuspended at a concentration of 1.5–2.0 mg/ml in imidazole/EDTA buffer (25 mM imidazole, 1 mM EDTA, 10% sucrose, pH 7.5). The enzyme was stored at −25°C.

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Digestion: The limited proteolysis of (Na^+,K^+)-ATPase by trypsin was performed essentially according to the method of Castro and Farley (6). The protein was digested by *N*-tosyl-L-phenylalanine chloromethyl ketone–trypsin at a trypsin/enzyme weight ratio of 1:100 in a buffer containing 25 mM imidazole and 1 mM EDTA at pH 7.5. The suspension was incubated at 35°C for 30 min. Proteolysis was stopped by the addition of trypsin inhibitor at fivefold weight excess over trypsin. The mixture was transferred to ice for 10 min and centrifuged in a Beckman Airfuge (Beckman Instruments, Inc., Palo Alto, CA) at full speed for 10 min, and the pellet was resuspended in imidazole/EDTA buffer. The extent of the proteolysis was monitored by SDS PAGE.

Crystallization: The formation of crystalline sheets was induced by a variety of inorganic salts, namely $NaVO_3$, Na_3VO_4, K_2HPO_4, and K_3CrO_4. The crystallization buffer used was 10 mM Tris, 1 mM $MgCl_2$, 10 mM KCl, and one of the inducing salts listed above, with pH adjusted to 7.5 with HCl. The concentration of $NaVO_3$, Na_3VO_4, and K_3CrO_4 was 0.25 or 0.5 mM and that of K_2HPO_4, 5 or 10 mM. The enzyme was resuspended in this solution at a protein concentration of 1.0–1.5 mg/ml. Bacterial growth was inhibited either by the addition of 0.02–0.1% NaN_3 to the buffer, or by filtering it through a 0.22-μm Millipore filter (Millipore Corp., Bedford, MA), and using sterile conditions for the crystallization. The suspension was held at 4°C, and the progress of crystallization was followed by electron microscopy.

Electron Microscopy: Specimens for microscopy were deposited onto ultrathin carbon support films rendered hydrophilic by glow discharge at low pressure in air. Negative staining was with aqueous uranyl formate (0.75%) and uranyl acetate (1.0%) following standard procedures (1). Freeze-drying and heavy metal shadowing with platinum-carbon or tungsten-tantalum at an elevation angle of 30° was performed as described by Smith (22) and Fowler and Aebi (8). Micrographs were recorded on 4489 film (Kodak) in a JEOL 1200EX or a Philips 301 electron microscope at nominal magnifications of × 75,000 and × 71,000, respectively. Microscope magnification was calibrated internally using the 4.1-nm striations of T-even phage tails (19) and the 8.6-nm pseudo-repeat of catalase crystals (23) prepared on the same grid with the ATPase crystals.

Image Processing: Image processing and image filtration employed procedures essentially the same as those described by Aebi et al. (1). Micrographs were screened by optical diffraction, and those showing the highest resolution were selected for computer processing. Images were digitized on an Optronics Photoscan (P1000) drum scanner on a 25-μm raster, corresponding to a distance of ~0.4 nm on the specimen. The micrographs were aligned so that they were scanned parallel on the *b* axis of the crystalline sheet. Areas of at least 150 unit cells were averaged in the process of generating a filtration. Filtered images were written onto film using an Optronics Photomation (P1700).

RESULTS

Enzyme Purification

The enzyme was purified from the dissected outer medulla of dog and pig kidneys as described in Materials and Methods. The purity of the enzyme was checked by SDS PAGE (Fig. 1 *a*) and by enzyme activity assays. The measured activity of the enzyme was 1,100–1,400 (μmol P_i/mg protein per hour) from pig and 1,300–1,600 (μmol P_i/mg protein per hour) from dog. The SDS PAGE and activity results indicated that the preparations were 70–85% pure.

Micrographs of negatively stained preparations of the purified enzyme (Fig. 2 *a*) show large (up to 600 nm diam) intact or broken vesicles, bearing an abundance of particles uniformly distributed over the membrane. This enzyme preparation was used directly either for crystallization experiments or for the tryptic digestion experiments described below.

Partial Proteolysis of the Enzyme

Partial proteolysis of the enzyme was carried out as described in Materials and Methods. SDS PAGE of the cleaved enzyme (Fig. 1 *b*) shows that up to 90% of the alpha subunit has been digested. The most prominent proteolytic fragment appears as a band at 77,000 dalton. The other bands are due to the further fragmentation of the alpha subunit or its 77,000-dalton piece. There is no apparent digestion of the beta subunit.

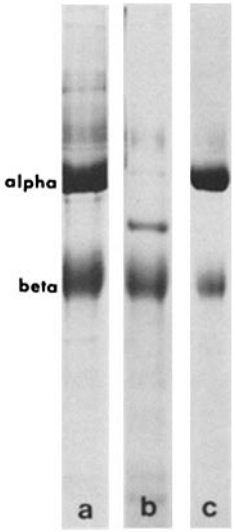

FIGURE 1 SDS polyacrylamide gel of (Na^+,K^+)-ATPase. (*a*) pure ATPase; (*b*) trypsin-digested ATPase; (*c*) crystallized ATPase. SDS PAGE was on a 7.5–12.0% gradient slab gel and staining was with Coomassie Blue.

Crystallization of the Enzyme

Micrographs of the purified enzyme (Fig. 2 *a*) show membrane patches covered uniformly with particles, presumably the ATPase molecules. After initiation of crystallization by the addition of an inducing salt, these particles cluster to form small arrays. An example is shown in Fig. 2 *b* where a small patch of a dimeric crystal is forming. Clustering into small patches of monomeric crystals is also observed. The clusters continue to grow and reorganize to form crystalline sheets (Fig. 2 *c*). SDS PAGE of the crystallized enzyme (Fig. 1 *c*) shows a pattern identical to that of the uncrystallized material (Fig. 1 *a*). The average crystalline sheet has an area of 200 × 300 nm. Since the crystalline patches are formed as a result of the ordering of the enzyme molecules in the membrane sheets, their size is limited by the number of particles in the membrane fragments obtained during purification. Regular arrays with identical crystal forms and lattice parameters are formed in enzyme obtained from both dog and pig kidneys. The crystallization procedure routinely generates ordered arrays in >90% of the membrane fragments when $NaVO_3$ (meta) or Na_3VO_4 (ortho) is used. The former had been employed previously (21) where it induced crystals over a period of 4–6 wk. The crystallization time can be reduced to 2–6 d by using Na_3VO_4 (ortho) (18). Crystallization took considerably longer (>7 wk in some cases) with K_2HPO_4 and K_3CrO_4, and was neither as reproducible nor as extensive as with the vanadate salts. Experimentation with a range of pH of the crystallization buffer showed that crystal formation was facilitated in the pH range 6.8–7.2, relative to pH 7.5 used for enzyme purification.

There was considerable variation in the stability of the crystals generated from preparation to preparation, even when apparently identical crystallization conditions were used. In some cases crystals remained ordered for periods of two and a half months at 4°C, whereas in other cases disorder began to occur in the arrays in as little as a week after full crystallization was achieved.

Crystal Forms of the Enzyme

Two types of crystals of the purified enzyme were induced by the meta- and ortho-vanadate. One form has an alpha-beta monomer (Fig. 3 *a*) and the other has an $(alpha\text{-}beta)_2$ dimer (Fig. 3 *b*) in the unit cell. The monomeric form is most

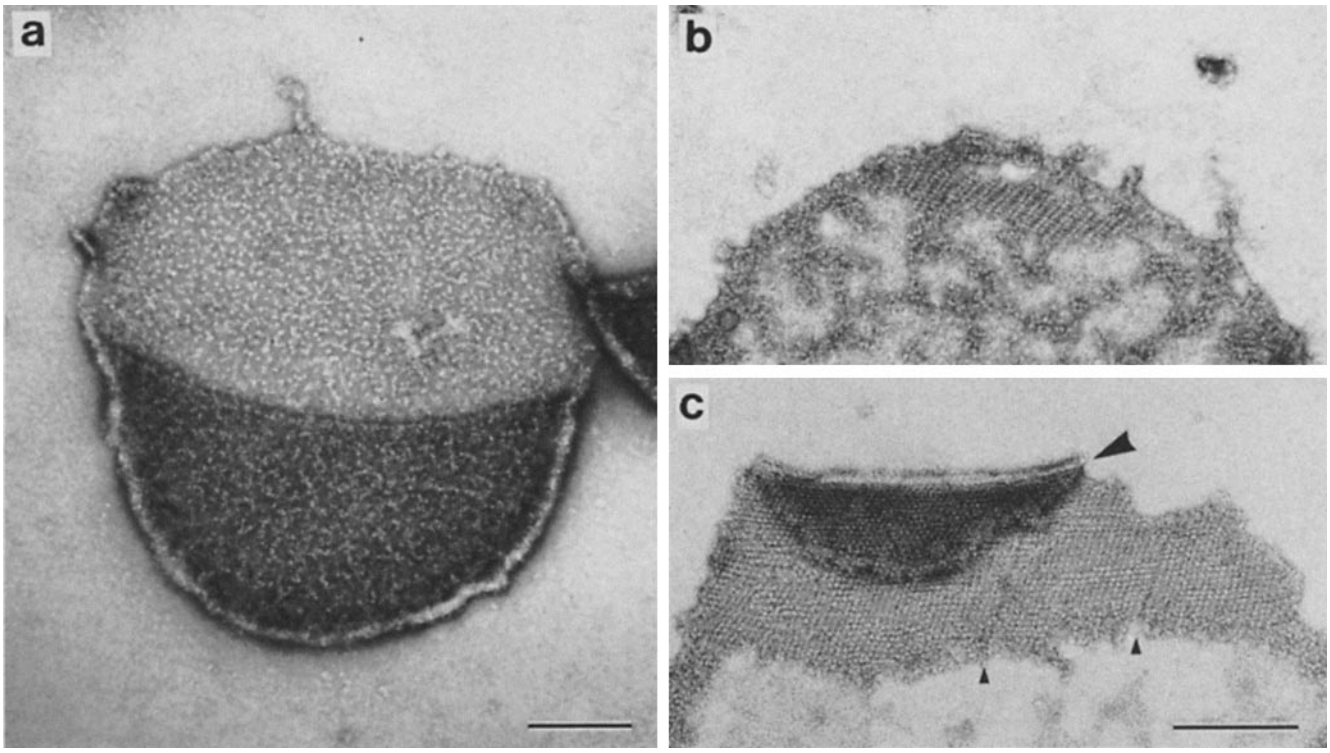

FIGURE 2 (a) Membrane fragments containing pure ATPase. (b) Early in the crystallization (4 d) induced by sodium orthovanadate, when clusters of particles and small dimeric arrays can be seen. (c) Two-dimensional crystals of the enzyme: the large arrowhead points to a segment of the sheet that has folded over. The small arrowheads indicate "faults" in the crystalline array. Bars, 100 nm.

commonly observed. While both vanadate salts can induce two-dimensional arrays in the preparations of the digested enzyme, so far the only crystalline form observed has been monomeric.

The unit cell dimensions of the crystalline sheets were calculated from the reciprocal lattice measurements on the optical diffraction patterns (Fig. 3, lower panels) recorded from their micrographs. Individual examples of all crystal forms showed diffraction spots to a maximum resolution of ~2.0 nm, but reproducible structural information could only be obtained at a resolution of 2.7 nm. The lattice constants are: $a = 5.9 \pm 2$ nm, $b = 5.6 \pm 2$ nm, $\gamma = 70°$ (monomeric); $a = 16.1 \pm 3$ nm, $b = 5.5 \pm 3$ nm, $\gamma = 66°$ (dimeric); and $a = 5.6 \pm 3$ nm, $b = 4.3 \pm 3$ nm, $\gamma = 66°$ (digested). The values represent averages over 30 micrographs for the monomeric form, and 10 for the dimeric and digested forms. It should be noted that while the dimension of the unit cell of the digested form is only 5% smaller than that of the monomeric form in the a direction, it is 25% smaller in the b lattice direction.

Freeze-drying

Freeze-dried and shadowed preparations (Fig. 4) allow visualization of the two surfaces of the crystalline sheets of the enzyme. The two surfaces show different morphologies; one surface has regular striations, the other appears smooth (Fig. 4*a*). Discernment of the regular structure in the freeze-dried and shadowed preparations of ATPase sheets proved to be more difficult than in other specimens studied by this method (e.g., references 8 and 22). Using tungsten-tantalum as the shadowing material, in the best examples (e.g., Fig. 4*b*) structures similar to those seen in negative stain could be observed.

Image Processing

The computer-filtered images of the monomeric and the dimeric crystal forms (Fig. 5, *a* and *b*) reveal essentially the same outer envelope for the molecule. The image of the molecule shows an asymmetrical mass distribution with two major structural features; a massive "body" with a less massive "hook" protruding from it. The back of the body, which is the side opposite to the hook, appears somewhat flattened. In the monomeric form the hooks point in the same direction, but in the dimeric form the molecules are paired hook to hook, with a twofold axis of symmetry between them. The flattened backs leave a narrow but distinctive stain-filled trough between each pair which contrasts with the broader trough through the zone occupied by the paired hooks. The alternating broad and narrow troughs are a prominent feature of the dimer sheet. Similar troughs are frequently observed as "faults" in large crystalline patches of the monomeric form where they may demarcate zones of crystal "twinning."

The digested molecule (Fig. 5*c*) shows similar structure to that of the monomeric crystals of the whole enzyme but the body is significantly less massive relative to the hook. Furthermore, the lattice constant is smaller in the b direction which is the direction in which body-to-body contacts are made.

DISCUSSION

In this paper we have presented the results of an electron microscopic study of crystalline sheets of (Na^+,K^+)-ATPase. Computer image processing was used to provide an enhanced view of the negatively stained crystals. We generated crystal-

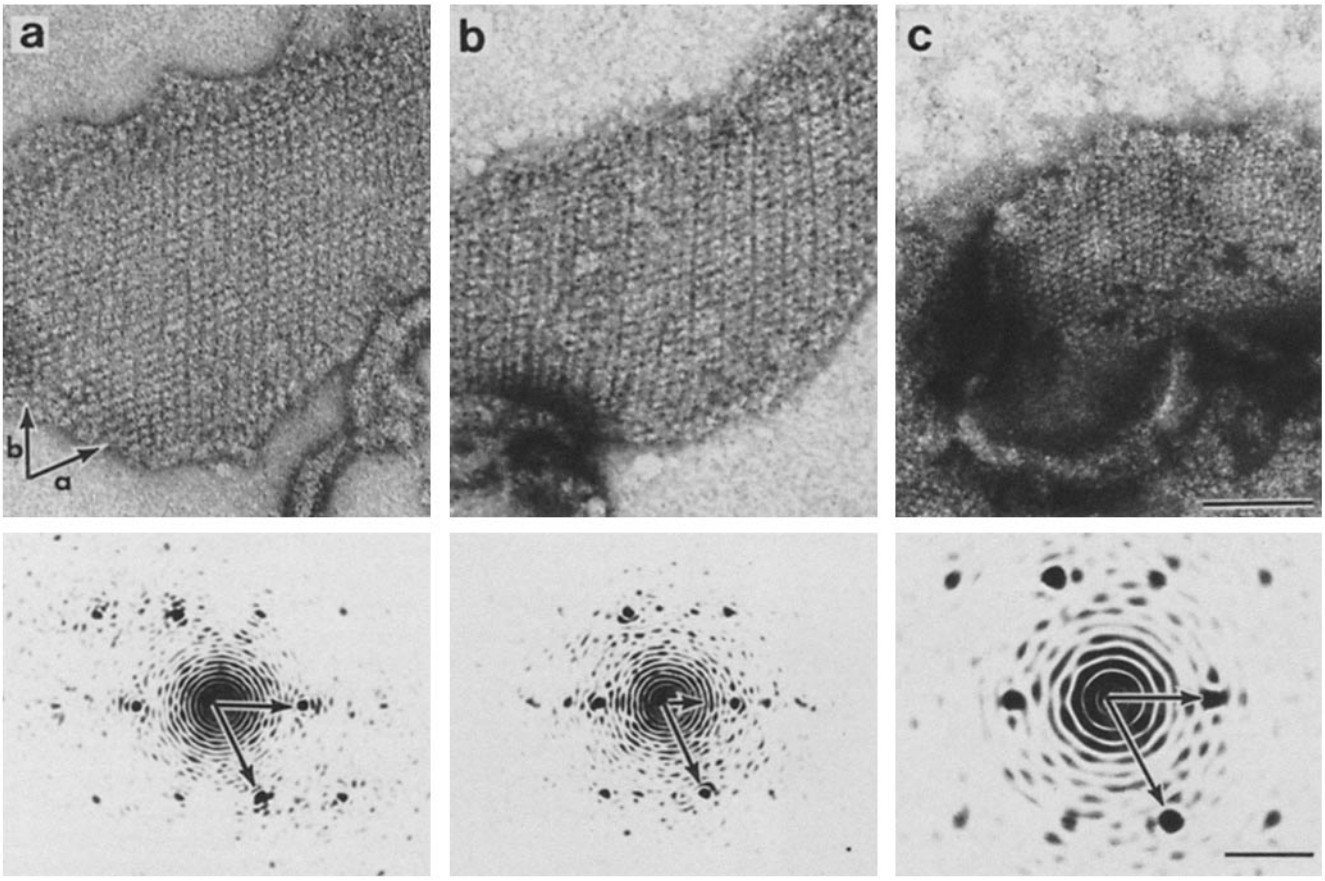

FIGURE 3 Crystalline sheets of ATPase (*upper panels*) with their corresponding optical diffraction patterns (*lower panels*). (*a*) The monomeric crystal form with one (alpha-beta) monomer per unit cell. (*b*) The dimeric crystal form with $(alpha\text{-}beta)_2$ per unit cell. (*c*) The trypsin-digested enzyme. These crystals were induced using sodium meta-vanadate. The arrows on the micrograph indicate the approximate directions of the *a* and *b* lattice vectors for all lattices: γ is the included angle between *a* and *b*. The arrows on the diffraction patterns indicate the directions and approximate lengths of the *a'* and *b'* reciprocal lattice vectors: these are perpendicular to the corresponding real space vectors. Bars: micrographs, 50 nm; diffraction patterns, $(5\ nm)^{-1}$.

line sheets of the whole enzyme using a number of inducing salts and developed conditions for fast and reproducible crystal formation. We observed one monomeric and one dimeric form for the molecular packing of the enzyme and in both cases the same basic subunit shape was observed. This consists of a relatively large body, with a hook extending from it (Fig. 5). In the monomeric form the hooks point in the same direction, but in the dimeric form the subunits are paired hook-to-hook.

Hebert et al. (9) have reported three different forms of the (Na^+,K^+)-ATPase crystals, two monomeric and one dimeric. Their monomer sheets were obtained using meta-vanadate as the inducer and differ in their lattice constants. The dimeric form was obtained with magnesium phosphate. There is excellent correspondence in the structural features of the dimeric form we have obtained (Fig. 5*b*) and the dimeric crystals of Hebert et al. This is particularly interesting since the crystals were formed with the two vanadates in the case we describe, and with phosphate in the case they report. There is good agreement between the lattice parameters of our monomeric form and one of the monomeric crystal forms they describe (Fig. 3*B* in reference 9). However, the reconstruction they present shows subunits with a less structured outline, reflecting the fact that their data is at a lower resolution. Their other monomeric form (Fig. 3*A* in reference 9) has not been observed following the procedures we have employed.

As described above, the tryptic digestion of the (Na^+,K^+)-ATPase produces a membrane-bound fragment that can be crystallized using meta- or ortho-vanadate inducing salts. It has been demonstrated that the proteolysis of (Na^+,K^+)-ATPase by trypsin (6, 13) and chymotrypsin (6) in the presence of various ligands results in different fragmentation patterns of the catalytic subunit. Subsequent extraction of the digested ATPase with alkali showed that in most cases all the major proteolytic fragments could be recovered, suggesting that these fragments were embedded in the membrane (6). The limited proteolysis of (Na^+,K^+)-ATPase by trypsin described here results predominantly in the cleavage of the catalytic subunit into a membrane-bound 77,000-dalton piece which has been shown to retain the ATP hydrolysis site, with no apparent effect on the beta subunit: SDS PAGE of our digested preparations (Fig. 1*b*) is in good agreement with those reported by Castro and Farley (6). The results presented here from image analysis of the digested enzyme support the earlier contention that a substantial mass of the enzyme remains associated with the membrane after fragmentation. Both comparison of the images of the intact molecule with those of the digested form (Fig. 5) and the difference in lattice constants for the two forms suggest that only a relatively small fraction of the molecule has been removed by proteolysis.

The digested enzyme viewed in projection shows a charac-

teristic asymmetrical mass distribution (Fig. 5*c*) which corresponds for the most part with the mass distribution of the whole molecule (Fig. 5, *a* and *b*). However, in the digested form the body region is relatively less massive than that of the intact molecule, and the lattice is compressed in the direction of the body-to-body contacts. Although we do not yet have three-dimensional structural information, we can reasonably infer that the mass lost by partial proteolysis has been removed from the body region of the whole enzyme, given the marked change in the lattice dimensions and the shape of the molecule. Since mass is only lost from the alpha subunit by proteolysis, this permits the identification of the body of the monomer with the alpha subunit of the molecule.

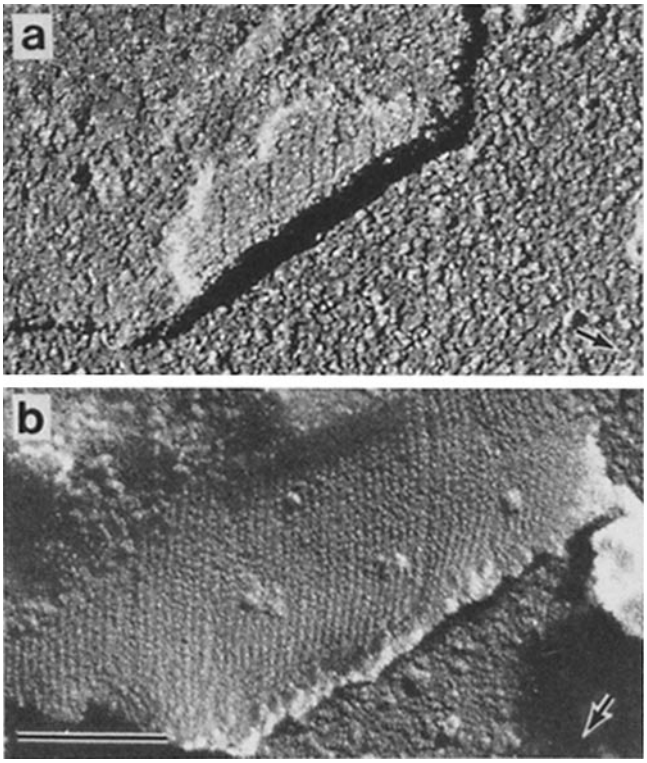

FIGURE 4 Freeze-dried and shadowed crystals. *a* shows a region of the crystalline sheet that has folded over, indicating that one side of the crystalline sheet has regular striations and the other is relatively smooth: shadowing with platinum-carbon. *b* presents an area of tungten-tantalum–shadowed crystal which allows visualization of higher resolution structure. The handedness of the lattice relative to the negatively stained crystals has yet to be determined. The arrows indicate the direction of metal deposit: metal is light; absence of metal is dark. Bar, 100 nm.

The preliminary freeze-drying and shadowing results depict the different morphologies of the two surfaces (Fig. 4*a*) and the crystalline appearance of one of them (Fig. 4*b*). These results are consistent with the biochemical findings that indicate that the molecule protrudes extensively on the cytoplasmic side of the membrane, and less so on the exoplasmic surface (14), suggesting that the two surfaces should indeed possess different structures. Studies currently under way in this laboratory aim to identify the two surfaces of the membrane using freeze-drying and metal shadowing of crystals labeled with lectin.

It is interesting to consider the role of vanadate as an inhibitor of the enzyme and as an inducer of its crystallization. Vanadate inhibits not only a number of ATPases but also several other enzymes involved in the transfer or release of phosphate (3, 20). Since ortho-vanadate and chromate [a milder inhibitor of (Na^+,K^+)-ATPase] ions have a structure and charge analogous to those of the phosphate ion, it is reasonable to assume that their inhibition of activity occurs as the result of a reaction at the phosphate binding site, and that this replacement would not cause more than a minimal conformational change in the enzyme. The two-dimensional arrays of (Na^+,K^+)-ATPase induced by vanadate, phosphate, and chromate ions all have similar structures. This similarity is particularly striking in the case of the dimeric crystals induced with vanadate described above, and those induced with phosphate (9), implying that crystal formation by these ions is induced via analogous mechanisms. In one possible mechanism, the ions could bind to the enzyme molecules and lock them into the "phosphorylated" conformational state that would facilitate their subsequent assembly into crystalline arrays. Alternatively, these inorganic ions may participate directly in the formation of specific interactions between molecules. Irrespective of the details of the mechanism, crystal formation by these ions must somehow involve a reaction at the phosphorylation site, suggesting that the enzyme in the crystal has the same structure as the phosphorylated form, which under the crystallization conditions is in the E_2-p conformation (4).

There is indirect evidence in support of these hypotheses; the partially proteolysed enzyme, which has been shown to

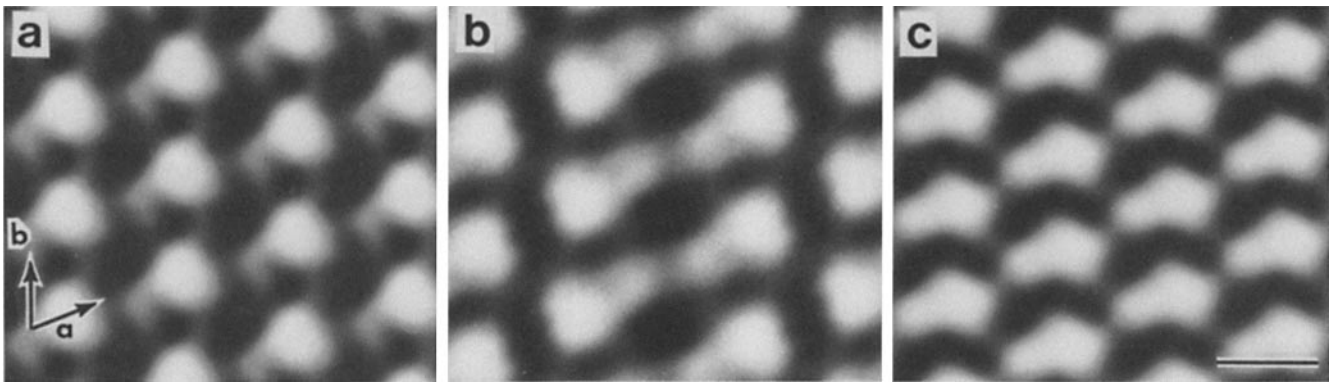

FIGURE 5 Computer-filtered images of the enzyme corresponding to the crystalline sheets shown in Fig. 3. *a* shows the monomeric, *b* the dimeric, and *c* the digested form. The image in *b* has been twofold symmetrized which caused a 2% loss of dynamic power (1). The arrows indicate the approximate directions of the *a* and *b* axes for all lattices. Bar, 5 nm.

retain the phosphorylation site (6), can be crystallized using the vanadate ion as an inducer. Furthermore, the calcium-ATPase, which has considerable structural similarity to the (Na^+,K^+)-ATPase, also forms crystals with both vanadate (2, 7) (rabbit skeletal muscle sarcoplasmic reticulum) and phosphate (5) (scallop muscle sarcoplasmic reticulum), and the two crystals show similar molecular structure. In addition, attempts to induce crystallization of (Na^+,K^+)-ATPase with ouabain, which inhibits ATPase activity by binding to the exoplasmic side of the membrane without involving the phosphorylation site, have been unsuccessful so far, indicating that crystallization is not a simple consequence of enzyme inhibition.

A comparison of the structure we observe for (Na^+,K^+)-ATPase and the structure of the calcium-ATPase is particularly instructive. The calcium-ATPase is an ion pump with a similar function to the (Na^+,K^+)-ATPase in that it uses energy from ATP hydrolysis to actively transport calcium and hydrogen ions in opposite directions through a membrane. It consists of a single subunit of M_r = 106,000 with structural similarities to the alpha subunit of the (Na^+,K^+)-ATPase (4). Calcium-ATPase forms phosphate- (5) and vanadate-induced (2) tubular structures with a crystalline surface lattice possessing a dimer in the unit cell. While the two different ATPases form dimeric crystals, there are significant differences in their structure. The *a* lattice dimension of the (Na^+,K^+)-ATPase is 40% longer than that of the calcium-ATPase which parallels its 40% higher molecular weight, owing to the presence of the beta subunit. The comparison of the filtered images of the two ATPases shows that in (Na^+,K^+)-ATPase the larger lattice dimension accommodates the hook structures which the calcium-ATPase lacks (2, 5). Thus, the hook structure could be attributed to the beta subunit of the enzyme, leading to a model for the (Na^+,K^+)-ATPase in which the body corresponds to the alpha subunit (from the digestion results) and the hook region to the beta subunit.

Our results have allowed us to draw tentative conclusions concerning the structure of the (Na^+,K^+)-ATPase, and the location of one of its subunits within the mass distribution demarcated by negative stain. Progress in the understanding of the enzyme from a structural point of view must come from higher resolution microscopical data, and also from three-dimensional reconstructions of the mass distribution from tilted views of the crystalline sheet. Both approaches are being employed in this laboratory. Additionally, the similarity between the calcium-ATPase and the (Na^+,K^+)-ATPase is likely to permit important insights into the workings of both enzymes, and possibly other transport systems as well.

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