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Two-dimensional crystals of membrane-bound gastric H,K-ATPase

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Two-dimensional crystallization of membrane-bound H,K-ATPase (EC 3.6.1.36) in vesicle preparations from parietal cells of hog gastric mucosa was induced by an imidazole buffer containing Mg^{2+} and VO_3^- ions. A continuous reorganizaiton of the protein molecules started within a few hours by the formation of linear arrays. At later stages confluent two-dimensional crystals were formed. Electron microscopy and image processing showed that these were of a single tetragonal type. The asymmetric unit consisted of one pear-shaped protein domain corresponding to a H,K-ATPase protomer. Through stain-deficient contact regions four adjacent protein units were connected forming a tetrameric structure.

Gastric mucosa; Biologically active transport; Hydrogen potassium adenosine triphosphate; Crystallization; Electron microscopy; Computer assisted image processing

1. INTRODUCTION

The parietal cells of the gastric exocrine glands are responsible for the secretion of hydrochloric acid into the gastric lumen. These cells undergo morphological changes upon hormonal stimulation whereby intracellular vesicles fuse with the plasma membrane. The vesicle membranes contain a proton translocating enzyme, K^+-stimulated adenosine triphosphatase or H,K-ATPase (EC 3.6.1.36) [1] which can be isolated either from the apical plasma membranes of stimulated cells or from the tubulovesicular membranes of resting cells. This enzyme is an integral membrane-bound protein and belongs to a family of E_1/E_2 transport ATPases. Other members of this group are Na,K-ATPase and Ca^{2+}-ATPase. Like the Na,K-pump, H,K-ATPase has two subunits; one large catalytic α-subunit with a molecular mass of about 94 kDa and a smaller glycosylated β-subunit. The amino acid sequence of the α-subunit has been determined from rat [2], pig [3] and human [4]. The β-subunit from rat, rabbit, pig and human has also been cloned recently [5–9]. During one pump cycle the enzyme alternates between specific conformations. The major conformational states are the E_1 and the E_2 forms with high affinity for H^+ and K^+ ions, respectively. Furthermore, it is known that specific ligands can be used to stabilize some of these conformations. For instance vanadate tends to form a stable E_2 form of the enzyme.

Stabilizing a protein is important for crystallization experiments since a rigid molecule may form periodic arrays more readily than a flexible one. Thus it was shown that vanadate could be used to form crystalline arrays of membrane-bound Na,K-ATPase [10]. This has enabled structural studies of the Na,K-pump utilizing electron microscopy and image processing, for a review see Maunsbach et al. [11]. In the present work we have used a similar method to obtain two-dimensional membrane crystals of H,K-ATPase. The successive formation of such arrays is described and a projection structure for H,K-ATPase is presented. A comparison is made to previous reports concerning crystalline arrays found in H,K-ATPase-containing vesicle preparations [12,13].

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2. MATERIALS AND METHODS

Vesicular membranes from pig gastric mucosa containing H,K-ATPase were prepared essentially as described previously [14]. The corpus mucosa from pig stomachs was flooded with a saturated NaCl solution for 3–5 min, and the mucusa was wiped off. Scrapings of the mucosa were homogenized in 0.25 M sucrose containing 1 mM ethyleneglycol tetraacetic acid (EGTA). The homogenate was centrifuged at 20,000 × *g* for 40 min. The supernatant was then centrifuged at 75,000 × *g* for 1.5 h, and the resulting microsomal pellet was resuspended in sucrose-EGTA and layered on top of a 0.25 M sucrose plus 7.5% (w/v) Ficoll cushion and centrifuged at 75,000 × *g* for 1 h. H,K-ATPase-enriched vesicular membranes were obtained from the top of the sucrose–Ficoll cushion. Loosely attached contaminating proteins were released by washing once in 0.13% (w/v) *n*-octylglucoside. Coomassie staining after gel electrophoresis was performed and showed that H,K-ATPase constitutes the major protein in this preparation [14]. When the specimen was treated with glycosidase the β-subunit appeared as a Coomassie-stainable 32 kDa protein after gel electrophoresis.

The purified H,K-ATPase preparations, 13 mg/ml protein in 0.25 mM sucrose and 0.5 mM EGTA, were incubated with 10 mM $Mg(NO_3)_2$, 0.5 mM NH_4VO_3, 50 mM imidazole, pH 6.8, 7.0 or 7.2 at

a protein concentration of 0.1 mg/ml for 1–7 days at +2°C. In some experiments 100 mM KNO_3 was also included in the crystallization solution. Controls were made by adding merely 50 mM imidazole to the H,K-ATPase preparations. Aliquots (5 μl) were collected and applied on glow-discharged, carbon-coated grids. Blotting with a piece of filter paper was made after 1 min followed by a rinse and simultaneous staining on a drop of 1% Uranyl acetate placed on a teflon plate.

The specimens were examined in a Philips EM420 or an EM400 electron microscope at 50,000× calibrated magnification and under controlled dose conditions. The quality of the micrographs were judged by optical diffraction and crystalline areas with little astigmatism and drift and underfocused such that the first zero of the contrast transfer function was at frequencies higher than $1/20 Å^{-1}$ were selected for processing. An Eikonix 1412 equipped with a Rodenstock fixed focus objective was used to digitize the micrographs with a pixel size of 20.1 μm corresponding to 4.0 Å on the specimen level. Projection maps were obtained by correlation averaging essentially as described by Saxton and Baumeister [15] using the EM-system [16] linked to the SunVision program package running on a Sun SPARCstation 2.

3. RESULTS

Vesicle preparations in sucrose after incubation in imidazole buffer showed a distribution of membrane fragments in negative stain ranging in size from 1 μm in diameter and less (Fig. 1). Most of them were densely stained with a lighter rim. Within the fragments randomly distributed particles were observed. Specimens kept in imidazole for up to 7 days did not change in morphology with time.

A few hours after incubating the specimens with the crystallization solution containing $Mg(NO_3)_2$ and NH_4VO_3 characteristic arrays of particles were observed (Fig. 2). These can be described as randomly oriented flexible 'beads on a string' structures. The density of these strings increased with time. However, after about 1–2 days instead domains with periodically arranged particles were observed (Fig. 3). Also the overall morphology of the membrane fragments changed and they became disrupted. Two types were of particular interest since they were characteristic, frequent and almost always contained crystalline arrays of particles. One type had a round, stain-deficient part from which the protein dense domains extended (Fig. 3a). The other frequently observed subtype of membrane domains consisted of a disrupted perimeter with an 'opening' (Fig. 3b). With few exceptions these membranes contained an enriched protein density towards the opening forming a crystalline array. These two types of membrane fragments could be identified at low magnification thus simplifying the use of low dose methods. Crystalline arrays were also observed in membrane fragments with other less-defined morphologies but these were more rare. The addition of KNO_3 in the crystallization medium did not influence the crystallization significantly and pH 7.0 was found to give the highest density of crystalline arrays.

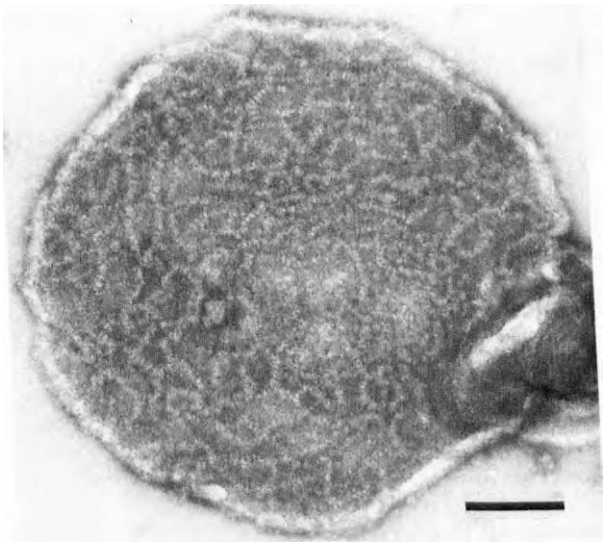

Fig. 2. Linear arrays of particles found in vesicle preparations incubated in 50 mM imidazole, 10 mM $Mg(NO_3)_2$, 0.5 mM NH_4VO_3, pH 7.0, for 1 day. Bar = 100 nm.

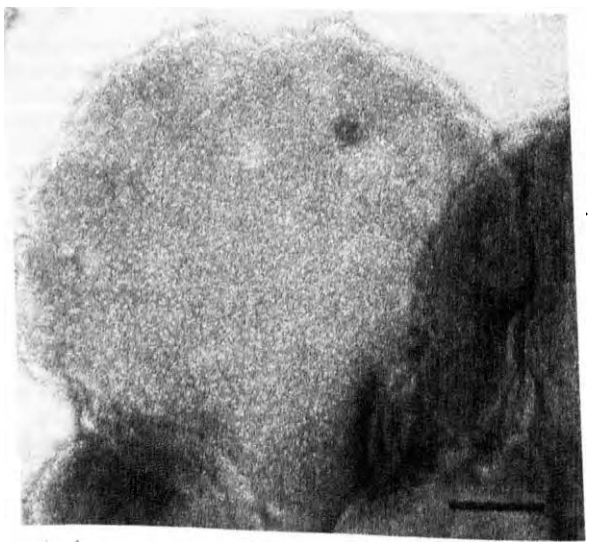

Fig. 1. An intact vesicle from a parietal cell left in 50 mM imidazole, pH 7.0, for 3 days. Bar = 100 nm.

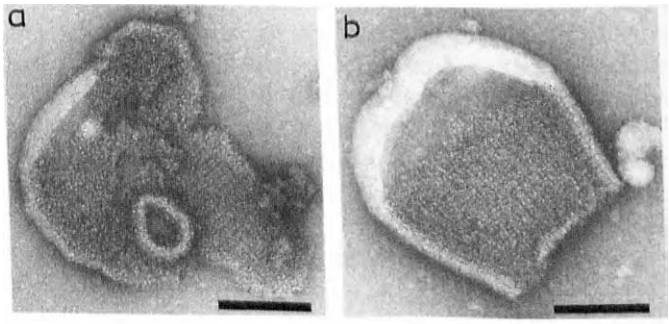

Fig. 3. Two-dimensional crystalline arrays of H,K-ATPase found in preparations incubated with 50 mM imidazole, 100 mM KNO_3, 10 mM $Mg(NO_3)_2$, NH_4VO_3, pH 7.0, for (a) 4 days and (b) 1 day. Bar = 100 nm.

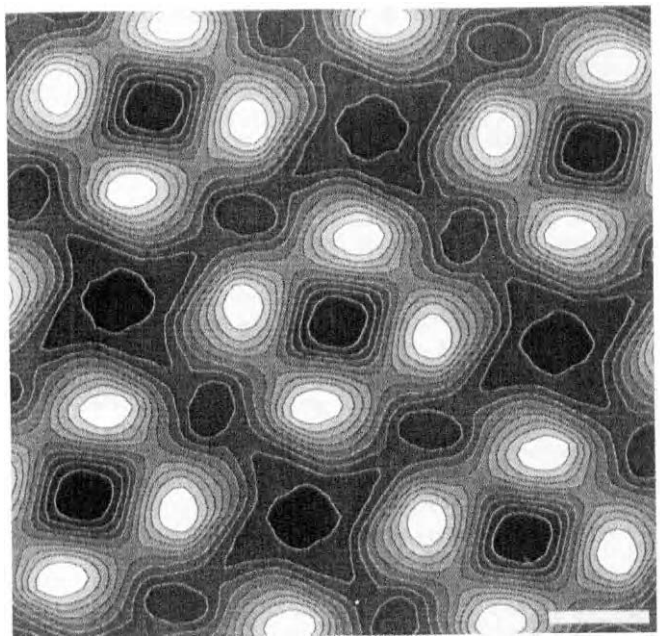

Fig. 4. Correlation averaged projection map of H,K-ATPase in tetragonal two-dimensional crystals, White represents high protein density. Bar = 40 Å.

The two-dimensional membrane crystals observed in the present work are all of the same type with p4 projection symmetry. The average cell edge is 115.1 Å (n=4). The variation is less than for vanadate-induced crystals of Na,K-ATPase, which in addition forms two different types of crystals with this ligand [17]. The unit cell contains four slightly pear-shaped asymmetric protein units (Fig. 4). The size of one of these is approximately 50 × 30 Å^2. The four- and two-fold axes of the structure are positioned at locations with high stain density. Protein–protein interactions are present along the directions of the unit cell and a relatively compact structure is formed by the four protein domains.

4. DISCUSSION

In the present work we have demonstrated crystal formation in tubulovesicle membranes from parietal cells containing purified H,K-ATPase following incubation in an imidazole buffer with Mg^{2+} and VO_3^- ions. Control experiments with imidazole alone were completely devoid of aggregated protein. During crystallization a continuous rearrangement of protein particles from a random distribution to a periodic arrangement could be observed with bent linear polymers as an intermediate step. The originally intact vesicle membranes became more disrupted as the incubation went on. This was probably coupled to local changes of the lipid/protein ratio. Fragments with typical morphologies (Fig. 3a and b) often showed crystalline arrays that presumably contained a relatively low amount of lipid.

A previous report on H,K-ATPase [12] showed crystalline areas with a wide distribution in organization and structure. In contrast to the present work crystallization was not easily reproducible from preparation to preparation. When studying membrane vesicles from hog stomachs, Mohraz et al. [13] found crystals with tetragonal symmetry interpreted as H,K-ATPase. Later these were shown to be identical to the regular surface layer of the Gram-negative bacteria *Comamonas acidovorans* as determined by Engelhardt et al. [18,19].

Although several proteins are associated with the tubulovesicular membranes H,K-ATPase is by far the most abundant and it is not likely that the crystalline domains are formed by any of the minor components. Moreover, vanadate, which was crucial to the formation of these arrays, is known to be a specific ligand to the enzyme stabilizing the E_2 conformation. This ligand has also been empolyed in inducing cyrstal formation of membrane-bound Na,K-ATPase [10] and Ca^{2+}-ATPase from the sarcoplasmatic reticulum [20]. A similar behaviour is not surprising since these enzymes all belong to a family of E_1/E_2 transport ATPases. In fact the catalytic subunits of Na,K-ATPase and H,K-ATPase show sequence homoloy and similarities in hydropathy plots based on the amino acid sequences determined from cDNA [2,21]. Sequence identities are particularly found around the phosphorylation site in the region between transmembrane domains two and three and in a part preceeding transmembrane fragment five which has been proposed to be part of the ATP-binding site [22]. Lane et al. [23] found that antibodies raised against the α-subunit of lamb kidney Na,K-ATPase cross-reacted with the catalytic subunit of pig gastric H,K-ATPase. The information accumulated concerning the 2D- and 3D structures of Na,K-ATPase [11] allows a structural comparison also of the low resolution tertiary structure.

As for H,K-ATPase linear arrays were frequently observed after treatment of Na,K-ATPase with the E_2 stabilizing ligand vanadate, in particular at early stages of crystallization [24]. However, the Na,K-ATPase molecules formed polymers resembling ladders with unique building blocks consisting of pairs of protein particles in a symmetrical arrangement. In the case of H,K-ATPase the linear arrays resemble more a 'beads on a string' structure suggesting that single H,K-ATPase molecules are the building blocks. For Na,K-ATPase, two major types of crystals with p1 and p21 symmetry were observed after vanadate treatment [17]. Subtypes could also be defined [25]. In the present work we have only observed one type of crystal with projection symmetry p4. Two-dimensional crystals with this symmetry have also been observed for Na,K-ATPase but after treatment with $Co(NH_3)_4ATP$, an ATP analogue which binds irreversibly to the enzyme and stabilizes a phosphorylated E_2 conformation [26]. However, there is a significant difference between that structure and the

H,K-ATPase crystals analyzed here. For Na,K-ATPase, each of the two-fold axes of the unit cell, positioned where the protein density is high, relates two ($\alpha\beta$)-protomers into a dimeric building block. The H,K-ATPase structure has the two-fold symmetries located in stain-rich domains and no dimeric arangement is formed (Fig. 4).

The unit cell dimension of the $Co(NH_3)_4ATP$-induced crystals of Na,K-ATPase was 141 Å [26] as compared to 115 Å for H,K-ATPase. This could reflect a lower lipid/ protein ratio in the crystalline regions of the membranes from the parietal cell vesicles resulting in a more compact packing of the protein molecules. The projection structure in most of the Na,K-ATPase studies shows a pear-shaped asymmetric protein domain. From 3D analysis this has been shown to correspond to an ($\alpha\beta$)-protomer of the enzyme. Since the size and form of the asymmetric stain-deficient domain in the H,K-ATPase crystals is very similar to that of Na,K-ATPase it is likely that this corresponds to a protomeric unit of the enzyme. In the projection structure the contact regions between adjacent protomers are relatively protein-dense indicating strong protein-protein interactions and the formation of tetramers. Interestingly Soumarmon et al. [27,28] determined an approximate molecular weight of 390–420 kDa by gel filtration and glycerol gradient centrifugation from an active octylglucoside-solubilized enzyme preparation. These studies and radiation inactivation analyses of Schrijen et al. [29] have suggested that the active enzyme is a tetramer.

Among different methods that have been tried for two-dimensional crystallization of membrane-bound proteins the approach in this study can be classified as reorganization and stabilization in intact membranes by specific ligands. In general this method yields relatively small crystalline domains. The size of the membrane fragments sets an ultimate limit. Moreover, lattice defects are often present. One approach to obtain large and well-ordered two-dimensional membrane crystals, which we are currently investigating, is to solubilize and reconstitute the protein into lipid vesicles.

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