Volume 330, number 1, 19–22 FEBS 12888 September 1993

Ultrastructure of membrane-bound Na,K-ATPase after extensive tryptic digestion

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Received 30 June 1993

Membrane-bound Na,K-ATPase was digested with trypsin in the presence of Rb^+ to form the stable 19-kDa and smaller fragments of the α-chain known to preserve occlusion of Rb^+ (K^+) or Na^+. The trypsinized membranes obtained from pig kidney and shark rectal gland were analyzed by electron microscopy. Tryptic digestion preserved general membrane structure but removed both the surface particles observed by negative staining and the protruding cytoplasmic portion of the α-subunit identified in thin sections. However, intramembrane particles defined by freeze-fracture were preserved after trypsinization suggesting that the remaining membrane spanning protein fragments retain the native structure within the lipid bilayer after proteolysis.

Na,K-ATPase; Electron microscopy; Tryptic digestion; 19-kDa fragment, Occlusion; Two-dimensional crystal

1. INTRODUCTION

Extensive trypsinization of membrane-bound Na,K-ATPase leaves a large 19-kDa fragment and smaller fragments (6–11 kDa) of the α-chain in the membrane [1]. Occlusion of Rb^+ (K^+) as well as Na^+ is retained within these membranes while ATP-dependent functions are lost [1,2]. Electron microscopy of native Na,K-ATPase has shown that parts of the enzyme protein protrude outside the lipid bilayer [3], while other parts are demonstrable as intramembrane particles (IMPs) by freeze-fracture [4], and that the enzyme is asymmetrically inserted in the lipid bilayer [5]. In the present work we have analyzed the trypsinized membranes by electron microscopy to determine the location of membrane-associated tryptic fragments. This preparation is ideal for analysis of the membrane-embedded parts of the Na,K-ATPase since the large cytoplasmic loop (residues 337 to 771, see [2] for discussion) as well as other extramembranous parts are removed by trypsin.

2. MATERIALS AND METHODS

Trypsin (T-8642), trypsin inhibitor (T-9128) and molecular weight markers (MW-SDS-17S and MW-SDS-70) and phospholipase A_2 (P-2509) were obtained from Sigma.

2.1. *Preparation of pig kidney enzyme*

Na,K-ATPase was isolated in membrane-bound form from outer medulla of pig kidney by the method of Jørgensen [6]. The enzyme was stored at −20°C in 250 mM sucrose, 12.5 mM imidazole, and 0.5 mM EDTA at pH 7.5. The specific ouabain-inhibitable Na,K-ATPase activity was 2300 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$ protein at 37°C. Two-dimensional crystals of the enzyme were induced with vanadate [7]. The membranes were first incubated with 0.33 μg/ml phospholipase A_2 [8] and then dialyzed at 4°C overnight against 10 mM imidazole buffer, pH 7.5, containing 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM NH_4VO_3.

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2.2. *Preparation of shark rectal gland enzyme*

Na,K-ATPase from the rectal gland of *Squalus acanthias* was prepared as described by Skou and Esmann [9], but without the treatment with saponin. The Na,K-ATPase constituted about 70% of the protein (determined as the content of α- and β-subunits from SDS gel electrophoresis), and the specific activity was about 1700 μmol $P_i \cdot h^{-1} \cdot mg$ protein. Na,K-ATPase activity, phosphorylation capacity (2.9 nmol/mg protein) and protein content were determined as previously described [10].

2.3. *Digestion with trypsin*

Pig kidney or shark rectal gland Na,K-ATPase (about 3 mg/ml) was incubated with 0.5 mg/ml trypsin for 60 min at 23°C in a buffer containing 15 mM histidine, 5 mM CDTA and 10 mM RbCl (pH 7.0). The reaction was terminated by addition of an equal volume buffer containing 1.5 mg/ml trypsin inhibitor, and the membranes were washed by centrifugation three times at 60,000 rpm for 30 min. The final pellet was taken up in a buffer containing 15 mM histidine, 10 mM RbCl, 1 mM CDTA and 25% glycerol, and stored at −20°C. Control preparations were treated as above, omitting trypsin in the 60 min incubation ('native enzyme').

2.4. *Polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was done according to the method of Schagger and von Jagow [11] using 1-mm 12% gels. The pellets were dissolved in sample buffer, which contained 100 mM Na-phosphate (pH 7.7), 1% 2-mercaptoethanol, 2% SDS and 36% urea. Samples were heated to 100°C for 5 min before electrophoresis. Staining was done in a solution of 0.25% Coomassie blue R250/40% ethanol/10% acetic acid for 1 h, and destaining in 30% ethanol/10% acetic acid.

2.5. *Electron microscopy*

Membrane samples were negatively stained with 1% uranyl acetate. For freeze-fracture membrane samples were incubated in 30% glyc-

erol, frozen in liquid nitrogen, fractured in a Balzers freeze-fracture apparatus (BAF 300, Balzers AG) at −100°C and shadowed unidirectionally with platinum. Membranes were also fixed with 2% glutaraldehyde and 1% tannic acid, post-fixed in osmium tetroxide, stained en bloc with uranyl acetate, embedded in Epon; the thin sections were stained with uranyl acetate and lead citrate The specimens were examined in a JEOL 100CX electron microscope. Details of the preparation procedures for electron microscopy were as described before [12].

3. RESULTS

3.1. *Pig kidney Na,K-ATPase*

Purified, native Na,K-ATPase-membranes showed surface particles following negative staining (Fig. 1B) but after tryptic digestion the membranes showed essentially smooth surfaces without particles (Fig. 1A). In contrast, intramembrane particles observed by freeze-fracture electron microscopy appeared unchanged in frequency after trypsinization. Before trypsinization the frequency of IMPs was 3,400 ± 1240 (S.D.) particles/μm^2 (n = 23) on the particle-rich fracture face (P-face) and 880 ± 620 particles/μm^2 (n = 28) on the particle-poor fracture face (E-face), respectively. The frequency of IMPs after trypsinization, 3,570 ± 1290 particles/μm^2 (n = 52) on the P-face and 910 ± 540 particles/μm^2 (n = 51) on the E-face, were not significantly different from non-trypsinized controls. In thin sections control membranes appeared triple-layered and in most places asymmetric with a light (lipid) layer surrounded by two stained layers of different thickness (Fig. 2A). The total thickness of the membranes following the tannic acid fixation procedure was 130–150 Å. Trypsinized membranes were thinner and showed a symmetric appearance in most places with a total thickness of 90–120 Å (Fig. 2B).

Membrane crystals of pig Na,K-ATPase, that in controls showed linear arrays of protein units following negative staining as well as freeze-fracture, showed no crystalline arrays and no surface particles after digestion. IMPs on the contrary were present although randomly distributed (data not given).

3.2. *Shark rectal gland Na,K-ATPase*

Observations on shark enzyme were similar to those on pig kidney enzyme with the exception that the surface particles on shark membranes were less distinct in negative staining than on pig membranes. Notably, the IMPs were unchanged in frequency following digestion by trypsin (Fig. 3). Frequency of IMPs was 3,600 ± 1250 (S.D.) particles/μm^2 (n = 52) on the P-face and 780 ± 520 particles/μm^2 (n = 59) on the E-face in native membranes versus 3,830 ± 930 particles/μm^2 (n = 29) and 970 ± 750 particles/μm^2 (n = 44) in trypsinized membranes, i.e. no significant differences between control and trypsinized membranes. In thin sections native shark membranes were asymmetric with one thin and one thick stained layer bordering an unstained (lipid) layer. In contrast trypsinized membranes were thinner and symmetric (Fig. 4).

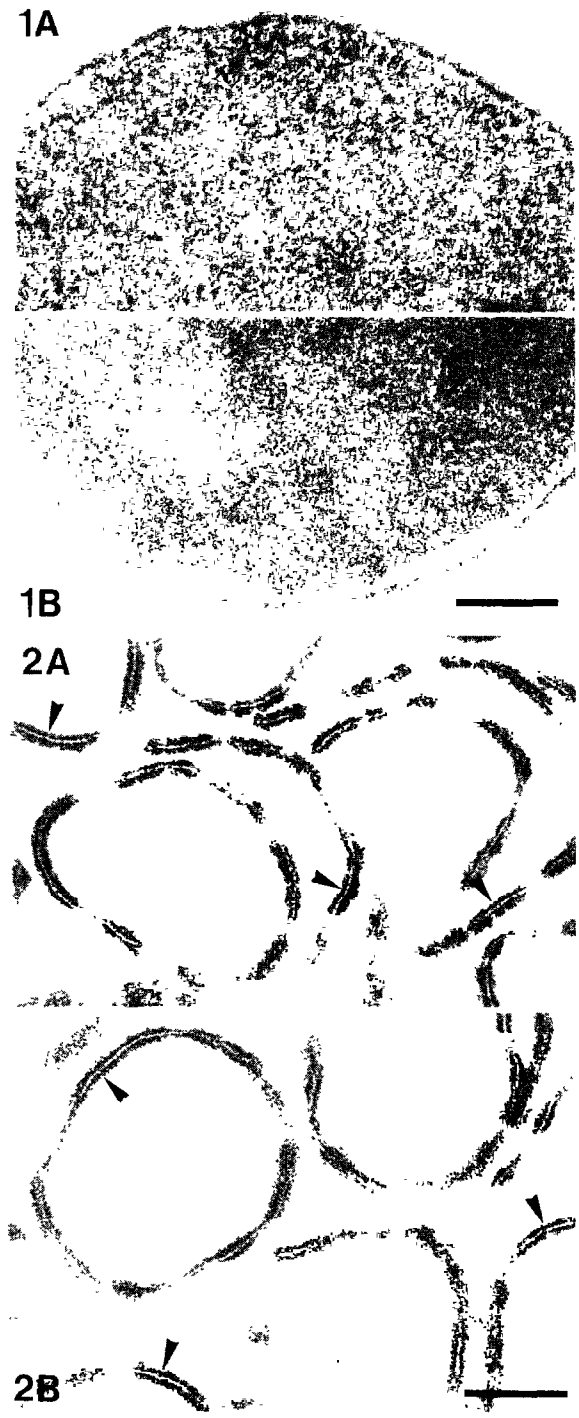

Fig. 1. Negatively stained Na,K-ATPase membranes from pig kidney before (A) and after (B) trypsinization. Bar = 0.1 μm.

Fig 2. In thin sections native Na,K-ATPase membranes from pig kidney are asymmetric in cross-sections (arrowheads in A) but symmetric (arrowheads in B) when fixed after trypsinization Bar = 0.1 μm.

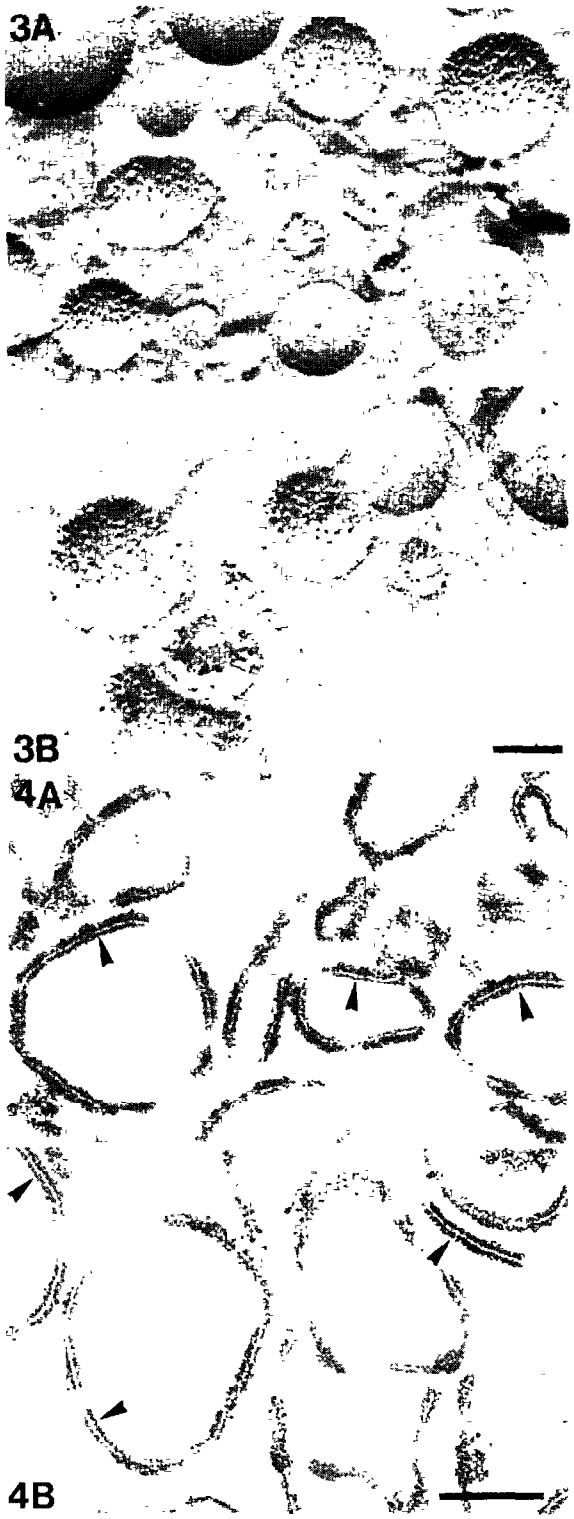

Fig. 3. Freeze-fractured Na,K-ATPase membranes from shark rectal gland before (A) and after (B) trypsinization show no difference with respect to the distribution of intramembrane particles. Bar = 0.1 μm.
Fig. 4. Na,K-ATPase membranes from shark rectal gland are asymmetric before (A) but symmetric after (B) trypsinization Bar = 0.1 μm.

3.3. *Electrophoretic analysis*

Pig kidney enzyme trypsinized in rubidium medium showed a dominant 19-kDa band, which was absent in control enzyme (Fig. 5). After trypsinization also the β-subunit was reduced giving rise to a faint 16-kDa band in pig membranes [13] while the β-subunit was virtually unaffected in shark membranes, cf. Fig. 5.

4. DISCUSSION

The present observations demonstrate that purified Na,K-ATPase even after extensive tryptic digestion remains in membrane-bound form. Surface particles on the Na,K-ATPase membranes were removed by tryptic digestion while significantly the freeze-fracture particles, which represent intramembrane aspects of the enzyme protein, remained intact. This demonstrates that trypsin only affected parts of the α-subunit protein located outside the lipid bilayer. The fact that the IMPs revealed by freeze-fracture remained suggests that the intramembrane parts of the enzyme system, including

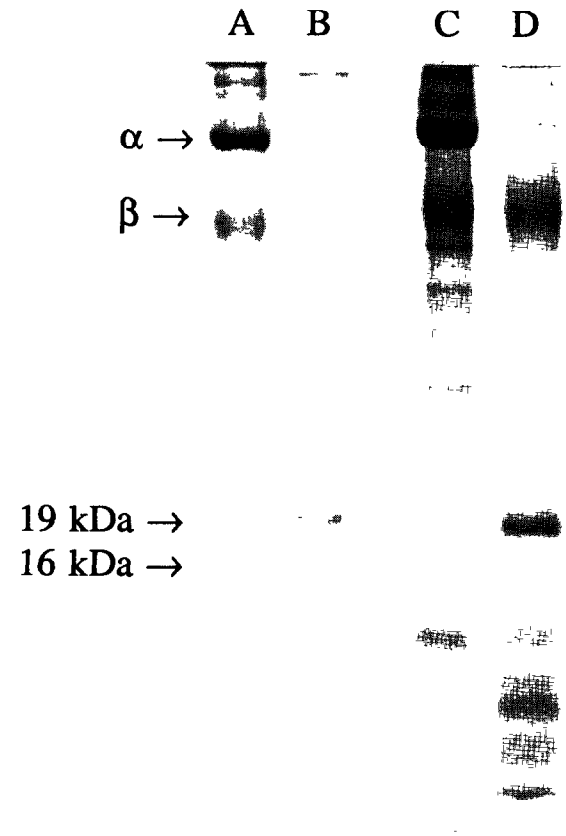

Fig. 5. Digestion of pig kidney and shark rectal gland Na,K-ATPase by trypsin. Lane A, pig kidney Na,K-ATPase (no trypsin added), Lane B, trypsinized pig kidney Na,K-ATPase. The positions of the α- and β-subunits are marked, as is that of the 19-kDa tryptic α-fragment and the 16 kDa tryptic β-fragment [13]. Lane C, shark Na,K-ATPase (no trypsin); lane D, trypsinized shark Na,K-ATPase [15]. Molecular weights were estimated using the markers described in section 2.

the 19-kDa fragment, were not dissociated during trypsinization, i.e. the intramembrane peptides formed by tryptic cleavage remained as integral entities within the lipid bilayer. This is consistent with results from electron spin resonance studies of covalently spin labelled enzyme where it is found that the overall rotational mobility of the trypsinized shark Na,K-ATPase is similar to that of the native Na,K-ATPase (Esmann et al., unpublished observations). On the contrary, the 19-kDa fragment is digested in the absence of Rb^+ or the presence of Ca^{2+}-ions [1] suggesting that parts of the 19-kDa fragment is exposed to the outside of the lipid bilayer under these conditions.

The α-subunit has major portions of its peptide chain protruding into the cytoplasm rather than into the extracellular space [2,14]. The present observations on ultrathin sections therefore identify the thick stained layer of the membrane as the cytoplasmic leaflet of the membrane since it is removed following tryptic digestion. This conclusion is particularly clear with respect to the shark membranes since here the β-subunit is virtually unaffected by the digestion procedure.

In membrane crystals of kidney Na,K-ATPase the ordered arrays of surface and intramembrane particles disappeared after trypsin digestion. This suggests that the formation and maintenance of the membrane crystals largely depend on conformationally sensitive protein-protein interactions between epitopes on α-subunits located outside the lipid bilayer. Following tryptic digestion of these interacting parts of the α-subunits the enzyme protein units disconnect and become randomly distributed in the lipid bilayer.

Acknowledgements. The authors wish to thank Karen Thomsen, Else-Merete Løcke, Birthe Bjerring Jensen and Angielina Tepper for valuable assistance. This work was supported by the Danish Medical Research Council, the Carlsberg Foundation and the Danish Biomembrane Research Center, University of Aarhus.

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