Coexistence of different forms of Na,K-ATPase in two-dimensional membrane crystals

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Two-dimensional membrane crystals of renal Na,K-ATPase were analyzed by electron microscopy and image processing. The particular property of the crystals in this work was that they showed unit cell parameters similar to the previously studied p21 crystals but lacked the dyad axis as observed in nominal 0°-projections. A three-dimensional reconstruction revealed that structural differences between αβ-units of the enzyme gave rise to the asymmetry. A high degree of two-fold rotational symmetry was observed in the middle of the structure while the protein units had different three-dimensional shapes at levels above and below the central sections. The simultaneous coexistence of different forms of Na,K-ATPase suggests that the conformational flexibility of the enzyme plays an important role in the pumping process.

Na,K-ATPase; Two-dimensional crystal; Three-dimensional structure; Electron microscopy; Image processing

1. INTRODUCTION

Na,K-ATPase and related transport ATPases function through transitions between recognizable conformational states. This flexibility is reflected in the presence of two-dimensional membrane crystals of Na,K-ATPase with different symmetries and unit cell parameters. Vanadate and magnesium as well as other ligands favouring the E_2 conformation provides crystals with plane group symmetries p1 or p21 [1–3]. The unit cell parameters may vary significantly even within the same specimen batch. Modification of the membranes with phospholipase A_2 results in dimeric crystals [4]. Incubation with an ATP-analogue, $Co(NH_3)_4ATP$, which stabilizes a cobalt-tetramine-phosphoenzyme, gives rise to tetragonal p4 crystals [5].

For the first image analysis performed on two-dimensional crystals of Na,K-ATPase we used Fourier methods on arrays without any significant degree of lattice distortions [2]. The p21 symmetry was assigned with residual phase errors typically between 10° and 20°. Later we applied correlation averaging methods that allowed for some degree of bending of the lattices [6]. Minor deviation from two-fold symmetry was observed but it was not significantly above the resolution level of the data. Recently Beall et al. [7] interpreted the deviation from perfect two-fold symmetry as evidence for simultaneous dissimilarities in protein units. That work was limited to two-dimensional analysis of projections recorded along or at small angles to the pseudo-dyad axis.

Deviation from the two-fold symmetry could arise from an actual dissimilarity between the protein units or by an offset from the nominal tilt angle 0° which corresponds to the direction perpendicular to the membrane. To clarify this uncertainty we have performed a three-dimensional reconstruction of a crystal of Na,K-ATPase showing pronounced asymmetry of the nominal 0° tilt angle.

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2. MATERIALS AND METHODS

Na,K-ATPase was purified in membrane-bound form from outer renal medulla of pig kidney by incubation of a microsomal fraction with SDS and ATP according to Jørgensen [8]. The SDS treated membranes were isolated on a linear (15–40%, w/v) sucrose gradient at 27000 rpm for 3 h using an SW 28 rotor in a Beckman L8-70M ultracentrifuge [9]. The specific activity of the enzyme used in this experiment was 32 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein. Protein was determined with bovine albumin as standard [10]. The enzyme (0.5 mg/ml) mixed with 0.33 μg/ml phospholipase A_2 (Sigma) in 10 mM Tris-HCl, pH 7.5, were dialysed against a 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 1 mM NH_4VO_3 [11].

Electron micrographs were recorded using a Jeol 100CX electron microscope following negative staining with uranyl acetate. Projections were collected within the range of −60° to +60°. Digitization in 512 × 512 pixels of 20 μm^2 were performed with an Eikonix 1412 scanner. The pixel size corresponds to 3.7 Å on the specimen level at the 54000 × calibrated magnification. The projections were analyzed by correlation averaging methods [6,12]. A common origin for the complete set of averages was found by cross-correlation functions calculated between adjacent projections. To exclude the possibility of error propagation in this step controls were made by correlating averaged structures further apart. For some projections at high tilt angles it was difficult to start the correlation averaging process due

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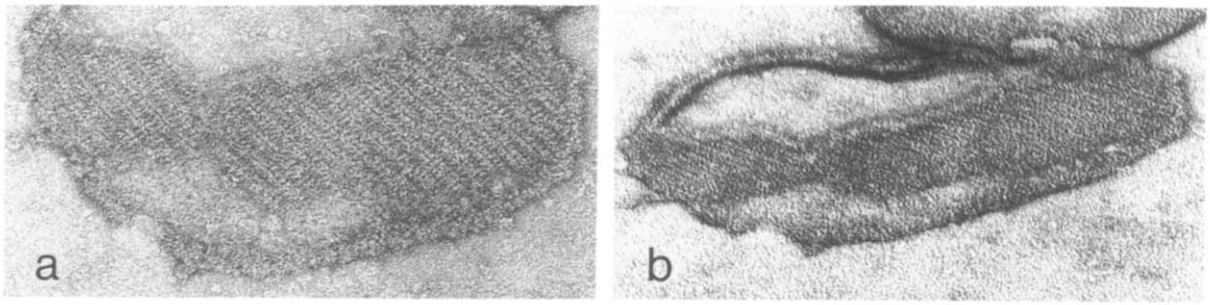

Fig. 1. Electron micrographs of a two-dimensional membrane crystal of Na,K-ATPase recorded at tilt angles (a) 0° and (b) +60°. The crystals were induced by vanadate and magnesium and negatively stained with uranyl acetate. Magnification: 172000 ×.

to a low signal-to-noise ratio in the micrographs. It was then useful to introduce a neighbouring average as a reference after proper resampling. The experimental lattice line data in Fourier space was collected and smooth curves were adapted according to Shaw [13]. Sampling points at 0.0084 $Å^{-1}$ intervals provided the complete 3D data set in Fourier space. The density volume obtained after inverse transformation was displayed as shaded surfaces on a graphics display and plotted as *xy*-sections through the 3D volume. The surface rendering algorithm [14] provides a high degree of flexibility concerning viewing direction, threshold level, definition of light sources, etc.

3. RESULTS

The two-dimensional membrane crystals of Na,K-ATPase obtained in different experiments showed variability in appearance. This was confirmed after optical diffractometer analysis of electron micrographs giving a spread in unit cell dimensions. Correlation averaging of nominal 0°-projections, i.e. projections perpendicular to the plane of the membrane, showed in some cases deviation from two-fold symmetry. Even relatively strong low resolution peaks in the Fourier transform had significant imaginary contributions. The crystalline domain shown in Fig. 1 was selected for a three-dimensional analysis.

Data above the noise level was observed on 32 lattice lines. This corresponds to a resolution of 25 Å in directions parallel to the membrane. No phase restoration was performed since all measurements of the data set within this limit were contributing with the correct phase. Resampling of the digitized areas to compensate for lattice distortions was considered irrelevant since only small deviations were detected when fitting least-squares lattices. As a control nominal 0°-projections were recorded at the beginning, in the middle, and at the end of the series. No significant deterioration of the structure could be observed in the correlation averages calculated from these micrographs.

The unit cell of the nominal 0°-projection of the crystal in Fig. 1 was a = 146 Å, b = 51 Å, γ = 98° (Fig. 2). These values are close to those of type I p21 crystals analyzed earlier [15]. However, the correlation average shows significant deviation from two-fold symmetry due to dissimilarities between protein units (Fig. 2). The relationship between the microscope coordinate system with the *xy*-plane perpendicular to the electron beam and the coordinate system of the crystal with its *xy*-plane parallel to the plane of the membrane was found by a program SNURR [14] with the adapted lattice parameters of the projections as input data. The result showed that the tilt offset of the crystal was 4° from the microscope coordinate system. This small angle demonstrates that the dissimilarities observed in Fig. 2 could not arise from projection offset.

A surface representation of the three-dimensional model is shown in Fig. 3. The height of the model is about 90 Å. The volume of the protein is consistent with assigning two $\alpha\beta$-units of Na,K-ATPase to the unit cell. Sections of the model in *xy*-planes (Fig. 4) show that the centre of the structure has a significant dyad axis (Fig. 4a,c). At increasing distances from the center of the volume this two-fold rotational symmetry is deteriorated (Fig. 4b,d). Moreover, the mass distribution relatively to the central *xy*-section is dif-

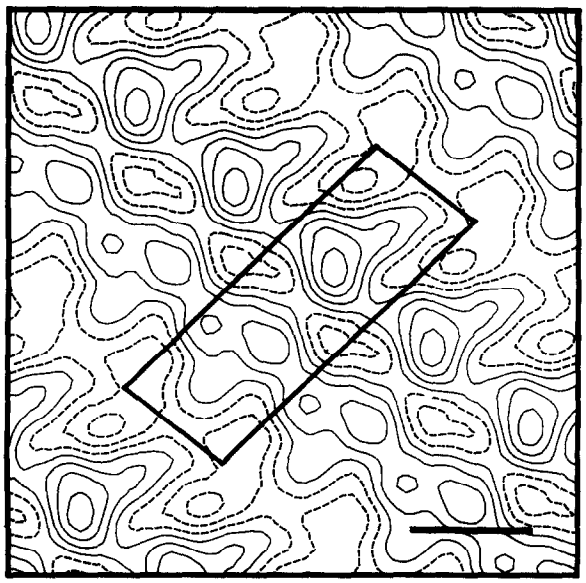

Fig. 2. Correlation-averaged structure of the nominal 0°-projection. The unit cell contains two components with different projection structures. Bar = 50 Å.

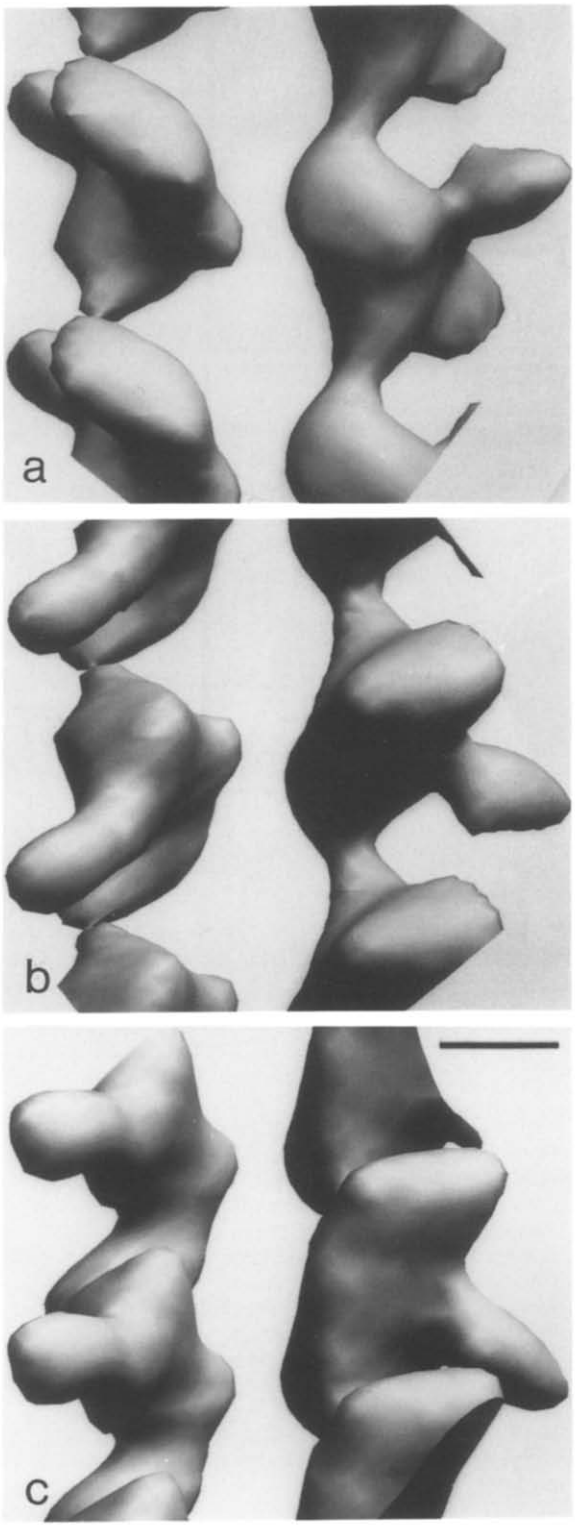

Fig. 3. Three-dimensional surface model of Na,K-ATPase from the crystalline domain shown in Fig. 1. The viewing directions are: (a) perpendicular to the membrane plane in the negative z-direction, (b) perpendicular to the membrane plane in the positive z-direction, (c) tilted 30° as compared to the direction in (b). Bar = 25 Å.

ferent in the two $\alpha\beta$-units although their individual volumes are equal. The protrusion of one of them is more pronounced on the cytoplasmic side, while the other extends more on the extracellular side of the membrane.

The intermolecular contact as delineated by the stain is primarily between units in rows parallel to the b-axis of the crystal. Adjacent rows of $(\alpha\beta)_2$-units are in contact in the center of the structure through the small domains extending from the body of the protein (Fig. 4a).

4. DISCUSSION

Previous three-dimensional structure analyses of membrane-bound Na,K-ATPase have been made from crystals showing two-sided plane group symmetries p1 with one $\alpha\beta$-protomer in the unit cell [16], p21 of two types with unit cells containing $(\alpha\beta)_2$-dimers of the enzyme [17,18] and recently p4 with four $\alpha\beta$-protomers in the unit cell [19]. When rotational symmetry was present $\alpha\beta$-protomers were related by the crystallographic symmetry so that the asymmetric unit always consisted of one $\alpha\beta$-protomer of the enzyme. At the resolution level of these studies and before imposing symmetry it was not possible to detect any significant structural difference between the $\alpha\beta$-protein domains.

In the present work we have demonstrated that a divergence from an expected two-fold symmetry can be attributed to three-dimensional structural differences between $\alpha\beta$-units of Na,K-ATPase. An alternative explanation that the asymmetry could arise from an unknown tilt offset of the specimen in the electron microscope can be excluded from the complete three-dimensional reconstruction of the crystalline array we present here. The analysis of the lattice parameter change upon tilting resulted in an offset of approximately 4° which is negligible when interpreting the nominal 0°-projection. Correlation averaging of this micrograph alone shows a high degree of asymmetry (Fig. 2).

The results presented here, which were obtained at a resolution level similar to previous studies, have led up to a retrospective analysis of our previous data. By analysis of the phases of significant Fourier components from different crystalline domains we conclude that at the resolution level obtained from the experimental data most of the expected p21 crystals (as judged by the unit cell dimensions) show the presence of a dyad axis. For $Co(NH_3)_4$-ATP induced tetragonal crystals the residual phase error is even smaller [19]. However, for some vanadate/magnesium induced crystals, in particular after treatment with phospholipase A_2, the two-fold rotation is absent as shown here. The modification of lipids by phospholipase A_2 may influence the lipids close to the Na,K-ATPase molecules. The present observations suggest that the degree of symmetry can vary between

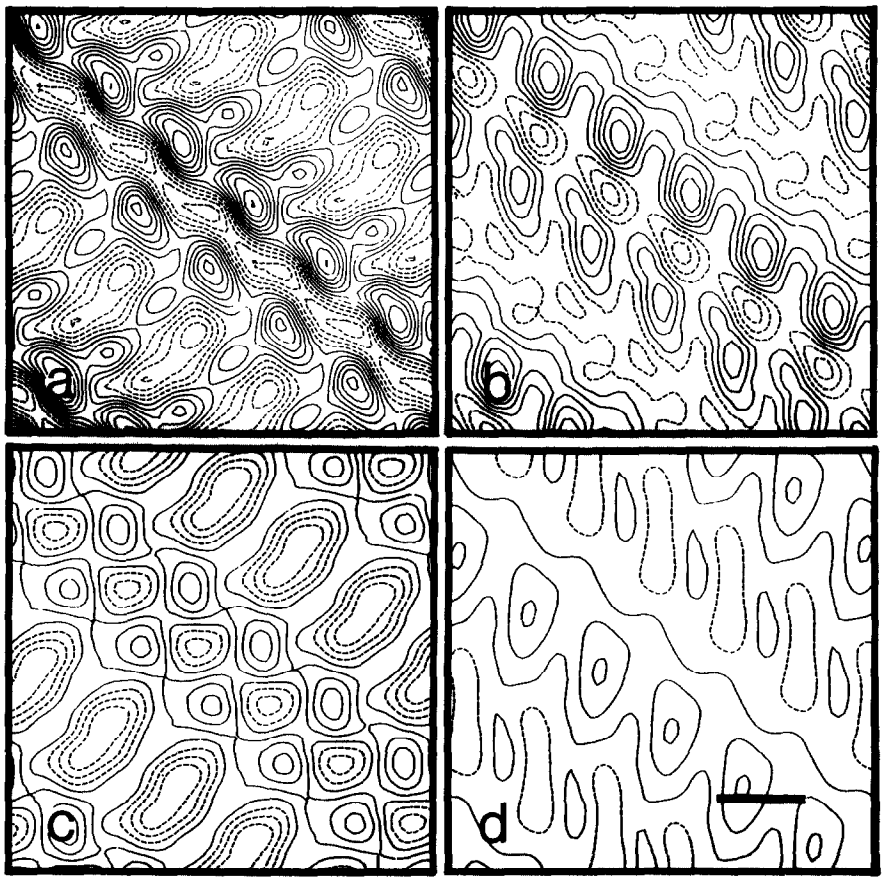

Fig. 4. Sections through the three-dimensional volume parallel to the membrane plane at (a) 0 Å, (b) −15 Å, (c) +20 Å and (d) +45 Å from the central section of the model. Bar = 50 Å.

different crystalline domains, in particular for vanadate/magnesium induced crystals with two $\alpha\beta$-units in the unit cell.

The demonstration that Na,K-ATPase molecules as delineated by negative stain can adopt different three-dimensional shapes is likely to have some casual connection to the structure at the chemical level. The volume of both $\alpha\beta$-units analyzed here is similar suggesting that the composition of the protein domains is constant. An interesting possibility is that the structural differences are associated with defined conformational states of the enzyme. Since the protein units form crystals and since they show two distinct structures at the present resolution level it is likely that they represent two different states rather than a continuous distribution. Furthermore the two $\alpha\beta$-units are localized differently relatively to the lipid bilayer. This observation may be relevant for the functional properties of the enzyme since there is evidence that parts of the α polypeptide chain change positions relatively to the lipid bilayer in connection with conformational changes related to functional alterations [20].

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