Three-dimensional structure of $(Na^+ + K^+)$-ATPase revealed by electron microscopy of two-dimensional crystals

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Prolonged incubation of membrane fragments containing homogeneous $(Na^+ + K^+)$-ATPase with Mg^{2+}, K^+ and VO_3^- at 4°C resulted in formation of two-dimensional crystals of this enzyme with unit cell parameters: $a = 66$ Å, $b = 118$ Å, $\gamma = 108°$. The crystals correspond to the two-sided plane group p21. By combining tilted electron microscopic views of the crystals, a three-dimensional structure of $(Na^+ + K^+)$-ATPase was calculated at ~20 Å resolution. The unit cell is formed by two $(\alpha\beta)$-promoters which are in contact in their central parts. The structure was compared with chemical modification and immunochemical data; the arrangement of intra- and extramembrane domains was proposed.

$(Na^+ + K^+)$-ATPase *Two-dimensional crystal* *Electron microscopy* *Image processing* *Fluorescamine labelling* *Protein structure*

1. INTRODUCTION

For many years numerous investigations were focused on $(Na^+ + K^+)$-ATPase. Structural information about this enzyme is a prerequisite for understanding molecular mechanisms of Na^+ and K^+ active transport across cell membranes. Two types of the subunits (α and β) in an equimolar ratio compose the enzyme molecule. Their molecular masses are 96 kDa for the α-subunit and 40 kDa and 7 kDa for the protein and carbohydrate moieties of the β-subunit, respectively [1]. In freeze-fracture studies, $(Na^+ + K^+)$-ATPase was identified as 90–120 Å diameter intramembrane particles, consisting of 4 α- and 4 β-subunits [2]. Such a membrane oligomeric structure was confirmed by enzyme affinity modification experiments with an alkylating ATP-analog [3]. The crystallization of $(Na^+ + K^+)$-ATPase in two-dimensional arrays [4,5] gives a possibility of obtaining more detailed structural information, using electron microscopy and computer image processing techniques.

2. MATERIALS AND METHODS

$(Na^+ + K^+)$-ATPase was isolated from pig kidney outer medulla as described in [1]. 'Inside-out' proteoliposomes were prepared from homogeneous $(Na^+ + K^+)$-ATPase and chromatographically pure egg lecithin by the cholate dilution method [6]. Fluorescamine labelling (0.1 μmol · mg^{-1} protein) was carried out in 0.25 M sucrose-20 mM $NaHCO_3$ buffer, pH 8.0. After SDS-PAGE of the labelled protein, part of the gel containing the enzyme subunits was sliced, homogenized in 1% SDS–0.1 M $NaHCO_3$, pH 8.5, and the fluorescence in the eluate was measured on a Hitachi spectrofluorimeter PF-1 [7].

Two-dimensional crystals of $(Na^+ + K^+)$-ATPase were prepared according to [5]. Negative staining of the crystals was performed by 2% uranyl acetate aqueous solution using collodion/carbon coated 400-mesh Cu grids.

The crystal thickness was determined by unidirectional Pt/C shadowing of freeze-dried samples using a Balzers BAF-301 device.

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Projection images of the crystals within the ± 60° tilt angle range with the 15°-step were registered on a JEOL JEM-100CX-II electron microscope equipped with a side entry goniometer. Radiation doses during crystal exposure to the electron beam were about 10 electrons/Å^2.

Electron micrographs were preliminary analysed on an optical diffractometer according to [8]. The best micrographs were scanned on a SYNTEX AD-1 microdensitometer at a step size of 32 μm (256 × 256 points), which is equivalent to 7 Å spacing with respect to the original object. Structure factors were computed using software originally written at the MRC Laboratory of Molecular Biology, in Cambridge, England (courtesy of Dr Linda Amos). Calculations were carried out on a Data General Eclipse C-330 minicomputer equipped with a Hewlett-Packard HP 7470 A pen plotter.

3. RESULTS AND DISCUSSION

Firstly two-dimensional crystals of $(Na^+ + K^+)$-ATPase were prepared by prolonged incubation (about 4 weeks) of membrane-bound enzyme fraction with Mg^{2+} and VO_3^- [4]. An essential improvement of the crystal quality was obtained upon a slow rate (~0.1°C/h) of sample cooling from 37 to 4°C. Depending on experimental conditions $(Na^+ + K^+)$-ATPase can be crystallized into three different forms [5]. A dominant type of the crystals formed in the presence of Mg^{2+}, VO_3^- and K^+ was selected for three-dimensional reconstruction (fig.1). Such crystals had cell dimensions: a = 66 Å, b = 118 Å, γ = 108° corresponding to the two-sided plane group p21 [5,9]. The crystal thickness determined by metal shadowing was 120 ± 20 Å. The optical diffraction and Fourier analysis of the crystal images showed the lateral resolution to be about 20 Å. As previously shown [5], the unit cell was formed by two $(\alpha\beta)$-promoters.

Three-dimensional reconstruction based upon 5 independent tilt series of crystal images was performed in space group P2. The resulting structure of the three-dimensional unit cell is presented in fig.2. The unit cell consists of two identical stain excluding regions which must be a dimer of $(\alpha\beta)$-promoters. The calculated volume of the dimer is 340000 Å^3. If one assumes the average partial pro-

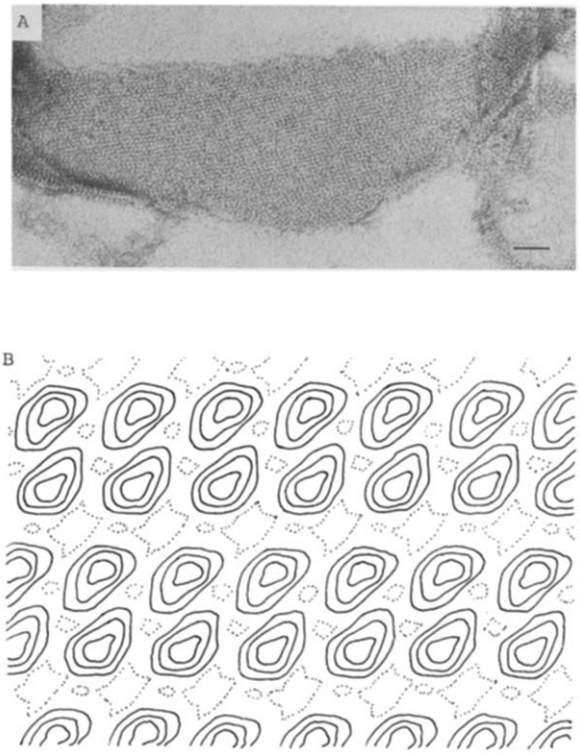

Fig.1. Electron micrograph of negatively stained two-dimensional crystal of the $(Na^+ + K^+)$-ATPase (A) and computer reconstructed image of the same crystal (B). Bar corresponds to 500 Å.

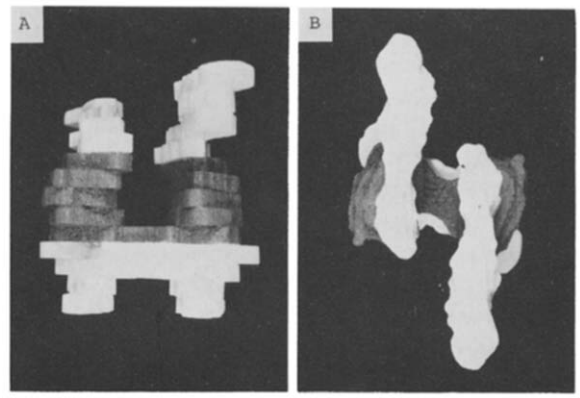

Fig.2. Three-dimensional model of $(Na^+ + K^+)$-ATPase crystal unit cell. Views parallel (A) and perpendicular (B) to the membrane plane. Intramembrane section of the protein is dark.

tein volume equals 1.3 Å^3/Da [10], the molecular mass of the dimer corresponds to 260 or 130 kDa per promoter. This value is in good agreement with the sum of α- and β-subunit molecular masses (140 kDa) [1].

Since the protein purification and crystallization were achieved without solubilization of the membranes the promoters had identical orientation relative to the membrane. In the unit cell promoters were linked by a 2-fold rotation axis normal to the membrane plane. Two promoters have a contact area approximately in their middle parts, which is about 20 Å in height. Vertical dimension of the promoter is about 100 Å, which is much bigger than the normal thickness of lipid bilayers. So, significant parts of the protein should be exposed at the membrane surfaces.

Relative mass values of the extra- and intracellular domains of the enzyme molecule were determined by means of amino group specific fluorescamine labelling of pure $(Na^+ + K^+)$-ATPase in open membrane fragments and 'inside-out' proteoliposomes. Large amounts of lysine residues in the α- and β-subunits, equal to 46 and 22, respectively [1], and high rate of the fluorescamine hydrolysis ($t_{1/2} < 1$ min) ensure random and uniform distribution of the label along

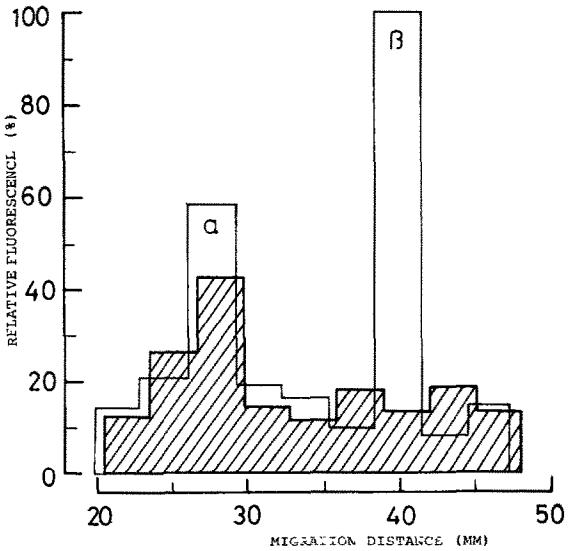

Fig.3. Fluorescamine labelling of α- and β-subunits of $(Na^+ + K^+)$-ATPase in membrane fragments (open bars) and 'inside out' proteoliposomes (stippled bars).

hydrophilic regions of the polypeptide chains. As shown in fig.3, both subunits were accessible for modification in open membrane fragments. No incorporation of the reagent in the β-subunit was observed in proteoliposomes, whereas the α-subunit retained 75% labelling. Therefore the hydrophilic part of the β-subunit is exposed only on the outer surface and cytoplasmic domain of the α-subunit is about 3-times larger than its extracellular region. This result is very similar to that observed upon modification of $(Na^+ + K^+)$-ATPase with Bolton-Hunter reagent [11], and both results are consistent with immunochemical data on localization of antigenic determinants of the α-subunit on both sides of the membrane and those of the β-subunit only on the outer membrane surface [12]. [^{3}H]Adamantane diazirine labelled transmembrane fragments of the α-subunit include over 24% of its total mass [13] and both subunits have the same relative content of the membrane-embedded regions [14]. All mentioned results on spatial organization of $(Na^+ + K^+)$-ATPase subunits on the membrane allow us to propose the most probable arrangement of the intra- and extramembrane domains in the model. The intramembrane portion of the promoter is about 40 kDa and includes 25% of the mass of each subunit; 75% of the total mass of the α-subunit is distributed among extra- and intracellular regions of the promoter as 3:1. So, both hydrophilic domains are of 50 kDa each. Since the total volume of the promoter (170000 Å^3) corresponds to 140 kDa, such hydrophilic domains occupy 4 cross-sections from one side and 4 from the opposite one of the model (one cross-section is of 7.5 Å height). So, the 5 retained cross-sections form a hydrophobic portion of ~40 Å in height (fig.2).

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