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Bulk properties of the lipid bilayer are not essential for the thermal stability of Na,K-ATPase from shark rectal gland or pig kidney

Aida S. Hansen, Kristian L. Kraglund, Natalya U. Fedosova, Mikael Esmann *

Department of Physiology and Biophysics, Aarhus University, Ole Worms Allé 6, DK-8000 Aarhus, Denmark

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ABSTRACT

The thermal stability of Na,K-ATPase from pig kidney is markedly greater than that of Na,K-ATPase from shark salt glands. The role of the lipid bilayer is studied by solubilisation of the membrane-bound enzyme in the nonionic detergent octaethyleneglycoldodecylmonoether ($C_{12}E_8$), addition of excess dioleylphosphatidylcholine (DOPC) or palmitoyloleylphosphatidylcholine (POPC) and reconstitution of membranes by removal of detergent. At 54 °C the reconstituted enzymatically active pig enzyme retains a high thermal stability, and reconstituted shark enzyme retains a low thermal stability, even with a 9-fold excess of DOPC. This result suggests that the origin of the difference in thermal stability is not related to bulk lipid properties of the native membranes.

1. Introduction

The sodium pump, or Na,K-ATPase, is a member of the P-type ATPase family, and performs the active transport of sodium and potassium ions across the cell plasma membrane. The enzyme is highly conserved in almost all eucaryotic cells [1–3]. The thermal stability of Na,K-ATPase isolated from rectal salt glands of shark (*Squalus acanthias*) is markedly smaller than that of enzyme isolated from pig kidney [4,5]. Pig enzyme is also more resistant towards denaturation by urea than shark enzyme [6]. Both enzymes are complexes of α- (Mr. 110 kDa), β- (Mr. 35.5 kDa) and γ-subunits (Mr. 10 kDa) and the overall architecture of the two enzymes is very similar as recently shown by crystallography [7,8]. The sequences of the subunits are similar, 87% identity for the α-subunit, 66% for the β-subunit and only 23% for the γ-subunit. A difference in thermal stability could be related to the relatively few differences in the overall sequence.

The lipid compositions of the pig and shark membranes are, however, also different, with a larger degree of unsaturation in the shark membrane lipids than in pig membrane lipids, pointing to a larger fluidity of the shark membranes at a given temperature (see [4] for a discussion). The present experiments were designed to test if the origin of the greater thermal stability of pig kidney enzyme compared to shark rectal gland enzyme is due to this difference in bulk lipid composition of the native membranes of the two sources.

The membranous enzymes were solubilised in detergent and excess phospholipid was added prior to reconstitution of the membranes by detergent removal. It was expected that with a large excess of exogenous lipid the bulk properties such as the fluidity of the reconstituted membranes would be similar for shark and pig, masking differences between the residual native lipids. It is found that even with a 9-fold excess of the phospholipid DOPC in the reconstituted membranes, the thermal stability of pig enzyme is still much larger than that of shark enzyme, suggesting a very minor role of the bulk properties of the lipid bilayer in thermal stabilization.

2. Materials and methods

2.1. Preparation of native Na,K-ATPase

Membranous Na,K-ATPase was prepared from the salt gland of *S. acanthias* according to the method of Skou and Esmann [9], but omitting the treatment with saponin. The shark membranes were stored in 20 mM histidine (pH 7.0) and 25% glycerol.

Na,K-ATPase from pig kidney microsomal membranes was prepared using pretreatment with SDS and purified by differential centrifugation [10,11]. The pig membranes were stored in 20 mM histidine (pH 7.0), 1 mM EDTA and 250 mM sucrose.

Abbreviations: Na,K-ATPase, Na^+- and K^+-transporting adenosine triphosphatase; K-pNPPase, K^+-*p*-nitrophenylphosphatase; DOPC, dioleylphosphatidylcholine; POPC, palmitoyloleylphosphatidylcholine; P0, P4, P10, reconstituted pig preparations; S0, S4, S10, reconstituted shark preparations; pNPP, *p*-nitrophenylphosphate; ρ_G, density of fractions from sucrose gradients; Buffer A, 20 mM histidine (pH 7.0) containing 150 mM KCl and 4 mM $MgCl_2$; $C_{12}E_8$, octaethyleneglycoldodecylmonoether.

* Corresponding author. Fax: +45 8612 9599.
E-mail address: me@biophys.au.dk (M. Esmann).

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The Na,K-ATPase constituted typically 70% of the total protein (determined as the content of α- and β-subunits from SDS gel electrophoresis). Protein concentrations were determined using the Lowry method [12].

2.2. Activity assays of native and reconstituted preparations

The steady-state Na,K-ATPase activity was assayed at 37 °C (130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP (tris salt), 20 mM histidine (pH 7.4), 0.33 mg/mL albumin and 0.2 mM EGTA in the assay medium) by measuring phosphate liberation from ATP with colorimetric methods [13,14]. Steady-state K-pNPPase activity (150 mM KCl, 20 mM $MgCl_2$, 20 mM histidine (pH 7.4), 0.33 mg/mL albumin, 0.2 mM EGTA and 10 mM p-nitrophenylphosphate in the assay medium) was assayed at 37 °C by following the increase in absorbance at 410 nm from liberation of p-nitrophenol [14]. The enzymatic activity of $C_{12}E_8$-solubilised enzyme was determined as previously described [15].

2.3. Solubilisation

Native pig or shark membranes (3.2 mL) were mixed with 0.4 mL of a solution containing 1500 mM KCl and 40 mM $MgCl_2$, and thereafter the membranes were solubilised by addition of 0.4 mL $C_{12}E_8$ to a final detergent/protein ratio of 2 (w/w). The solubilised enzymes were centrifuged for 60 min at 180,000*g* at 10 °C. The supernatant contains the solubilised Na,K-ATPase and is used for reconstitution.

2.4. Reconstitution

A concentrated DOPC micellar suspension was made as follows: to 50 mg DOPC was added 100 mg $C_{12}E_8$ in a buffer containing 20 mM histidine (pH 7.0), 150 mM KCl and 4 mM $MgCl_2$ (Buffer A). This solution was sonicated for about 2 h in a Metason 200 g sonicator. Reconstitution was done as follows: DOPC and supernatant enzyme were mixed to give a protein concentration of about 0.5 mg/mL and DOPC concentrations of 0, 4 or 10 mg DOPC/mg protein. These are in the following abbreviated P0, P4 and P10 for reconstituted pig enzymes and S0, S4 and S10 for shark. The P0 and S0 samples were made using Buffer A without DOPC and $C_{12}E_8$, i.e. a simple dilution of the supernatant enzyme. All of these steps were done on ice.

Removal of $C_{12}E_8$ from the samples were done by addition of BioBeads to a ratio of about 12 mg BioBeads/mg $C_{12}E_8$. The treatment with BioBeads proceeded for 12 h at about 4 °C. The reconstituted membranes were separated from the BioBeads and then centrifuged for 60 min at 260,000*g* at 10 °C. The pellet containing the reconstituted preparation was resuspended and homogenized in 20 mM histidine (pH 7.0) and 25% glycerol for shark and 20 mM histidine (pH 7.0), 1 mM EDTA and 250 mM sucrose for pig enzyme, and stored at −20 °C. In a smaller set of experiments POPC was used (cf. Fig. 1).

2.5. Sucrose gradient centrifugation

Sucrose gradient centrifugation was performed in a Beckman SW40TI swing-out rotor, run at 27,000 rpm (55,000*g*) for about 17 h at 10 °C using a Beckman Coulter Optima LE 80 K ultracentrifuge. Gradients consisted of 5 layers of sucrose in Buffer A, with sucrose concentrations of 5–10–15–20–25% ("5/25-gradients") or 10–20–30–40–50% ("10/50 gradients"). Each layer had a volume of 1.9 mL, and a 0.5 mL sample of Na,K-ATPase (typically containing 0.4 mg protein) in the same buffer was layered on top. After centrifugation 0.5 mL fractions were collected from the top of the gradient, placed on ice and immediately assayed for pNPPase activity. This was done by dilution of 30 μL into 0.97 mL pNPPase reaction medium at 25 °C and allowing the reaction to proceed for 5 min at 37 °C. The refractive index and protein concentrations were also measured on the fractions.

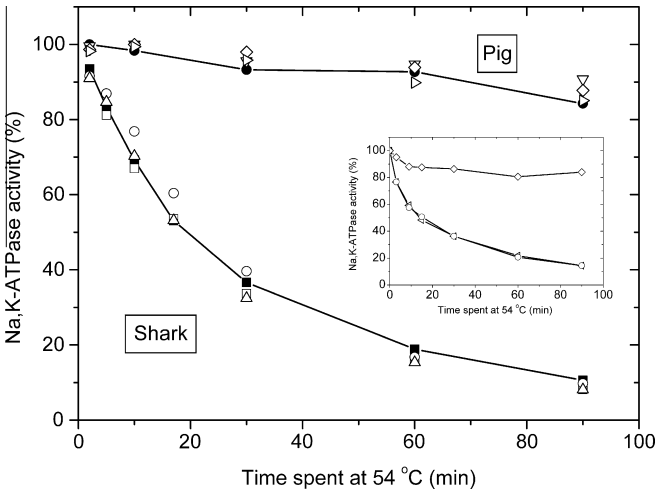

Fig. 1. Thermal stabilities of native and reconstituted Na,K-ATPase from shark and pig. Shark enzymes (native enzyme (■); reconstituted preparations S0 (□); S4 (○); S10 (open up-triangles) and pig enzymes (native enzyme (●); reconstituted preparations P0 (open down-triangles); P4 (◇); P10 (open right-triangle)) were incubated at 54 °C for the indicated periods and the residual Na,K-ATPase activity is given in % of the activity at time zero for each of the 8 samples. The specific activities of the preparations are given in Table 1. The inset shows similar stability experiments with shark and pig Na,K-ATPase reconstituted with POPC at a ratio of 4 mg POPC/mg protein (◇ for pig, ○ for shark) and at a ratio of 1 mg POPC/mg protein for shark enzyme (open left-triangle).

2.6. Calculation of the lipid/protein ratio in native and reconstituted samples

The refractive index of samples from sucrose gradient centrifugations was used to calculate the density (ρ_G) from standard tables [16].

The density of membranes reconstituted with DOPC in the absence of protein was estimated as follows: 1 mL of a solution with reconstituted DOPC in Buffer A was mixed with 1 mL of sucrose at concentrations between 0 and 4% (also in Buffer A), and the samples were centrifuged at 55,000*g* for 16 h (see above). At a final concentration of 0.5% sucrose a pellet of reconstituted DOPC was observed, and at 1.5% a clear layer of DOPC on top of the sucrose solution was observed. With 1% sucrose there was neither flotation nor clear pellet formation of DOPC, and we thus estimate the density of the phospholipid to be about 1.0039 mg/mL, i.e. that of a 1% sucrose solution.

The density of Na,K-ATPase protein was calculated from the amino acid composition by the method of Cohn and Edsall [17] to 1.365 mg/mL. The lipid/protein ratio (w/w) was calculated as $(1 - \rho_G/1.365)/(\rho_G/1.0039-1)$, where it is assumed that the value 1.0039 mg/mL is representative of all the lipid components of the reconstituted membranes.

Figures were produced using Origin 6.0 (Microcal Software).

3. Results and discussion

3.1. Solubilisation and reconstitution

Shark and pig Na,K-ATPase membranes were solubilised with $C_{12}E_8$ at a ratio of 2 mg $C_{12}E_8$/mg protein, and the enzymatic activity is not altered by this procedure [18]. Centrifugation of the solubilised enzyme lead to a clear supernatant containing all the