FORMATION OF TWO-DIMENSIONAL CRYSTALS IN PURE MEMBRANE-BOUND Na^+,K^+-ATPase

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1. Introduction

The aim of this work has been to induce two-dimensional crystals in preparations of purified membrane-bound Na^+,K^+-ATPase since information about the structure of integral membrane proteins can be obtained by electron microscopy of membrane crystals [1,2]. Na^+,K^+-ATPase is responsible for active Na^+,K^+-transport in kidney tubules and it can be purified in membrane-bound form without perturbing lipoprotein associations [3]. The organization of its proteins in the membrane had been examined by electron microscopy after negative staining and freeze-fracture [4–6]. The enzyme is asymmetrically oriented in the membrane and observed as protein units protruding above the plane of the membrane bilayer after negative staining. Here, we have used negative staining to monitor the aggregation of the protein units during exposure to different combinations of the specific ligands of Na^+,K^+-ATPase. We report the induction of two-dimensional crystals in membrane fragments of purified Na^+,K^+-ATPase during incubation with vanadate and magnesium, while crystalline arrays are rare with other ligand combinations. Information is also presented on the mode of assembly of the membrane crystals. It is suggested that transition to the vanadate-bound E_2-form of the enzyme protein favours immobilization of the units in crystalline arrays.

2. Materials and methods

Na^+,K^+-ATPase was isolated from the outer medulla of the pig kidney by selective extraction of plasma membranes with sodium dodecyl sulphate (SDS) in the presence of ATP followed by isopycniczonal centrifugation in a Ti 14 Beckman zonal rotor [3]. The specific activity was 32–40 μmol P_i . min^{-1} . mg $protein^{-1}$. The preparations were stored at 0°C in 25 mM imidazole–HCl, 1 mM EDTA–Tris (pH 7.5). Prior to incubation the enzyme was sedimented by centrifugation for 10 min at 100 000 rev./min in a Beckman Airfuge. The pellet was resuspended to 1.2 mg protein/ml in 10 mM Tris–HCl (pH 7.5) (20°C) and the following ligand combinations: (a) 1 mM $MgCl_2$; (b) 1 mM $MgCl_2$ and 2 mM P_i–Tris; (c) 1 mM $MgCl_2$ and 0.25 mM sodium monovanadate ($NaVO_3$, Merck); (d) 3 mM $MgCl_2$ and 12.5 mM P_i–Tris; (e) 10 mM Tris–HCl without additions. All solutions were sterilized before use by filtration through 0.1 μm Millipore filters. The suspensions were stored at 4–6°C. After periods from 2 h to 4 weeks, aliquots were examined by electron microscopy after negative staining with 0.5% uranyl acetate using hydrophilic carbon films supported by 400 mesh copper grids. The specimens were examined in a Jeol 100 CX electron microscope at 80 kV and micrographs of two-dimensional crystals recorded at magnifications of 50 000 or 66 000X.

3. Results and discussion

Incubation of membrane-bound Na^+,K^+-ATPase with sodium vanadate in the presence of magnesium gradually and in a reproducible fashion induced the formation of two-dimensional membrane crystals of the enzyme (fig.1,2). In strictly parallel incubations with 1 mM Mg^{2+} or with 1 mM Mg^{2+} and 2 mM P_i almost all membranes were devoid of crystals. The membranes instead showed regions with close packing of particles (fig.3) comparable to the pattern of clus-

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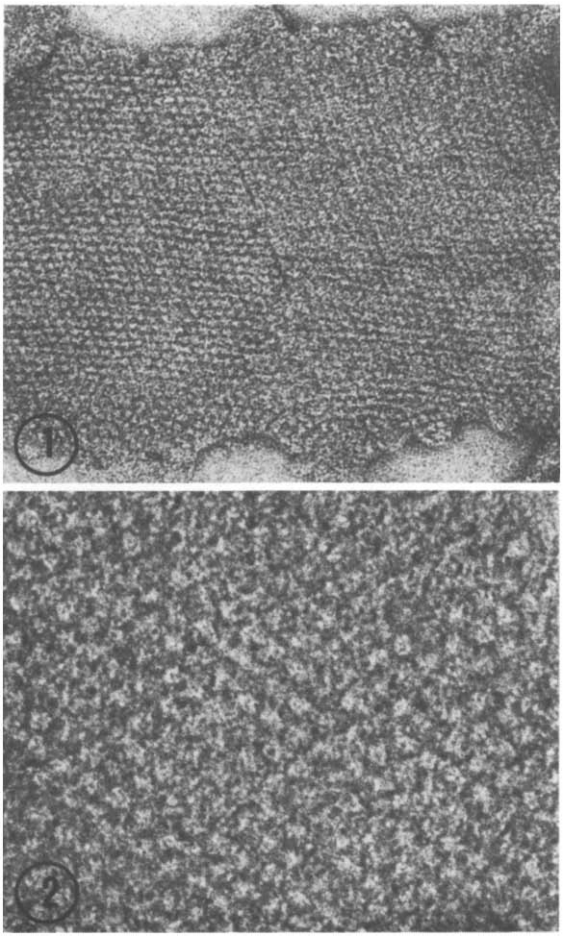

Fig.1,2. Two-dimensional crystals in membrane-bound Na^+,K^+-ATPase incubated for 4 weeks with 0.25 mM $NaVO_3$ and 1 mM $MgCl_2$: fig.1, × 300 000; fig.2, × 800 000.

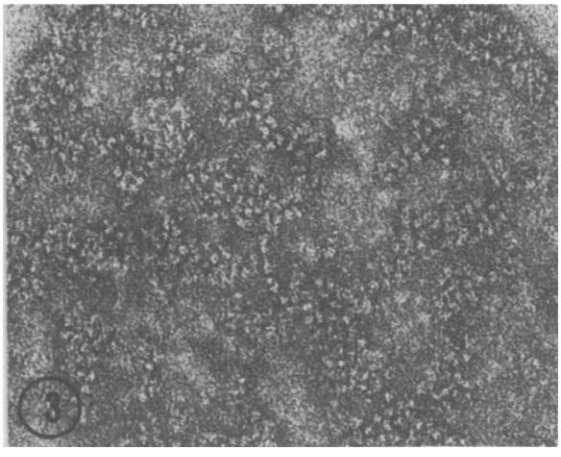

Fig.3. Membrane-bound Na^+,K^+-ATPase incubated for 4 weeks with 1 mM $MgCl_2$ and 2 mM P_i; × 300 000.

ters and strands in [4,5] and only after prolonged incubations were occasional crystalline regions observed. Membrane crystals were also infrequent after incubation in 3 mM Mg^{2+} and 12.5 mM P_i and were never observed with 10 mM Tris·HCl alone. Thus, vanadate was by far the most efficient ligand with respect both to the time required for formation of crystals and to the proportion of surface particles that formed membrane crystals. The possibility that crystals are formed by impurities or contaminants can be excluded. The preparations show a high degree of purity with respect to protein composition, enzymatic assays and electron microscopy [5,7]. Since crystal formation in vanadate media was extensive and present in almost all membrane fragments it can therefore safely be concluded that the crystals are formed by the proteins of the Na^+,K^+-ATPase.

Vanadate-induced membrane crystals showed a non-orthogonal lattice with basic repeats of about 47 Å and 54 Å and an average angle between the lattice lines of 66° (fig.1,2). In small membrane fragments usually only one membrane crystal was formed but in large fragments two or more lattice systems were often present. Many crystals showed a high degree of order and preliminary examination by optical diffraction performed by H. Hebert (in preparation) showed reflections to about 25 Å resolution. The induction of membrane crystals therefore opens the possibility for further structural analysis of Na^+,K^+-ATPase similar to those done on other intrinsic membrane proteins [2]. If completely regular crystals can be formed, information at better resolution may be obtained by the low-dose electron microscopy technique developed in [1].

The vanadate-induced membrane crystals formed gradually over days and weeks. It is important to observe the pattern of assembly of the protein units into crystalline arrays since this may provide information about subunit interactions. In the present system formation of linear polymers with the shape of ladders consisting of paired units was observed in some membranes already within hours after start of the incubation (fig.4). Later these linear polymers seemed to associate laterally (fig.5,6) to form two-dimensional crystals with a lattice of the type shown in fig.1. The confluent crystals gradually increased in size over the following weeks. Formation of the crystals appeared to involve a separation of protein and lipid within the membrane (compare fig.1 with fig.4–6). It is not possible to decide if the primary event in the assembly is

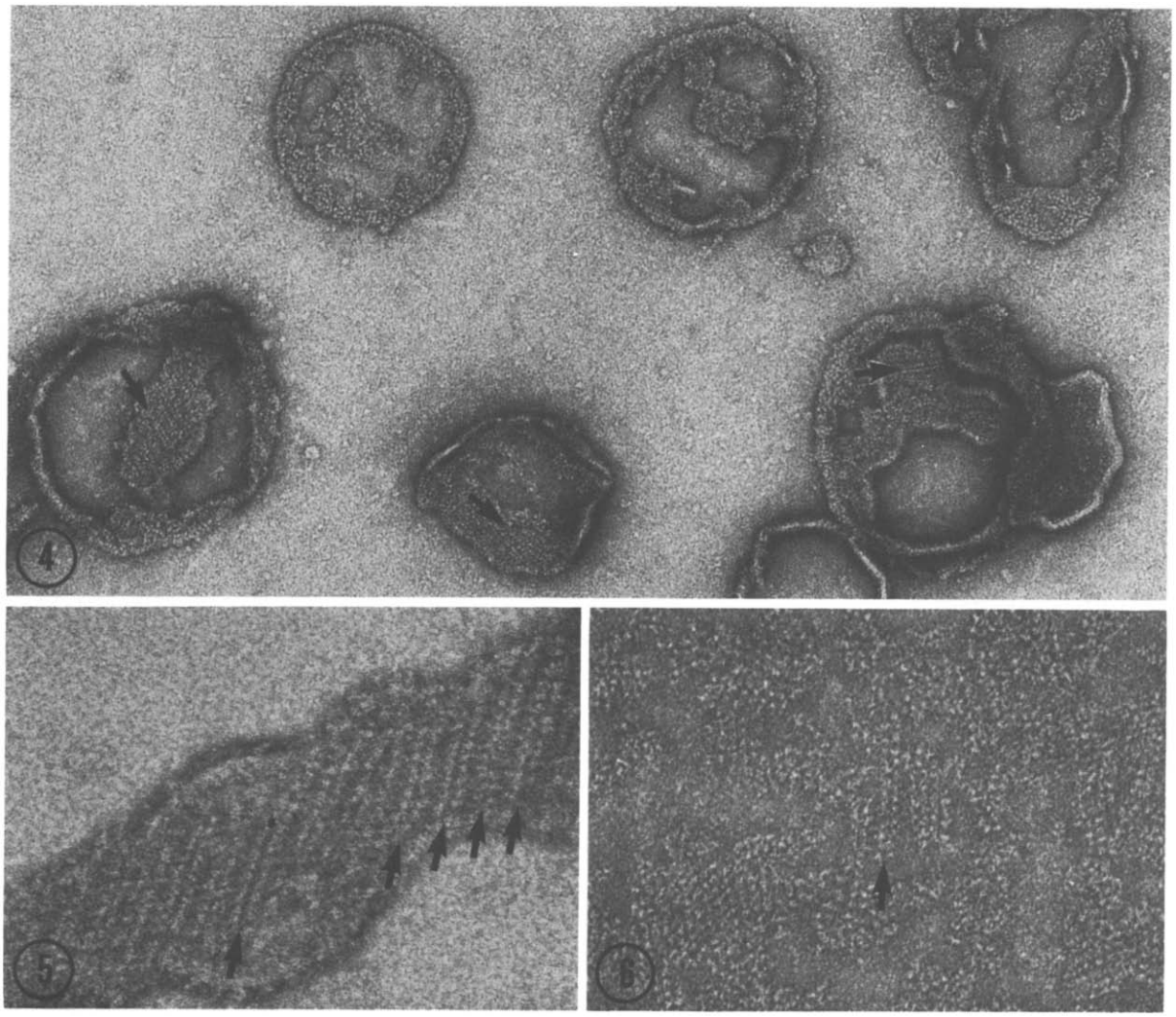

Fig.4–6. Linear dimeric polymers (arrows) in membrane fragments incubated for 2 h (fig.4), 1 week (fig.5) and 3 weeks (fig.6) in 0.25 mM $NaVO_3$ and 1 mM $MgCl_2$. Fig.5 also shows small membrane crystals. Fig.4, × 100 000; fig.5,6, × 300 000.

the formation of longitudinal or lateral bonds within the linear polymer but the energy of bonds within the linear polymers are likely to be higher than the energy of the bonds between the ladder-like polymers [8].

Our observations allow the conclusion that interaction between protein units in the membrane is favoured when the protein is in the E_2-conformation which is stabilized by vanadate. At 0.25 mM vanadate and 1 mM $MgCl_2$ the equilibrium between E_1- and E_2-forms of the protein will be poised heavily in favour of the E_2-form due to the very high affinity of Na^+,K^+-ATPase for vanadate [9,10]. It remains to be examined if the bonds between protein units in the crystal are formed between hydrophilic portions in the aqueous phase or between portions of the protein that are embedded in the membrane bilayer.

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